BIOPROCESS DEVELOPMENT FOR THE 2,3-BUTANEDIOL PRODUCTION BY *Paenibacillus peoriae* NRRL BD-62

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Engenharia Química, COPPE, da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Engenharia Química.

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Ciência resistente é ciência consciente!

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DESENVOLVIMENTO DO BIOPROCESSO DE PRODUÇÃO DE 2,3-BUTANODIOL POR *Paenibacillus peoriae* NRRL BD-62

Daniel Tinôco Campos Neto

Outubro/2022

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Programa: Engenharia Química

A produção biotecnológica de 2,3-butanediol (2,3-BDO), um versátil químico de plataforma, é ainda limitada à escala de laboratório, embora apresente vantagens sobre a rota química industrial como a alta pureza óptica isomérica e a menor demanda de energia. Este estudo teve como objetivo desenvolver as etapas *upstream* e *mainstream* do bioprocesso de 2,3-BDO para uma produção segura, eficiente e potencialmente aplicável em larga escala por um não-patogênico *P. peoriae* NRRL BD-62, recentemente isolado. As condições ótimas de fermentação de 32 °C, pH=5 e kLa~7,5 h^{-1} obtidas por planejamento fatorial 2^4 com réplica no ponto central a C/N= 8.5 g/g permitiram uma produção de 2,3-BDO de 39,4 g/L, com rendimento de 0,43 g/g, e seletividade *levo:meso*-isômeros de 1.9:1, sem acúmulo de acetoína em batelada alimentada por pulso. A batelada alimentada a taxa constante de 1,3 mL/h manteve o rendimento de 2,3-BDO acima de 0,40 g/g, valor comumente reportado por produtores microbianos patogênicos. Melaço de cana e NH_4Cl foram usados para substituir a glicose e o extrato de levedura (YE) comerciais em batelada, respectivamente, resultando em uma economia de aproximadamente 75,2% do meio de cultura, cujo preço final foi igual a 4,74 US$/$kg_{2.3\text{-}BDO}$. O aumento da escala de produção de 2,3-BDO a partir de glicose e YE cervejeiro foi baseado na taxa de transferência de oxigênio constante, que reproduziu as condições microaeróbicas necessárias à síntese de *levo*-2,3-BDO. Cerca de 0,9 g/L/h e 0,40 g/g de 2,3-BDO foi produzido em batelada a 70 L. Os fatores limitantes da produção de 2,3-BDO, identificados na inicial prospecção técnico-científica, foram controlados experimentalmente, contribuindo, assim, para a viabilidade industrial do bioprocesso no futuro.

Abstract of Thesis presented to COPPE/UFRJ as a partial fulfillment of the requirements for the degree of Doctor of Science (D.Sc.)

BIOPROCESS DEVELOPMENT FOR THE 2,3-BUTANEDIOL PRODUCTION BY *Paenibacillus peoriae* NRRL BD-62

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The production of bio-based 2,3-butanediol (2,3-BDO), an important platform chemical used in different industrial applications, is still limited to lab scale, although it has advantages over industrial chemical routes such as high isomeric optical purity and lower energy demand. This study aimed to develop the upstream and mainstream steps of the 2,3-BDO bioprocess for a safe, efficient and potentially applicable large-scale production by a newly wild-type and non-pathogenic *P. peoriae* NRRL BD-62. The optimal fermentation conditions of 32 °C, pH=5, and kLa~7.5 h^{-1} obtained by factorial design at C/N= 8.5 g/g allowed a 2,3-BDO production of 39.4 g/L, a yield of 0.43 g/g, and *levo:meso*-isomers selectivity of 1.9:1 with no acetoin accumulation in pulse-fed batch cultures. Fed-batch fermentation at constant feeding at 1.3 mL/h kept the 2,3-BDO yield above 0.40 g/g, commonly reported for pathogenic microbial producers. Sugarcane molasses and NH_4Cl replaced commercial glucose and yeast extract (YE) in batch cultures, respectively, resulting in a saving of approximately 75.2% of the culture medium, whose final price was 4.74 US$/$kg_{2,3\text{-}BDO}$. Bio-based 2,3-BDO production scale-up from glucose and brewer's YE was based on the constant oxygen transfer rate, which reproduced the microaerobic conditions for the absolute *levo*-2,3-BDO synthesis. About 0.9 g/L/h and 0.40 g/g of 2,3-BDO was produced in the batch culture at 70 L. The limiting factors of bio-based 2,3-BDO production identified from an initial technical-scientific prospection were experimentally controlled, thus contributing to the industrial bioprocess viability in the future.

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LIST OF SYMBOLS

%	Percentage
°C	Celsius degree
a	Interfacial gas-liquid area
C	Oxygen concentration
Cs	Saturated oxygen concentration
C/N	Carbon:Nitrogen ratio
D_i	Diameter of impeller
F	Feed rate
g, kg	Gram, kilogram
h	Hour
H	Height
H^+	Protons
J, kJ	Joule, kilojoule
k_L	Local mass transport coefficient
kLa	Volumetric oxygen transfer coefficient
L, mL	Liter, milliliter
M, mM	Molar, millimolar
m, cm, μm, nm	Meter, centimeter, micrometer, nanometer
N	Agitation rate
N_p	Impeller power number
P	Power
P_g/V	Gassed power/culture volume ratio
pH	Potential of Hydrogen
Q_{ar}	Aeration rate per medium volume
q_{Glu}	Glucose uptake rate
q_{O2}	Specific Oxygen Uptake Rate
q_p	Specific production rate
q_s	Specific substrate consumption
R^2	Determination coefficient
R^2_{adj}	Adjusted determination coefficient
rpm	Rotations per minute
S	Substrate
set	Preprogrammed
T	Temperature
t	Time
t	Tonne
US$	The United States dollar
v or V	Volume
vvm	Volume of air/volume of medium/minutes ratio
W	Watts
w	Weight
X	Cell biomass
$Y_{P/S}$	2,3-BDO yield from glucose
$Y_{P/X}$	Specific 2,3-BDO yield
$Y_{X/S}$	Cell biomass yield from glucose
α	Significance level

Δ	Variation
g	G-Force or relative centrifugal force
μ	Specific cell growth rate
π	Pi number

LIST OF ABBREVIATIONS

1,2-EDO	1,2-ethanediol
1,2-PDO	1,2-propanediol
1,3-PDO	1,3-propanediol
2,3-BDO	2,3-butanediol
ADP	Adenosine DiPhosphate
ALDS	α-Acetolactate decarboxylase
ALS	α-Acetolactate dehydrogenase
ANOVA	Analysis of Variance
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine TriPhosphate
BCAA	Branched-Chain Amino Acids
BDH	Butanediol dehydrogenase
C4-compounds	Compounds of four carbons
CAGR	Compound Annual Growth Rate
CCR	Carbon Catabolite Repression
CIP	Clean-in-place
CLAs	Colloidal Liquid Aphrons
CMBM	Chicken Meat and Bone Meal
CO	Carbon monoxide
Co.	Company
CO_2	Carbon dioxide
COVID-19	Coronavirus disease (2019)
CSL	Corn Steep Liquor
CV	Coefficient of Variation
DAR	Diacetyl reductase
DCCR	Design of Central Composite Rotational
DCW	Dry Cell Weight
df	Degrees of freedom
DO	Dissolved Oxygen
DoE	Design of Experiments
DSP	Separation and Purification
EPS	Exopolysaccharide
EtOH	Ethanol
FAN	Free Amino Nitrogen
FDH	Formate dehydrogenase
FOB	Free-on-board
GC	Glucose consumption
GDH	Glycerol DeHydrogenase
GRAS	Generally Recognized As Safe
His	Histidine
HPLC	High-Performance Liquid Chromatography
ITS	Impeller Tip Speed
JAP	Jerusalem Artichoke Pomace
Ltd	Limited

MEED	Multi-Effect-Evaporation-assisted Distillation
MEK	Methyl Ethyl Ketone
Met	Methionine
MS	Mean Square
MSP	Minimum Selling Price
MTBE	Methyl Tert-Butyl Ether
N_2	Nitrogen gas
NAD	Nicotinamide Adenine Dinucleotide
NAD^+	Nicotinamide Adenine Dinucleotide in the oxidized form
NADH	Nicotinamide Adenine Dinucleotide in the reduced form
NADP	Nicotinamide Adenine Dinucleotide Phosphate
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate in the oxidized form
NADPH	Nicotinamide Adenine Dinucleotide Phosphate in the reduced form
O_2	Oxygen gas
OD	Optical Density
OPEX	Operational expenditure
OTR	Oxygen Transfer Rate
OUR	Oxygen Uptake Rate
RID	Refractive Index Detector
RQ	Respiratory quotient
RTD	Resistance Temperature Detector
SC	Substrate Consumption
SDGs	Sustainable Development Goals
Ser	Serine
SIP	Steam-in-place
SMB	Simulated Moving Bed
SMB	Simulated-Moving-Bed
SS	Sum of Squares
SSF	Simultaneous Saccharification and Fermentation
S-SF	Solid-State Fermentation
TAC	Total Annual Cost
TKN	Trevor Kashey Nutrition
TPC	Total Phenolic Content
TSB	Tryptic Soy Broth
UNO	United Nations Organization
USA	The United States of American
VB2	Riboflavin
VHP	Very High Polarity
YE	Yeast Extract
YPD	Yeast extract, peptone and dextrose media

CHAPTER 1

1. Introduction

1.1 Motivation

Dependence on oil, increasing environmental concerns, and conflicts in countries with fossil energy sources have stimulated the replacement of synthetic and petrochemical goods with bioproducts obtained by fermentation and integrated biorefinery processes (CHANG, HWANG, *et al.*, 2017, RAJENDRAN, HAN, 2022, WANG, Qiang, ZHAN, 2019). Despite a one-quarter increase in total renewable energy consumption between 2010 and 2019, the final energy consumption share is still low, around 17.7%. This share may be even lower in the coming years due to the delay in the commitment of countries to realize the energy transition to greener economies as set out in the Paris Agreement in 2015 (UNO, 2022).

Recently, this delay has been intensified by the humanitarian crises of the COVID-19 pandemic and the war in Ukraine, responsible for affecting the reach of the 17 Sustainable Development Goals (SDGs) proposed by the United Nations Organization (UNO), mainly the 7th (Affordable and clean energy) and the 12th (Responsible consumption and production). By 2021 alone, there was a 6% increase in energy-related CO_2 emissions, with a forecast to reach 14% by 2030. Furthermore, dependence on natural resources has been increasing in recent years by more than 65% from 2000-2019 (UNO, 2022). Therefore, developing bioprocesses based mainly on circular economy principles is vital in making global energy production and consumer goods more sustainable, eco-friendly, and economical.

In this context, the bio-based 2,3-butanediol (2,3-BDO) production, a highly versatile chemical platform used by different industries such as pharmaceuticals, cosmetics, food, solvents, fuel, and energy, has been investigated (MAINA, PRABHU, *et al.*, 2022). The interest in 2,3-BDO is due to its economic relevance and new industrial applications. The 2,3-BDO market is estimated to reach around US$300 million by 2030, with a 3.5% CAGR (compound annual growth rate) in 2020-2030. The recent interest in

2,3-BDO-based fertilizers and COVID-19 pathogen-killing plastic films has further stimulated the bio-based 2,3-BDO production, contributing to the growth forecast for the assessed period (TRANSPARENCY MARKET RESEARCH, 2022).

Although the bio-based 2,3-BDO production has advantages over the chemical route, such as low energy requirement, no greenhouse gas emissions, high isomeric optical purity, and cost-effectiveness and cheapness, its application is still limited to lab scales (AMRAOUI, NARISETTY, *et al.*, 2021, PRIYA, LAL, 2019). The main production and operational factors responsible for a techno-economically viable bio-based 2,3-BDO production capable of replacing the current naphtha cracking process include: (a) high titer, yield, and volumetric productivity of 2,3-BDO (KOUTINAS, VLYSIDIS, *et al.*, 2014), (b) microbial producers with biosafety, low energy cost and good performance (GE, LI, *et al.*, 2016), (c) low-cost culture medium and easy downstream processing (YANG, Yuling, DENG, *et al.*, 2022), (d) and investments in efficient scale-up processes (HAIDER, QYYUM, *et al.*, 2018).

Developing a bioprocess that meets all the above requirements depends on a detailed study of its constituent steps: upstream, mainstream, and downstream. The upstream step corresponds to all operations performed before the fermentation process, such as the microbial producer selection (biosecurity and performance), the determination of optimal fermentation conditions (temperature, pH, oxygen supply), and the nutritional composition of the culture medium (carbon and nitrogen sources, mineral salts, vitamins, and other growth factors). The mainstream step corresponds to carbon bioconversion, whose efficiency is measured by fermentation parameters (titer, yield, and productivity). The most commonly used bioprocess operation modes are batch, fed-batch, and continuous cultures, where cellular metabolism development occurs. Finally, the downstream step corresponds to the unit operations used for bioproduct recovery, separation, and purification, whose complexity depends on the fermentation broth composition (KOUTINAS, YEPEZ, *et al.*, 2016, SATISH KUMAR, RAO, *et al.*, 2022, TINÔCO, BORSCHIVER, *et al.*, 2020, XIE, LI, *et al.*, 2022).

The culture medium composition and the bioproduct recovery are the most critical factors for the bioprocess economy, as they can represent a significant portion of the final production costs (AMRAOUI, PRABHU, *et al.*, 2022, LÓPEZ-GARZÓN,

STRAATHOF, 2014, XIE, LI, *et al.*, 2022). For bio-based 2,3-BDO production, several studies have investigated renewable energy sources to replace commercial carbon sources, mainly agro-industrial wastes (HAZEENA, SHURPALI, *et al.*, 2022). Recently it has been reported to use Jerusalem artichoke tubers (GUO, NI, *et al.*, 2022), corn straw (WANG, Yue, WU, *et al.*, 2022), whey (FERNÁNDEZ-GUTIÉRREZ, VEILLETTE, *et al.*, 2022), crude glycerol (TINÔCO, DE CASTRO, *et al.*, 2021), food waste (OHAIR, JIN, *et al.*, 2021), crude bakery waste (MAINA, SCHNEIDER, *et al.*, 2021), sugarcane bagasse (KIM, YOO, *et al.*, 2020), cassava starch (KHUNNONKWAO, JANTAMA, *et al.*, 2021), soybean hull (CORTIVO, MACHADO, *et al.*, 2019), and sugarcane molasses (PSAKI, MAINA, *et al.*, 2019). Traditional technologies have been improved in the downstream step, and new methods have been developed for efficient 2,3-BDO recovery (CUELLAR, STRAATHOF, 2020, XIE, LI, *et al.*, 2022). As indicated by the group's technological evolution analysis, solvent extraction, salting-out, sugaring-out, membrane separation, ion exchange, and distillation have been the leading technologies used for 2,3-BDO recovery (TINÔCO, BORSCHIVER, *et al.*, 2020).

Both critical economic factors are closely related to the 2,3-BDO microbial producer. While the residual biomass use depends on the cellular capacity to metabolize it, the 2,3-BDO recovery depends on the isomer ratio and the by-products formed, which are related to the cellular metabolic capacity and microbial genetic traits (YANG, Zhiliang, ZHANG, 2019). The leading 2,3-BDO producers belong to the *Enterobacter*, *Klebsiella*, and *Serratia* genera, classified as Risk Group 2 pathogens by the World Health Organization (WHO) (CELIŃSKA, GRAJEK, 2009). The highest 2,3-BDO production reported to date, about 150 g/L, was achieved by a *K. pneumoniae* strain from glucose in fed-batch fermentation, whose yield and volumetric productivity were equal to 0.44 g/g and 3.95 g/L/h, respectively (MA, WANG, *et al.*, 2009). Although highly productive, these microorganisms pose a potential health risk and can increase the 2,3-BDO production costs on a large scale (CELIŃSKA, GRAJEK, 2009, JURCHESCU, HAMANN, *et al.*, 2013). Therefore, the GRAS (generally recognized as safe) strain application is desirable for safe and economical industrial scale-up (XIE, LI, *et al.*, 2022).

Bacteria of the *Paenibacillus* and *Bacillus* genera are the most common GRAS microorganism examples used for 2,3-BDO production (PETROV, PETROVA, 2021).

The best results achieved so far were 111 g/L (volumetric productivity of 2.05 g/L/h) by a wild-type *P. polymyxa* strain from sucrose, and 144.7 g/L (volumetric productivity of 1.14 g/L/h) by a wild-type *B. licheniformis* strain from glucose, both in fed-batch fermentation. A *levo*-isomer selectivity of 98% was achieved by this *P. polymyxa* strain, while a high 2,3-BDO yield of about 0.40 g/g was obtained by this *B. licheniformis* strain (HÄßLER, SCHIEDER, *et al.*, 2012, JURCHESCU, HAMANN, *et al.*, 2013).

Genetic improvement and the discovery of new 2,3-BDO microbial producers have been used to overcome the limited cell performance and thus make the bioprocess industrially competitive (JI, HUANG, *et al.*, 2011, LEE, BAE, *et al.*, 2022, MITSUI, YAMADA, *et al.*, 2022, XIE, LI, *et al.*, 2022, YANG, Zhiliang, ZHANG, 2019). Recently, a bacterium phylogenetically related to *P. polymyxa* for the 2,3-BDO production was isolated. *P. peoriae* NRRL BD-62 is a GRAS microorganism capable of producing *levo*- and *meso*-isomers from different carbon sources, including glucose, fructose, sucrose, xylose, and glycerol (data presented in Chapter 7). To date, no previous studies have reported the 2,3-BDO production by a wild-type *P. peoriae* strain. Therefore, a detailed investigation of the 2,3-BDO bioprocess steps is required to assess the *P. peoriae* NRRL BD-62 production capacity and its potential large-scale application.

This doctoral thesis aimed to develop the upstream and mainstream steps of the 2,3-BDO bioprocess by *P. peoriae* NRRL BD-62 to test the hypothesis of a safe, efficient, and potentially applicable large-scale production using a wild-type GRAS strain instead of a traditional microbial producer strain. Three main criteria were used to evaluate the proposed bioprocess performance, in this order, 2,3-BDO yield, isomeric optical purity (selectivity), and low-cost culture medium with low oxygen supply. The 2,3-BDO yield was used because it is a relative production measure, which allowed more reliable comparisons between the data obtained here and those reported in previous studies. Selectivity is related to the downstream step costs, while culture medium and oxygen supply are critical economic and production factors for industrial 2,3-BDO production. The results provided relevant information on the *P. peoriae* NRRL BD-62 metabolism and the entire biochemical engineering involved in the bio-based 2,3-BDO production, representing the main scientific contributions generated by this study. Therefore, a better

understanding of the critical economic and production factors related to the large-scale 2,3-BDO bioprocess was achieved here.

1.2 Objectives

1.2.1 General

Develop the upstream and mainstream steps of the bio-based 2,3-BDO process by *P. peoriae* NRRL BD-62 for potential large-scale application, using 2,3-BDO yield, selectivity (isomeric optical purity), and reduced culture medium costs at low oxygen supply as performance criteria.

1.2.2 Specifics

- Perform a techno-scientific prospection of the bio-based 2,3-BDO process and then construct a technology roadmap to represent the bioprocess maturity level concerning the upstream, mainstream, and downstream steps and the leading technology players;
- Perform a state-of-the-art of the main *Paenibacillus* strains and verify the novelty of *P. peoriae* NRRL BD-62 as a microbial 2,3-BDO producer;
- Investigate the 2,3-BDO production capacity of *P. peoriae* NRRL BD 62;
- Optimize fermentation conditions (temperature, pH, and oxygen supply) for high-yield and high-selectivity 2,3-BDO production by *P. peoriae* NRRL BD 62 and validate them in batch and fed-batch cultures;
- Investigate alternative nitrogen sources to reduce the culture medium costs: organic sources, inorganic sources, amino acids supplementation, C/N ratio, and the relationship between nitrogen and pH;
- Investigate different carbon sources: commercial and residual types;
- Perform a preliminary economic assessment of the culture medium costs using the selected nitrogen and carbon sources;
- Validate the 2,3-BDO production by *P. peoriae* NRRL BD 62 using selected nitrogen and carbon sources in batch and fed-batch cultures;

- Investigate different feeding strategies for 2,3-BDO production by *P. peoriae* NRRL BD 62 in fed-batch fermentation: pulsed, constant, and exponential profiles;
- Investigate the scale-up strategy based on maintaining a constant oxygen transfer rate for 2,3-BDO production by *P. peoriae* NRRL BD 62 in a 200 L pilot bioreactor.

1.3 Thesis structure

This doctoral thesis was structured in 10 chapters to achieve the proposed objectives. The information sequence was based on the standard bioprocess development, following the definitions previously presented for the upstream and mainstream steps.

In Chapter 1, a general introduction was presented, containing the motivation and the general and specific research objectives, the thesis structure, and scientific publications obtained from this study.

In Chapter 2, a techno-scientific prospection was performed to determine the technological maturity level of the bio-based 2,3-BDO process, highlighting the upstream, mainstream, and downstream steps. Technology roadmaps contained information on the leading technology players, trends and prospects, and the limiting factors for industrial bio-based 2,3-BDO production. The content of this chapter was published in Biofuels, Bioproducts, and Biorefining (Biofpr) by Wiley (TINÔCO, BORSCHIVER, *et al.*, 2020).

In Chapter 3, a state-of-the-art study was performed to determine the novelty of *P. peoriae* NRRL BD-62 as a microbial 2,3-BDO producer and its potential application on a large scale. Information on cell metabolism, fermentation conditions, process improvements, and recovery technologies related to the bio-based 2,3-BDO process development were obtained for *P. polymyxa*, a strain phylogenetically related to *P. peoriae*, and used to define the hypothesis and research objectives. The content of this chapter was published in ChemBioEng Reviews by Wiley (TINÔCO, PATERAKI, *et al.*, 2021).

In Chapter 4, the fermentation conditions were optimized using a design of experiments (DoE). The optimal temperature, pH, and oxygen supply values considering the volumetric oxygen transfer coefficient (kLa) by agitation and aeration adjustment were determined for *P peoriae* NRRL BD-62. The results found were validated in fed-batch fermentation and compared with previous studies. The content of this chapter is in the submission process.

In Chapter 5, different nitrogen sources were investigated to replace yeast extract (YE) and reduce the culture medium costs. Initially, the C/N ratio was optimized using YE, which was then replaced with organic sources (YE brewery, YE Senai, corn steep liquor - CSL, and urea) and inorganic sources (sodium nitrate, ammonium chloride, ammonium sulfate, and ammonium sodium phosphate dibasic). The amino acids arginine (Arg) and asparagine (Asn) were used as nutritional supplements, and the relationship between nitrogen and pH on 2,3-BDO metabolism was evaluated at low oxygen supply. The results found were validated in batch fermentation. An initial attempt at 2,3-BDO production scale-up was carried out in fed-batch fermentation. The costs of the new culture medium were determined by considering the global market prices of the inputs used for bio-based 2,3-BDO production. The content of this chapter is in the submission process.

In Chapter 6, three substrate feeding strategies in fed-batch fermentation were investigated: pulsed, constant, and exponential profiles. The fermentation conditions optimized in Chapter 4 and the inorganic nitrogen source selected in Chapter 5 were used for high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62. The relationship between the feeding profile and the nitrogen source nature was evaluated under different conditions, which included nutritional limitation by C/N ratio, and the specific cell growth rate used to control continuous fermentation feeding. The content of this chapter is in the submission process.

In Chapter 7, different commercial carbon sources were investigated for the 2,3-BDO production by *P. peoriae* NRRL BD-62. From the results, sugarcane molasses was selected as a residual and low-cost feedstock to replace commercial glucose. Two nitrogen sources (organic and inorganic) preliminarily selected in Chapter 5 to replace YE were used in a molasses-based medium to reduce culture medium costs further. The

costs of the new culture medium were determined considering a large-scale bio-based 2,3-BDO production. The cheaper culture medium was validated in fed-batch fermentation using the most efficient feeding strategy verified in Chapter 6. The content of this chapter is in the submission process.

In Chapter 8, the 2,3-BDO production scale-up by *P. peoriae* NRRL BD-62 was investigated. The strategy was based on maintaining a constant oxygen transfer rate by controlling kLa. Through this strategy, the geometric similarity criterion and the dimensional analysis were partially met, allowing the reproduction of optimal fermentation conditions in a 200 L bioreactor. The fermentation mode effects on the bio-based 2,3-BDO production on a large scale were evaluated, and the critical operational and fermentation parameters were determined. The content of this chapter is in the submission process.

In Chapter 9, the principal results and conclusions verified in each chapter were summarized. The main scientific contributions achieved were punctually highlighted.

In Chapter 10, an overall conclusion on the bio-based 2,3-BDO process development by *P. peoriae* NRRL BD-62 was presented. This conclusion was drawn from the results of the previous chapters to evaluate the hypothesis initially proposed in this doctoral thesis.

Finally, the references in Chapters 1, 9, and 10 (Introduction, Scientific contributions, and Conclusion) were presented following the Associação Brasileira de Normas Técnicas (ABNT) rules. The other references were presented at the end of each chapter, following the ABNT rules.

1.4 Scientific publications

1.4.1 Scientific articles

TINÔCO, D., BORSCHIVER, S., COUTINHO, P. L., *et al.* "Technological development of the bio-based 2,3-butanediol process", **Biofuels, Bioproducts and Biorefining**, v. 2, p. 1–20, 2020. DOI: 10.1002/bbb.2173.

TINÔCO, D., PATERAKI, C., KOUTINAS, A. A., *et al.* "Bioprocess Development for 2,3-Butanediol Production by Paenibacillus Strains", **ChemBioEng Reviews**, n. 1, p. 1–

20, 2021. DOI: 10.1002/cben.202000022.

1.4.2 Full papers published in conference proceedings

TINÔCO, D., COUTINHO, P.L., FREIRE, D.M.G. Capacidade de produção de 2,3-butanodiol por *Paenibacillus peoriae* NRRL BD-62. In: ANAIS DO 23° CONGRESSO BRASILEIRO DE ENGENHARIA QUíMICA, 2021, Gramado. Anais eletrônicos... Campinas, Galoá, 2021. Disponível em: <https://proceedings.science/cobeq/cobeq-2021/papers/capacidade-de-producao-de-2-3-butanodiol-por-paenibacillus-peoriae-bd62> Acesso em: 23 set. 2022.

This study was recently invited to be published in Brazilian Journal of Chemical Engineering.

1.4.3 Simple abstracts published in conference proceedings

TINÔCO, D., FREIRE, D.M.G. Influence of pH and oxygen supply on 2,3-butanediol production by *Paenibacillus peoriae* NRRL BD-62. In: ANAIS DO XXIII SINAFERM & XIV SHEB & ENZITEC 2022, 2022, Armação dos Búzios. Anais eletrônicos... Campinas, Galoá, 2022. Disponível em: <https://proceedings.science/sinaferm/sinaferm-sheb-enzitec-2022/papers/influence-of-ph-and-oxygen-supply-on-2-3-butanediol-production-by-paenibacillus-peoriae-bd-62> Acesso em: 23 set. 2022.

1.4.4 Publications related to the thesis

TINÔCO, D., COUTINHO, P.L., FREIRE, D.M.G. Aproveitamento de glicerol bruto para produção microbiana de 2,3-butanodiol. In: 5º CIBIO - CONGRESSO INTERNACIONAL DE BIOMASSA - ONLINE - Online, 2020. Disponível em: <https://www.doity.com.br/anais/cibio2020/trabalho/171295>. Acesso em: 23/09/2022

TINÔCO, D., DE CASTRO, A. M., SELDIN, L., *et al.* "Production of (2R,3R)-butanediol by *Paenibacillus polymyxa* PM 3605 from crude glycerol supplemented with sugarcane molasses", **Process Biochemistry**, v. 106, n. March, p. 88–95, jul. 2021. DOI: 10.1016/j.procbio.2021.03.030.

TINÔCO, D., FREIRE, D.M.G. Bio-based 2,3-butanediol production from crude glycerol by *Paenibacillus polymyxa* PM 3605 in batch bioreactor. In: Anais do V Simpósio Internacional de Microbiologia e Biotecnologia. Anais...Viçosa(MG) Online, 2021. Disponível em: <https//www.even3.com.br/anais/simb/414272-BIO-BASED-23-BUTANEDIOL-PRODUCTION-FROM-CRUDE-GLYCEROL-BY-P-POLYMYXA-PM-3605-IN-BATCH-BIOREACTOR>. Acesso em: 23/09/2022

TINÔCO, D., FREIRE, D.M.G. 2,3-Butanediol production by *Paenibacillus polymyxa* PM 3605 from crude glycerol in a surface aeration bioreactor. In: Anais do XXIII SINAFERM & XIV SHEB & ENZITEC 2022, 2022, Armação dos Búzios. Anais eletrônicos... Campinas, Galoá, 2022. Disponível em:

<https://proceedings.science/sinaferm/sinaferm-sheb-enzitec-2022/papers/2-3-butanediol-production-by-paenibacillus-polymyxa-pm-3605-from-crude-glycerol-in-a-surface-aeration-bioreactor> Acesso em: 23 set. 2022.

1.4.5 In the submission process

Paper 1: Optimization of culture conditions as a metabolic control strategy for a high-yield and high-selectivity 2,3-butanediol production by a newly *Paenibacillus peoriae* NRRL BD-62

Paper 2: Effects of the nitrogen source and pH on the 2,3-butanediol metabolism and culture medium costs by *Paenibacillus peoriae* NRRL BD-62 under microaerobic conditions

Paper 3: Relationship between feeding strategies and different nitrogen sources in the high-yield 2,3-butanediol production by *Paenibacillus peoriae* NRRL BD-62 in microaerobic fed-batch fermentation

Paper 4: Production of optically pure (R,R)-2,3-butanediol from molasses using a low-cost salt medium by *Paenibacillus peoriae* NRRL BD-62

Paper 5: Scale-up of 2,3-butanediol production by *Paenibacillus peoriae* NRRL BD-62 using constant oxygen transfer rate-based strategy

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CHAPTER 2

Contextualization

In this chapter, the technology maturity level of the bio-based 2,3-BDO process was investigated by collecting information in scientific articles, technical patents, market reports, and other sources, such as company websites and news, to build technology roadmaps used to visualize the gaps and opportunities for future bioprocess performance. The leading players and limiting factors for large-scale implementation were presented. Furthermore, a technological evolution analysis was performed, highlighting the most relevant aspects of the bioprocess's upstream, mainstream, and downstream steps.

Therefore, the specific objectives of this chapter were:

- Identify the maturity and technological implementation level of the bio-based 2,3-BDO process at an industrial scale;
- Identify the main limiting factors in the industrial bio-based 2,3-BDO production, considering the bioprocess's upstream, mainstream, and downstream steps;
- Identify the primary technology holders (clusters), technological trajectory, applications, and market economics of the bio-based 2,3-BDO process;

Relevance

Through the investigation carried out in this chapter, it was possible to understand:

- The main bio-based 2,3-BDO process production factors such as the microorganism, raw material and methods used, culture medium nutritional supplementation, recovery technologies, isomeric optical purity, and industrial applications;
- The leading companies and academic research institutions that have carried out studies on the bio-based 2,3-butanediol process;
- The scientific novelty of the bio-based 2,3-BDO process technology roadmap and its application as a tool for making decisions for future research planning and development.

TECHNOLOGICAL DEVELOPMENT OF BIO-BASED 2,3-BUTANEDIOL PROCESS

Abstract

Biotechnological production of 2,3-butanediol (2,3-BDO) has economic and environmental advantages over the chemical route, although its large-scale implementation has not yet been consolidated. To establish the technological maturity of the bio-based 2,3-BDO process and the limiting factors of its industrial processing, a technology roadmap was built from patents and scientific articles, in which four drivers were identified: Production Process, Recovery Process, Product, and Application. Production Process driver was reported in 53.25% of documents, marked by studies on substrate, engineered microorganisms, efficient production methods, and nutritional supplementation. Recovery Process driver was investigated in 23.5% of studies, with conventional distillation and solvent extraction being the main technologies performed on a large scale. Product and Application drivers accounted for 9.25% and 14% of studies, respectively, highlighting the market segments: chemical intermediates, plastics, food additives, and cosmetics. Few companies like LanzaTech and GS Caltex Corporation have stood out at the current stage, while academic centers like the Yancheng Institute of Technology have mastered the bioprocess knowledge in the long term, showing that the technology is still concentrated in the laboratory environment. Therefore, this study can help to understand and establish the necessary strategies to consolidate the industrial production of bio-based 2,3-BDO in the future.

Keywords: Bio-based 2,3-butanediol, technology roadmap, technological maturity, industrial production, large-scale process.

1. Introduction

2,3-Butanediol (2,3-BDO) is a versatile platform chemical used in the manufacture of drugs, cosmetics, food additives, fuels, and solvents (JI, HUANG, *et al.*, 2011). Its synthesis can occur by the chemical and biochemical vias. The biological route provides environmental and economic advantages to the process, due to the use of low-cost renewable carbon sources, reduction of the greenhouse gas emissions, and selective production of 2,3-BDO isomers (PRIYA, LAL, 2019) (Fig. 2.1).

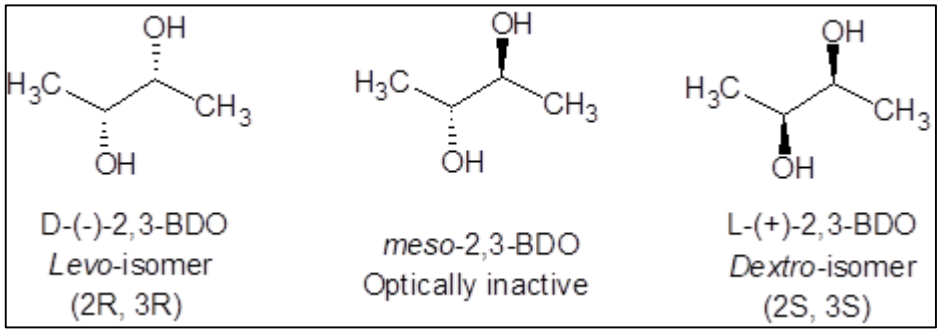


Figure 2.1: Isomeric forms of 2,3-BDO.

Several microorganisms can produce 2,3-BDO, including the GRAS (generally recognized as safe) bacteria such as *Bacillus* and *Paenibacillu*s, the risk class 2 bacteria such as *Klebsiella*, *Enterobacter*, and *Serratia*, (JI, HUANG, *et al.*, 2011) and some engineered yeasts such as *Saccharomyces cerevisiae* (LEE, SEO, 2019). The best 2,3-BDO titers were achieved using metabolically engineered strains such as *S. cerevisiae* (178 g/L) (LEE, SEO, 2019), *E. cloacae* (152 g/L) (LI, Lixiang, LI, *et al.*, 2015), and *K. oxytoca* (130 g/L) (CHO, KIM, WOO, KIM, *et al.*, 2015), and highly productive wild strains such as *K. pneumonia* (150 g/L) (MA, Cuiqing, WANG, *et al.*, 2009), and *P. polymyxa* (111 g/L) (HÄßLER, SCHIEDER, *et al.*, 2012).

Despite these high 2,3-BDO titers, the large-scale implementation of bioprocess is not yet consolidated. Some limiting factors for the scale-up of bio-based 2,3-BDO are: safety process, isomer purity, raw material costs, and downstream step (recovery processes) (JI, HUANG, *et al.*, 2011, YIN, LI, *et al.*, 2015). The main 2,3-BDO producers are the risk class 2 bacteria. Industrially, bioprocess safety requires stricter control using these strains, which can increase the operating costs. Alternatively, safe microorganisms can be used. However, a previous cell improvement step must be carried out to obtain

highly productive microorganisms. Genetic modifications and metabolic flow control have been developed to ensure the strain improvement process (YIN, LI, *et al.*, 2015). Furthermore, a 2,3-BDO stereospecific mixture is normally produced by biological route, which requires efficient purification steps. Each 2,3-BDO isomer has a characteristic chemical property and, consequently, a specific industrial application, including cosmetics, drugs, polymers, fuels, and energy (BIAŁKOWSKA, 2016). In particular, *levo*-2,3-BDO has a low freezing point (-60 °C) and a high capacity to interact with water through hydrogen bonds, which gives it antifreeze properties (CELIŃSKA, GRAJEK, 2009). Hence, it can be applied in coolant formulations for combustion engines. Carbon source costs can also limit the scale-up of the bio-based 2,3-BDO process, since the substrate is responsible for up to 50% of production costs (HAZEENA, SINDHU, *et al.*, 2020). The use of renewable feedstocks from agro-industrial processes is an economy circular strategy employed to reduce substrate costs and make the bioprocess eco-friendlier (PRIYA, LAL, 2019). 2,3-BDO recovery is another very expensive step, responsible for up to 50% of the total bioprocess costs (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015). Bio-based 2,3-BDO production results from cell metabolism in which carbon is directed to the 2,3-BDO pathway and parallel pathways, such as ethanol and organic acids (lactic, acetic, succinic, and formic). Therefore, the fermentation broth complexity can significantly affect the downstream step, increasing the industrial production costs of bio-based 2,3-BDO (HARVIANTO, HAIDER, *et al.*, 2018).

Around US$ 220 million is planned for the 2,3-BDO market in 2030 (TRANSPARENCY MARKET RESEARCH, 2022). Therefore, the industrial production of bio-based 2,3-BDO is expected to consolidate in the coming years. The market trends of a process can be verified by technology roadmaps, resulting from patent and scientific informations (HAKIZIMANA, MATABARO, *et al.*, 2020). This management tool can establish the technological maturity of a process and its development over time, contributing to multidimensional views used in decision making (VASCONCELOS LOUREIRO, BORSCHIVER, *et al.*, 2010). Technology roadmap has been widely used by companies, industries, and public and private research centers to prospect information and manage the results found. Through it, action plans, resource allocation, management support (PHAAL, FARRUKH, *et al.*, 2004), and identification of new business opportunities (BORSCHIVER, S., SILVA, 2016) can be carried out.

This study aimed to establish the technological maturity of the 2,3-BDO bio-based process and the limiting factors of its industrial processing, through the construction of a technology roadmap, based on four drivers: Production Process, Recovery Process, Product, and Application. The limited number of companies carrying out industrial production of bio-based 2,3-BDO at the current stage and the dominance of research centers in the long term demonstrated that this biotechnology is still concentrated in the laboratory environment, depending on studies related to the substrate, microorganisms, efficient fermentation methods, and downstream step, to become economically viable on a large scale. Therefore, this study can help to understand and establish the necessary strategies to consolidate the industrial production of bio-based 2,3-BDO in the future.

2. Materials and methods

2.1 Technological prospecting methodology

The methodology used in this study was based on the grouping of scientific and technological documents prospected from keywords correlated by Boolean operators (and, or, not) and wildcards (*, +, ( ), -, ~, " ") (BORSCHIVER, Suzana, VASCONCELOS, *et al.*, 2019). The following steps were carried out: choosing the database, defining the keywords, and determining the most suitable correlation between the keywords.

2.1.1 Patent prospecting

Patents were selected from the Patentscope database, which belongs to the World Intellectual Property Organization - WIPO (www.wipo.int). Patentscope's search service covers more than 76 million documents, including granted and applied patents, that are part of the collections of more than 192 Member-States connected to WIPO. The technological search based on the bibliographic data of the documents and using the correlation (butanediol and (ferment* or microb*)) resulted in 245 patents. Among this amount, the 30 most relevant patents on bio-based 2,3-BDO were selected. These patents corresponded to the period from 2008 to 2019.

2.1.2 Scientific articles prospecting

Scientific articles were selected from the sixteen databases of Web of Science (www.webofknowledge.com), a platform for searching bibliographic data for indexed scientific citations, covering more than 34000 journals from different knowledge areas. Prospecting was carried out from the titles of scientific articles and using the correlation (butanediol and (ferment* or microb*)). A total of 137 documents was founded, of which the 30 most relevant scientific articles were selected, from 2013 to 2019.

2.2 Classification of the selected documents

2.2.1 Macro, Meso and Micro analysis

The selected documents were classified into three levels of information details: Macro, *Meso*, and Micro analyzes, defined according to our expertise and used to understand the bio-based 2,3-BDO process concerning the stages of its conception, conduction, and development. In Macro analysis, the documents were grouped according to the main patent filing countries and the origin country of the first authors of scientific articles. Patents have also been divided into granted patents and applied patents. In the *Meso* analysis, the documents were grouped into four main categories (drivers), according to the focus given in the study: Production Process, Recovery Process, Product, and Application. In Micro analysis, some subcategories were defined to better specify the bioprocess aspects reported in the documents. Production Process driver was divided into: Microorganisms, Substrate, Nutritional Supplementation, and Methods. Recovery Process driver was divided into: Distillation, Membrane Separation, Solvent Extraction, Ion Exchange, Salting-out, and Sugaring-out. Product driver (isomer), and Application driver (market segmentation) did not have subcategories (Table 2.1).

Table 2.1: Classification of documents based on the *Meso* and Micro Analysis

***Meso* Analysis**	**Micro Analysis**	**Description**
Production Process	Microorganism	Documents that report the application of genetically modified or recently isolated microorganisms with high potential for the bio-based 2,3-BDO production.
	Substrate	Documents that report the use of agro-industrial, urban, and glycerol residues for the bio-based 2,3-BDO production.
	Nutritional Supplementation	Documents that report the use of nutritional supplements, such as amino acids, vitamins, and minerals to increase the bio-based 2,3-BDO production.
	Method	Documents that report the application of methodologies to improve the bio-based 2,3-BDO production, such as saccharification and fermentation approaches, integration processes, operation modes, and optimization of cultivation conditions.
Recovery Process	Distillation	Documents that report the application of distillation and its improvements for the bio-based 2,3-BDO recovery.
	Membrane Separation	Documents that report the application of membranes for the bio-based 2,3-BDO recovery, such as pervaporation, nanofiltration, and reverse osmosis.
	Solvent Extraction	Documents that report the application of solvents for the bio-based 2,3-BDO recovery, such as liquid-liquid, reactive, and green solvent extractions.
	Ion Exchange	Documents that report the application of ion exchange columns for the bio-based 2,3-BDO recovery.
	Salting-out	Documents that report the application of solvents with the addition of salt for the bio-based 2,3-BDO recovery.
	Sugaring-out	Documents that report the application of solvents with the addition of sugar for the bio-based 2,3-BDO recovery.
Product	-	Documents focused on the 2,3-BDO purity concerning its isomers.
Application	-	Documents focused on new applications of 2,3-BDO isomers and its derivatives.

2.3 Technology roadmap

Technology roadmap for the bio-based 2,3-BDO process was built from the 60 selected documents (patents and scientific articles) consisting of the vertical and horizontal axes. The drivers defined in the *Meso* and Micro analyzes were arranged on the vertical axis, while the horizontal axis consisted of four periods: current stage, short term, medium term, and long term. The methodology developed by the NEITEC research group (www.neitec.com) - Center for Industrial Technological Studies - EQ/UFRJ was applied due to the difficulties in accurately determining the time corresponding to each period in a technology roadmap (BORSCHIVER, S., SILVA, 2016). According to this methodology, the current stage was defined based on information from the companies' websites, which have already carried out this bioprocess on a large scale, while the other periods were based on studies reported by patents and scientific articles. The patent classification was used to differentiate the knowledge applied in the industrial tests (granted patent – short term) from that still to be approved for large scale implementation (applied patent – medium term). Scientific articles have reported the experimental knowledge obtained in the laboratory environment, which should be used in future industrial applications to improve the scaling-up of the biotechnology (long term). Players were identified and distributed over time, according to the development stage of their activities. They were represented by companies and research centers (universities, private and public organizations). Player clusters have also been verified and used to understand the technology maturity of bio-based 2,3-BDO on an industrial scale. Finally, a technological trajectory analysis was carried out to identify the players who have acted in the different development stages of the bio-based 2,3-BDO process aiming to make it economically viable.

3. Results and discussion

3.1 Macro analysis

The selected patents were classified into granted patents (53%) and applied patents (47%). The similar number of these documents demonstrated the advance of the bio-based 2,3-BDO technology in industrial intellectual property, since they show studies already implemented (granted patents) or with real possibility of implementation (applied

patents) on a commercial scale. The main countries holding the knowledge ensured by patents was China, which received the largest number of patent registrations (67%), followed by the European Patent Office (10%), and the Republic of Korea (10%). An Asian supremacy was identified in the bio-based 2,3-BDO process, in which China, the Republic of Korea, and Japan together accounted for about 80% of the selected patents (Fig. 2.2).

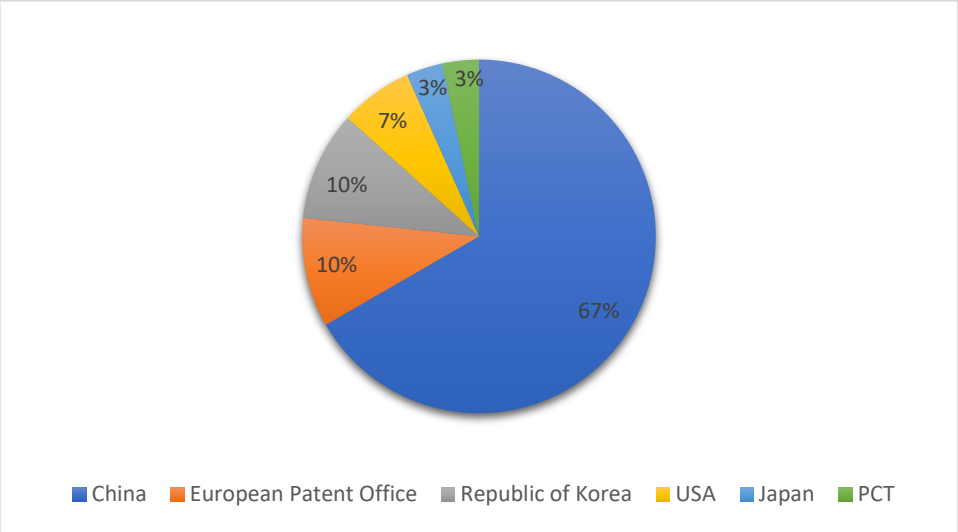


Figure 2.2: Patents on bio-based 2,3-BDO by countries and offices.

Notably, Asian countries are recognized as a reference in the field of biotechnology. This reality can explain the Asian supremacy also observed in 77% of scientific articles. About 31% of the studies were conducted by research institutions and companies located in China, 23% in the Republic of Korea, 14% in India, and 9% in Japan, Pakistan, and Taiwan, together (Fig. 2.3). As the scientific articles show the prospects for a long-term study, Asian countries must remain the protagonists in the development of the bio-based 2,3-BDO process, aiming to make it productively and economically viable on a large scale in the coming years.

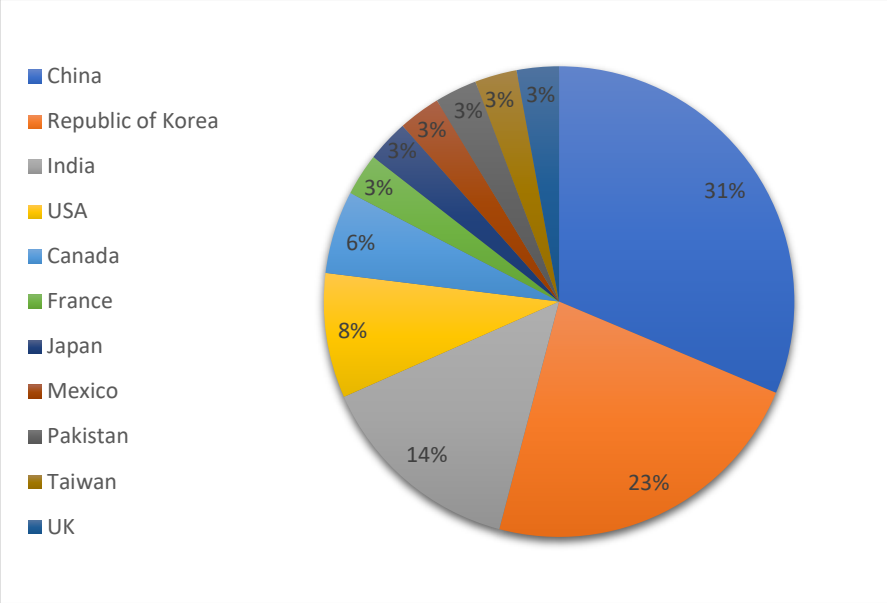


Figure 2.3: Scientific articles on bio-based 2,3-BDO by countries.

3.2 *Meso* analysis

The development of the bio-based 2,3-BDO process showed a change in driver focus over time, marked by 50% of studies on 2,3-BDO Applications in the current stage, 71% and 59% on Production Process in the short and medium terms, respectively, and 30% on Recovery Process in the long term (Fig. 2.4). The great focus on the Production Process, in approximately 53.25% of selected documents, was due to the interest in making the 2,3-BDO production sustainable and commercially viable. Traditionally, 2,3-BDO is produced by chemical catalysis of cracked gases from non-renewable oils at 800-900 °C, which requires a large amount of energy. In the naphtha cracking process, an immense amount of greenhouse gases is generated and, therefore, it is considered a non-eco-friendly process (PRIYA, LAL, 2019). Although the chemical route is the conventional via for the 2,3-BDO production, it is expensive and quite complex, being responsible for the generation of a racemic mixture of the three 2,3-BDO isomers, whose purification is costly (WOO, JONG, *et al.*, 2019). In turn, the biological route requires mild operating conditions, such as low temperatures and low pressures, and it is, therefore, energetically more interesting. Furthermore, 2,3-BDO can be produced with high optical purity from low-cost raw materials and high environmental availability (CELIŃSKA, GRAJEK, 2009). The use of wild and engineered microorganisms highly selective to one of the isomers favors certain industrial applications, since the formation

of a specific 2,3-BDO isomer is prioritized, which also contributes to the cost reduction of the downstream step (JI, HUANG, *et al.*, 2011). Biological route is eco-friendly and sustainable, with no greenhouse gas emissions (PRIYA, LAL, 2019).

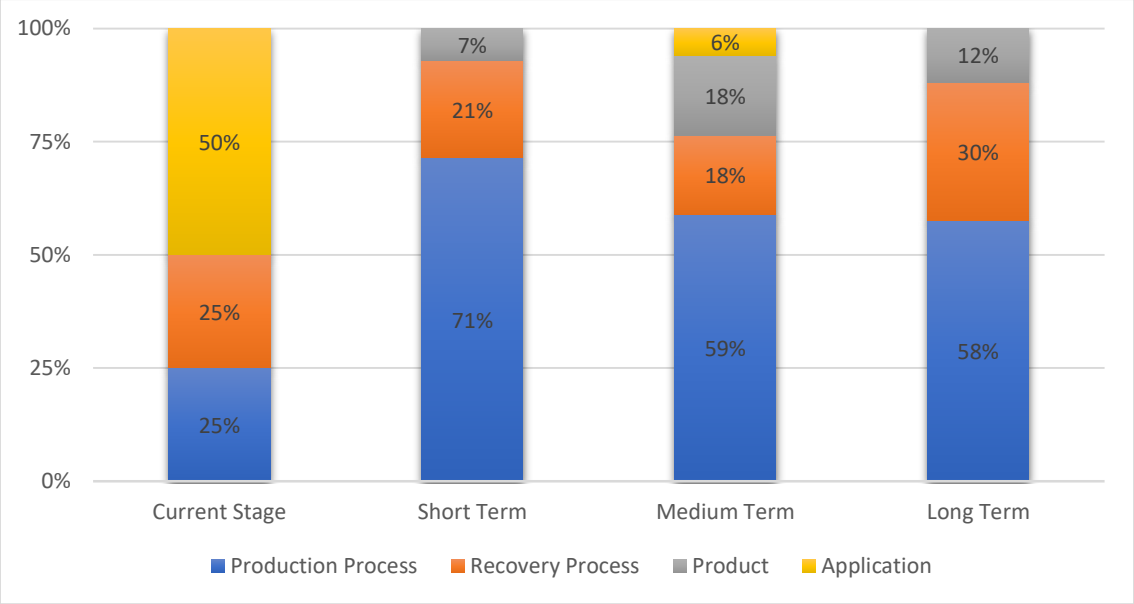


Figure 2.4: *Meso* analysis of the technological evolution of the bio-based 2,3-BDO process.

Recovery Process was the second most investigated driver, averaging 23.5% of the evaluated documents. The interest in 2,3-BDO recovery technologies was due to their costs, which can reach up to 50% of the total costs of the bioprocess (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015). Basically, the fermentation broth consists of 2,3-BDO, insoluble particles (proteins), soluble particles (inorganic salts: Na^+, K^+, Ca^{2+}, and Mg^{2+}, ethanol, and organic acids: acetic, succinic, lactic, and formic), and a large amount of water (HARVIANTO, HAIDER, *et al.*, 2018, WOO, JONG, *et al.*, 2019). Although there is no formation of azeotropes with water, which allows easy 2,3-BDO recovery by conventional distillation, the low 2,3-BDO titer around 9-10% (w/w), and its high solubility with water and other components make its purification more expensive (HARVIANTO, HAIDER, *et al.*, 2018). Furthermore, the high volume of wastewater generated from this bioprocess has a considerable cost that must be minimized (WOO, JONG, *et al.*, 2019). Hence, different recovery methods have been investigated, including the combination of technologies to reduce energy consumption and increase purification efficiency. However, the economic challenges (capital costs) and the low market value of

2,3-BDO have limited the scale-up of recovery processes (WOO, JONG, *et al.*, 2019). So far, only GS Caltex and LanzaTech have investigated promising 2,3-BDO recovery technologies at pilot and commercial scales, respectively. The GC Caltex process is divided into two stages: centrifugation/filtration (stage 1), and electrodialysis/ion exchange/evaporation/distillation (stage 2), leading to a purification of up to 99.5% of bio-based 2,3-BDO. Moreover, discoloration and deodorization processes can be used according to the final application of 2,3-BDO (WOO, JONG, *et al.*, 2019). The LanzaTech process is based on the simulated moving bed (SMB), a special type of large-scale chromatographic separation (HARVIANTO, HAIDER, *et al.*, 2018). Regardless of the technology used, different problems and limitations can be observed and, therefore, the optimization of the Recovery Process must be performed.

Product driver was related to the purity of the 2,3-BDO isomer, being investigated on average in 9.25% of the analyzed studies. The purity of 2,3-BDO is a determining factor, capable of influencing the Recovery Process and Application drivers, and, consequently, the bioprocess economy. Notably, a mixture of 2,3-BDO stereoisomers is produced biologically (JI, HUANG, *et al.*, 2011). Few microorganisms can selectively produce one of the isomeric forms. Therefore, it is necessary to design strains capable of synthesizing the optically pure 2,3-BDO-isomer (ZENG, SABRA, 2011). The patent granted to the Yancheng Institute of Technology in 2015 claimed intellectual property over a genetic modification carried out on a *P. polymyxa* strain, naturally capable of producing up to 98% of *levo*-2,3-BDO (NAKASHIMADA, MARWOTO, *et al.*, 2000). The gene of the diacetyl reductase, an enzyme responsible for producing *meso*-2,3-BDO, was eliminated from the cell metabolism by the homologous recombination method. As a result, the purity of *levo*-2,3-BDO was enhanced to 99.99%. (JIAN, HONG, *et al.*, 2014) The yeast *Pichia pastoris* was also designed for the *levo*-2,3-BDO production. Three genes related to the key-enzymes of the 2,3-BDO metabolism: α-acetolactate dehydrogenase (ALS), α-acetolactate decarboxylase (ALDS), and butanediol dehydrogenase (BDH), were integrated into the *P. pastoris* genome. As a result, the purity of *levo*-2,3-BDO reached a value of 99% (YANG, Zhiliang, ZHANG, 2018).

Application driver was reported on average in 14% of the studies evaluated, being associated with the following consolidated segments of the global 2,3-BDO market:

intermediate chemicals, plastics, food additives, and cosmetics (TRANSPARENCY MARKET RESEARCH, 2022). Bio-based 2,3-DO has also been used as an antifreeze agent, agricultural pesticide, pharmaceutical component, synthetic rubber precursor, and fuel additive (especially as a high-quality aviation fuel precursor) (BIAŁKOWSKA, 2016, JI, HUANG, *et al.*, 2011). Among the selected documents, the following application examples were identified: cosmetics, pesticides, and polymers for plastics, solvents, and paints (current stage), and production of methyl ethyl ketone (MEK), an important aviation fuel (medium term). The cosmetic application was reported by a patent from the Republic of Korea (KIM, JW, 2015), while the MEK production by the patent from Tsinghua University in China (ZHAO, CUI, *et al.*, 2017). In the short and long terms, there were no technological novelties for bio-based 2,3-BDO.

3.3 Micro analysis

3.3.1 Production process driver

The application of high-performance microorganisms, resulting from metabolic-genetic modifications, and the use of agro-industrial and urban residues were the main strategies verified to improve the Production Process, followed by the application of more efficient fermentation methodologies, related to the nature of the substrate (Fig. 2.5). The successful transition from the petroleum-based industry to the bio-based industry depends on these strategies (PRIYA, LAL, 2019), which were also identified in the scale-up issues of the bio-based 2,3-BDO process (YANG, Zhiliang, ZHANG, 2019).

Strain improvement to enhance the fermentation yield, reported on average in 32.5% of the selected documents, is a production strategy carried out through screening processes, random mutagenesis, and metabolic engineering systems (YIN, LI, *et al.*, 2015). Metabolic engineering has gained prominence in recent years as an important biological tool capable of promoting changes in existing biochemical routes through gene overexpression and by deleting genes encoding stereospecific enzymes by genetic engineering (YANG, Taowei, RAO, *et al.*, 2017). Through these strategies, an increase in the NADH pool and can be achieved, consequently, in the $NADH/NAD^+$ ratio, thus enhancing the 2,3-BDO yields (DAI, Jun-Jun, CHENG, *et al.*, 2014). Metabolic engineering can also control the formation of NADH-dependent by-products, prioritizing

the major pathway for the 2,3-BDO formation over the parallel metabolic pathways (BAO, ZHANG, *et al.*, 2015). The main modified and recently isolated microorganisms reported in the evaluated documents were: *Klebsiella pneumoniae* (GUO, WANG, *et al.*, 2017, JEON, KIM, *et al.*, 2014), *K. oxytoca* (CHO, KIM, WOO, LEE, *et al.*, 2015, MOON, KIM, *et al.*, 2018, SHARMA, NAIN, *et al.*, 2018), *Paenibacillus polymyxa* (JIAN, HONG, *et al.*, 2014, LI, Z, CAN, *et al.*, 2016, XUE, TONG, *et al.*, 2017), *Bacillus subtillis* (LIN, Q, RAO, *et al.*, 2010), *Clostridium* sp. (KOEPKE, NAGARAJU, *et al.*, 2013, SIMPSON, TRAN, *et al.*, 2013), *Enterobacter cloacae* (PRIYA, DUREJA, *et al.*, 2016, ZHANG, WU, *et al.*, 2019), *Escherichia coli* (CHEN, HUANG, *et al.*, 2017, FERNÁNDEZ-GUTIERREZ, VEILLETTE, *et al.*, 2020), *Raoultella* sp. (BUSTAMANTE, SANCHIS, *et al.*, 2019, KIM, Taeyeon, CHO, *et al.*, 2016), *Zymobacter* sp. (ISOBE, SAWAI, *et al.*, 2016), *Pichia pastoris* sp. (YANG, Zhiliang, ZHANG, 2018), *B. licheniformis* (LI, Lixiang, CHEN, *et al.*, 2014), *B. amyloliquefaciens* (RAO, YANG, *et al.*, 2015, YANG, Tao-Wei, RAO, *et al.*, 2013), *Serratia marcescens*(SHEN, ZHOU, *et al.*, 2009, SHI, GAO, *et al.*, 2014), and *Saccharomyces cerevisiae* (HUANG, GENG, 2020, LEE, SEO, 2019). Cellular modifications also aim at the process safety, considered another important requirement for the large-scale 2,3-BDO production. Although bacteria such as *Klebsiella*, *Enterobacter*, and *Serratia* are widely used in the 2,3-BDO production, their pathogenicity is a limiting factor for their industrial use (WOO, JONG, *et al.*, 2019). Except *K. oxytoca*, a safe microorganism capable of forming less capsular polysaccharide (advantage for the downstream step) (CHO, KIM, WOO, KIM, *et al.*, 2015). *Enterobacteriaceae* bacteria require greater virulence control, that can be achieved by eliminating pathogenic factors, such as lipopolysaccharides, polysaccharide capsules, and fimbrial adhesins (WOO, JONG, *et al.*, 2019). However, a reduction of at least 30% in the 2,3-BDO titer was found when this virulence control was performed (JUNG, JANG, *et al.*, 2013). Yeasts like *S. cerevisiae* can also be designed to produce 2,3-BDO by introducing bacterial pathways into its genotype. The YG01_SDBN strain received a heterologous NADH oxidase from *Lactococcus lactis* for redox control. The production of 178 g/L and 132 g/L of 2,3-BDO from glucose and cassava hydrolysate was achieved, respectively. These results make this yeast a potential candidate for large-scale fermentations (LEE, SEO, 2019). Therefore, for the selection of the industrial host, the following requirements must be considered for an economical and safe production of bio-based 2,3-BDO: high production efficiency and non-pathogenicity.

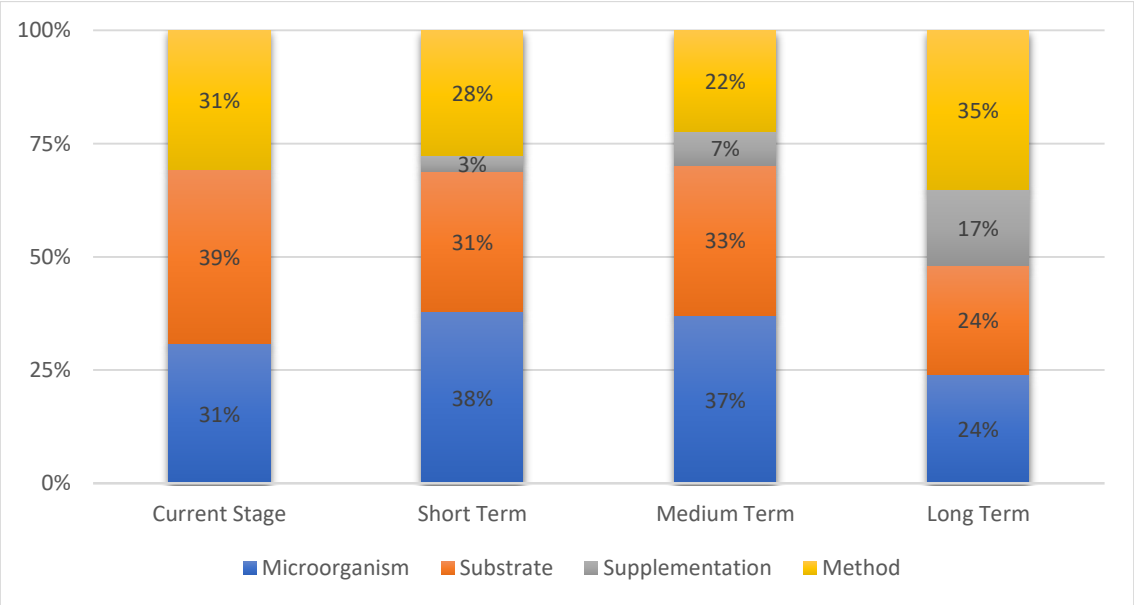


Figure 2.5: Micro analysis of the technological evolution of the bio-based 2,3-BDO process - Production Process driver.

Substrate was extensively investigated in all evaluated periods, being reported on average in 32% of the documents. Residual saccharin sugars, lignocellulosic materials, and the biodiesel-derived glycerol were the most used feedstocks in the bio-based 2,3-BDO production. The main raw materials verified were: artichoke biomass (CAO, ZHANG, *et al.*, 2017, GAO, J., JIANG, *et al.*, 2019, LI, Z, CAN, *et al.*, 2016, XIU, SUN, *et al.*, 2008), vinegar (XUE, TONG, *et al.*, 2017), corn hydrolysate (MA, Kedong, HE, *et al.*, 2018, XIAO, GU, *et al.*, 2016), xylose (GURAGAIN, VADLANI, 2017, JOO, LEE, *et al.*, 2016, MA, Cuiqing, WANG, *et al.*, 2009, PASAYE-ANAYA, VARGAS-TAH, *et al.*, 2019, SHARMA, NAIN, *et al.*, 2018, WANG, HU, *et al.*, 2016), whey (FERNÁNDEZ-GUTIERREZ, VEILLETTE, *et al.*, 2020, GUO, WANG, *et al.*, 2017), oil palm (HAZEENA, NAIR SALINI, *et al.*, 2019), cassava (CHEN, HUANG, *et al.*, 2017, MOON, KIM, *et al.*, 2018), and crude glycerol (XU, WANG, *et al.*, 2016, YANG, Tao-Wei, RAO, *et al.*, 2013).

Despite presenting a reduction in the long term, the study on the substrate must still be extensively carried out, due to its importance in the bioprocess economy on a large scale, since the substrate costs can represent up to 50% of the production costs (HAZEENA, SINDHU, *et al.*, 2020). Therefore, the use of cheap and abundant raw materials should be prioritized to enable industrial 2,3-BDO production (LEE, SEO,

2019). Furthermore, the limitation imposed by the carbon catabolic repression responsible for compromising the correct substrate assimilation requires further studies on the carbon behavior in the several cell metabolic pathways, aiming a greater bioprocess efficiency (YANG, Zhiliang, ZHANG, 2019). The use of agricultural biomass and crude glycerol is also an eco-friendly strategy, that can overcome its environmental disposal problems (PRIYA, LAL, 2019), combining sustainability and economy (circular economy). The process integration by biorefineries allows for the joint and sustainable production of fuels, energy (electricity and heat), and other high value-added chemicals (commodities) (DEMIRBAS, DEMIRBAS, 2010, HAZEENA, SINDHU, *et al.*, 2020). In particular, the 2,3-BDO biorefinery based on lignocellulosic feedstocks has great potential for large-scale implementation due to its advanced level of development, the result of several studies carried out to date (HAZEENA, SINDHU, *et al.*, 2020).

The investigation of the methods used for the bio-based 2,3-BDO production was correlated with the nature of the substrate used and the high-efficiency production required for a large-scale bioprocess. Approximately 29% of the evaluated documents reported strategies involving: solid-state fermentation (CAO, ZHANG, *et al.*, 2017), saccharification techniques and high-efficiency fermentation (HAZEENA, NAIR SALINI, *et al.*, 2019, HAZEENA, SINDHU, *et al.*, 2020, JOO, LEE, *et al.*, 2016, LI, Lixiang, CHEN, *et al.*, 2014, MOON, KIM, *et al.*, 2018), bioreactor operating modes (GUO, WANG, *et al.*, 2017, GURAGAIN, VADLANI, 2017, MA, Kedong, HE, *et al.*, 2018), and strategies for optimization and control of cultivation conditions (CHO, KIM, WOO, LEE, *et al.*, 2015, DEMIRBAS, DEMIRBAS, 2010, GUO, WANG, *et al.*, 2017, HAZEENA, SINDHU, *et al.*, 2020, KIM, Taeyeon, CHO, *et al.*, 2016, PRIYA, DUREJA, *et al.*, 2016, SHARMA, NAIN, *et al.*, 2018). Solid-state fermentation has been performed as an alternative channel in the use of agro-industrial waste to produce 2,3-BDO, with the following advantages: high productivity, high product stability, and low need for sterilization, due to its low water activity (CAO, ZHANG, *et al.*, 2017). Different processes of saccharification and fermentation, such as SSF - simultaneous, SHF - separated, and SScF - co-fermentation, have been extensively investigated due to the increased use of lignocellulosic materials as carbon sources that require an efficient solubilization step for substrate availability (HAZEENA, SINDHU, *et al.*, 2020).

Different operation modes such as batch, fed-batch, continuous cultivation, and their adaptations have also been investigated. Although the batch mode has allowed the determination of the cell capacity of the 2,3-BDO production and consequently of the bioprocess kinetics (RIPOLL, DE VICENTE, *et al.*, 2016), the fed-batch mode and continuous cultivation have been carried out to improve the 2,3-BDO yields, avoiding cell inhibition processes caused by excess substrate and product (RIPOLL, RODRÍGUEZ, *et al.*, 2020), and increasing its productivity (MA, Kedong, HE, *et al.*, 2018), respectively. Productivity is an important fermentation parameter related to the bioprocess economy. Volumetric productivities between 1 and 2 g/L/h allow energy consumption regardless of fermentation time and substrate concentration, which is reflected in a lower annual cost of the bioprocess (DHESKALI, KOUTINAS, *et al.*, 2020). The optimization of cultivation conditions such as pH, temperature, and oxygen availability (HAKIZIMANA, MATABARO, *et al.*, 2020) has also been performed, since these parameters can directly affect the 2,3-BDO metabolism and, therefore, its yield and productivity as well as the formation of by-products (JI, HUANG, *et al.*, 2011).

Nutritional supplementation was verified on average in 6.5% of the evaluated documents. The decrease in studies on substrate was offset by the increase in studies on nutritional supplementation in the long term, which can be explained by the knowledge consolidation about the carbon source and the need to improve the 2,3-BDO yield through control metabolism in terms of enzymes and endogenous reducing agents, such as NADH and NAD^+. The redox balance achieved by these reducing agents can affect the activity of ALS, ALDS, and BDH, key-enzymes of 2,3-BDO metabolism (JI, HUANG, *et al.*, 2011). The addition of vitamins C and E, composed by exogenous reducing agents, can increase the NADH/NAD^+ ratio and, consequently, the efficiency of the bioprocess (CHENG, YUAN, *et al.*, 2014, DAI, Jun-Jun, CHENG, *et al.*, 2014). Other nutritional supplements have also been identified, such as nitrogen complexes (CHO, KIM, WOO, LEE, *et al.*, 2015), and amino acids (GAO, J, CAO, *et al.*, 2017, GAO, J., JIANG, *et al.*, 2019), considered organic nitrogen sources. Typically, inorganic nitrogen sources are represented by nitrate and ammonium salts, whose costs are lower than those observed for organic nitrogen sources such as casamino acids (0.70 $/kg) and yeast extract (0.50 $/kg) (KOUTINAS, YEPEZ, *et al.*, 2016). Furthermore, the downstream processing using inorganic nitrogen in the culture medium is less complicated, which allows for more

efficient purification of 2,3-BDO (PRIYA, LAL, 2019). Supplements with acetic acid (HAZEENA, NAIR SALINI, *et al.*, 2019, WANG, HU, *et al.*, 2016), sodium bicarbonate (ZHANG, WU, *et al.*, 2019), and iron (SOLEM, JENSEN, *et al.*, 2017) have also been verified. Acetate is an important inducer of the key-enzymes of the 2,3-BDO metabolism, in which its yield can be increased by adding acetic acid/acetate to the culture medium (CELIŃSKA, GRAJEK, 2009). Supplementation with acetate in the 2,3-BDO fermentation from glucose in fed-batch with a molar ratio of 0.35 acetate: 1 glucose led to an improvement in the *levo*-2,3-BDO production by *P. polymyxa*, without reducing its optical purity (NAKASHIMADA, MARWOTO, *et al.*, 2000).

3.3.2 Recovery process driver

The main methodology for recovering 2,3-BDO investigated in the current stage, in the short and medium terms was conventional distillation, reported in 50% of the studies in each period. In the long term, solvent extraction was identified in 36% of the evaluated documents, showing an industrially promising methodology. Other processes such as membrane separation (21% on average), ion exchange (12.5% on average), salting-out and sugaring-out (27% in the long term, together) have also been verified (Fig. 2.6).

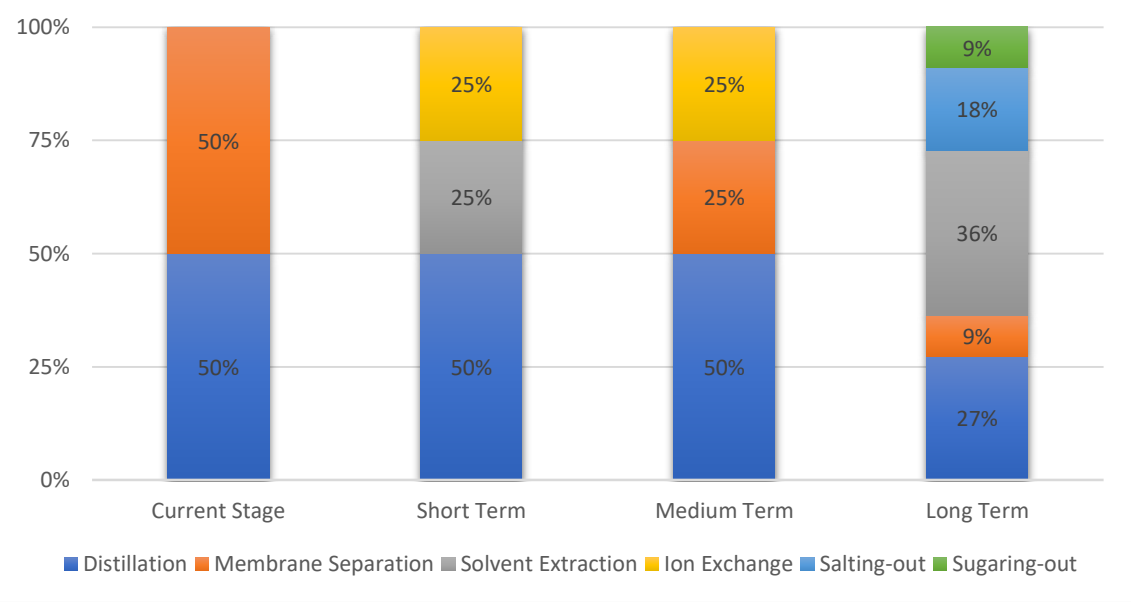


Figure 2.6: Micro analysis of the technological evolution of the bio-based 2,3-BDO process – Recovery Process driver.

Since no azeotropic mixture with water is observed, conventional distillation has been largely directed towards the industrial recovery of bio-based 2,3-BDO. However, distillation is an energy-intensive process due to the hydrophilic nature and the high boiling point of 2,3-BDO, around 180 °C (HAIDER, QYYUM, *et al.*, 2018, JI, HUANG, *et al.*, 2011). The costs of conventional distillation tend to be even higher with the large number of column stages required in the bioprocess (SONG, YOON, *et al.*, 2019). Furthermore, this high energy demand contributes to the increase in greenhouse gas emissions, another limiting factor for its scaling-up (HAIDER, QYYUM, *et al.*, 2018).

The following technologies: solvent extraction (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015, JIANYING, CHUBJIAO, *et al.*, 2014, LI, Yanjun, WU, *et al.*, 2016), membrane separation (DAVEY, HAVILL, *et al.*, 2016, KAWAMURA, MORITA, *et al.*, 2014), salting-out (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015, DAI, Jianying, WANG, *et al.*, 2018), sugaring-out (DAI, Jian-Ying, LIU, *et al.*, 2015), and ion exchange (ISOBE, SAWAI, *et al.*, 2016) have been investigated as alternatives to conventional distillation. Solvent extraction or liquid-liquid extraction is considered a promising technology for the industrial recovery of bio-based 2,3-BDO due to its advanced development (SHAO, KUMAR, 2009) and its low energy demand (DAI, Jian-Ying, LIU, *et al.*, 2015). This method can be highly efficient when solvents with high selectivity and high partition coefficient are employed. Moreover, environmental and economic benefits can be achieved with the use of green solvents and the reuse of organic extractors (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015). Reactive extraction is a special solvent extraction that is very effective to improve the bioproduct solubility in the organic phase via reaction, in complicated systems (LI, Yanjun, WU, *et al.*, 2016). However, it presents some difficulties due to the homogeneity and the corrosive properties of the catalyst used (usually an acid), although less solvent is used. For an industrial implementation, reactive extraction requires the addition of anti-corrosion devices, whose technology maturity is still early, and therefore limited to the laboratory environment (HARVIANTO, HAIDER, *et al.*, 2018).

The addition of salts to solvent extractions decreases the extractor amount required for the 2,3-BDO purification and increases the separation factor (HAIDER, QYYUM, *et al.*, 2018). Generally, selectivity and distribution coefficient is higher in

solvent extractions containing salts, whose 2,3-BDO recovery can reach 90-99%, with the removal of soluble proteins and organic acids (DAI, Jian-Ying, LIU, *et al.*, 2015). However, the presence of salt can affect subsequent refining steps, increasing the bioprocess capital costs (XIE, ZHANG, *et al.*, 2017). Salt recycling can also lead to scaling and blockage phenomena (HARVIANTO, HAIDER, *et al.*, 2018), which limit its large-scale application. To overcome the problems of salting-out, the use of sugars has been carried out through the sugaring-out processes. As sugar is a substrate, it can be reused without great difficulties by microorganisms after the extraction process (DAI, Jian-Ying, LIU, *et al.*, 2015).

Membrane separation can provide great savings for fermentation processes (KOROS, 2004). Thin-film polydimethylsiloxane membranes (PDMS) filled with silicalite have been used in pervaporation processes for continuous and preferential bio-based 2,3-BDO recovery, whose working principle is based on the solution diffusion theory. Fed-batch fermentations were used to confirm the technical and economic viability of purification by PDMS (SHAO, KUMAR, 2009). Nanofiltration and reverse osmosis membranes have also been reported. Both membranes can be used to replace part of the separation by distillation of low volatility organics in 2,3-BDO fermentations, allowing less energy expenditure and more sustainable separations (DAVEY, HAVILL, *et al.*, 2016).

Although simpler and more economical, most alternative technologies have limitations, responsible for compromising their application on a large scale (HAIDER, QYYUM, *et al.*, 2018). The efficiency of membrane separation tends to decrease with time due to the fouling and the swelling of the fibers, resulting from the increased complexity of the fermentation broth (HAIDER, QYYUM, *et al.*, 2018, HARVIANTO, HAIDER, *et al.*, 2018). This swelling can promote the membranes plasticization, which can be suppressed with fillers (SHAO, KUMAR, 2009). Reverse osmosis membrane may be more efficient than the nanofiltration membrane in the bio-based 2,3-BDO recovery due to the steric properties of its molecules and the lower presence of irreversible encrustations (DAVEY, HAVILL, *et al.*, 2016). Ion exchange is another recovery method that can be based on membranes. In this case, ion exchange membranes have the same

limitations as conventional membrane separation processes (ISOBE, SAWAI, *et al.*, 2016).

In the evaluated documents, the integration of recovery methodologies was also verified: alcohol precipitation and vacuum distillation (JEON, KIM, *et al.*, 2014), hybrid extraction-distillation (HARVIANTO, HAIDER, *et al.*, 2018, JEON, KIM, *et al.*, 2014), solvent extraction and pervaporation (SHAO, KUMAR, 2009), extraction and salting-out (SUN, JIANG, *et al.*, 2009), and nanofiltration membrane separation and ion exchange (ISOBE, SAWAI, *et al.*, 2016). The combination of recovery technologies is necessary due to the low purity achieved by the technologies conducted individually. If this association is not made, the process should be repeated for an efficient bio-based 2,3-BDO recovery (FREIDANK, AHRENS, 2014). This procedure can impact energy demand, increasing production costs. Therefore, the integration of recovery technologies must be carried out to optimize and economize the bioprocess.

3.3.3 The product and the application drivers

The applications of 2,3-BDO were related to its isomeric form (product), since the optical activity (chirality) is responsible for specific chemical properties, such as antifreeze (*levo*- and *dextro*-isomer) (BOUTRON, 1992), and antisepsis, humectance and emollience (*meso*-isomer) (WOO, JONG, *et al.*, 2019). Each property can be used in a distinct industrial segment, highlighting cosmetics, drugs, polymers, fuels, and energy (BIAŁKOWSKA, 2016). Compounds derived from 2,3-BDO can be obtained by dehydration, dehydrogenation, ketalization, and esterification (JI, HUANG, *et al.*, 2011), and their global market handles an average of about 32 million tons per year, with sales of US$ 43 billion (KÖPKE, MIHALCEA, *et al.*, 2011). The main applications of 2,3-BDO isomers and their derivatives, according to their characteristic properties, were summarized in Table 2.2.

Table 2.2: Applications and Market segments for 2,3-BDO isomers and its derivatives

Compound	Obtaining method	Application	Market segment	Reference
(2R,3R)-BDO	Direct	Asymmetric synthesis using boron esters	(2S,3S)-3-methyl-5-hexen-2-ol	MATTESON, CAMPBELL (1990)
		Synthesis of high-value drugs and liquid crystals	Pharmaceutical and polymer industry	ALEKSANDRIISKII, NOVIKOV, *et al.* (2019)
		Pest control agent (antibacterial)	Agriculture	CORTES-BARCO, HSIANG, *et al.* (2010), KONG, SHIN, *et al.* (2018), RYU, FARAG, *et al.* (2004)
(2R,3S)-BDO	Direct	Antiseptic, humectant and emollience	Cosmetics	BAEK, WOO, *et al.* (2016), SONG, YOON, *et al.* (2019)
(2R,3R)-BDO	Direct	Cryoprotectant for organ cryopreservation	Health	BOUTRON (1992)
(2S,3S)-BDO		Antifreezing	Polymer industry	JI, HUANG, *et al.* (2011)
(2R,3R)-BDO **(2S,3S)-BDO** **(2R,3S)-BDO**	Direct	Innate immunity enhancer, anti-inflammation, and biomarker	Pharmaceutical and food	HSIEH, LU, *et al.* (2007), LAI, CHANG, *et al.* (2012), RYU, FARAG, *et al.* (2004)
		Softening agents, plasticizers, fumigants, and printing inks	Polymer industry	BIAŁKOWSKA (2016)
1,3-Butadiene	Dehydration	Synthetic rubber precursor	Polymer industry	CELIŃSKA, GRAJEK (2009)
Methyl ethyl ketone (MEK)	Dehydration	Fuels, resins, paints, and solvents	Fuel and polymer industry	JI, HUANG, *et al.* (2011), LEE, J, KIM, *et al.* (2018), ZHAO, CUI, *et al.* (2017)
Diacetyl	Dehydrogenation	Flavoring agent and bacteriostatic food additive	Food	BARTOWSKY, HENSCHKE, (2004), XIAO, Zijun, XU (2007)
Acetoin	Dehydrogenation	Aroma carrier in flavors and essence	Food	XIAO, Zijun, XU (2007)
Tetramethyl (methyl tert-butyl ether – MTBE)	Ketalization	Gasoline blending agent	Fuel	VOLOCH, JANSEN, *et al.* (1985)
2,3-BDO diester	Esterification	Precursors of drugs and cosmetic, and effective plasticizers for thermoplastic polymers (cellulose nitrate, polyvinyl esters, polyacrylates, and polymethylacrylates)	Pharmaceutical, cosmetics, and polymer industry	VOLOCH, JANSEN, *et al.* (1985)
Polyurethane	Polymerization	Novel chain initiator and extender in the polyurethane intermediates manufacture	Polymer industry	SONG, YOON, *et al.* (2019)

3.4 Technology Roadmap

3.4.1 Current stage

At the current stage, the major players that have operated in the global commercialization of bio-based 2,3-BDO were: LanzaTech, Orochem Technologies Inc., Global Bio-Chem Technology Group, Biosyncaucho S.L., Intrexon, Tokyo Chemical Industry Co. Ltd., Merck KGaA, GS Caltex Corporation, and Praj Industries Ltd. (TRANSPARENCY MARKET RESEARCH, 2022). The small number of companies linked to the industrial production of bio-based 2,3-BDO indicates a little diversified market, with centralized innovation. These companies were identified at the current stage, while the research centers dominated the other evaluated periods (short, medium, and long terms).

LanzaTech New Zealand Limited (www.lanzatech.com) was the first company to announce the commercial production of bio-based 2,3-BDO on a pilot scale in 2012, and to implement the large-scale bioprocess in 2016. Headquartered in the USA, LanzaTech is a company based on the circular carbon economy, characterized by waste mitigation, resource efficiency, and added value. Its bio-based 2,3-BDO technology is characterized by the residual carbon monoxide (CO) gas fermentation from steelmaking processes by engineered *Clostridium* bacteria, in which ethanol is also produced (1:1 ratio). Bio-based 2,3-BDO is intended for the production of aviation fuel by conversion to MEK, and the production of synthetic fibers, plastics, and rubbers. According to LanzaTech's projections, an annual production of up to 100,000 gallons of 2,3-BDO and ethanol was expected with the integration of the pilot demonstration plant with the Baosteel plant in Shanghai, China. The main limitation of this biotechnology is the formation of the by-product such as butadiene and butenes. LanzaTech has heavily invested in the development of bio-based 2,3-BDO technology, establishing partnerships such as that observed with the Chinese Academy of Sciences to build a Bioenergy Research Center in China, where the bio-based 2,3-BDO production has been under investigation since then.

Orochem Technologies Inc. (www.orochem.com) is an American company recognized worldwide for its biological recovery technologies, with offices in USA and

India. It is responsible for the manufacturing of membrane filter plates, silica manufacture, silica bonding, solid-phase extraction products, and HPLC Columns. Moreover, Orochem company offers a chromatography system called Simulated Moving Bed (SMB), used for specific purification of nutritional supplements, fatty acids, and specialty sugars. In partnership with LanzaTech, the bio-based 2,3-BDO was recovered from the CO gaseous fermentation by SMB, with less energy and less operational expenditure (OPEX). According to information on the company's website, OPEX using SMB can be reduced by up to 10-fold compared to the conventional distillation process.

Global Bio-Chem Technology Group (www.globalbiochem.com) is a company localized in Hong Kong and a pioneer in refined and corn-based products in the Asia Pacific region. Its product mix consists of amino acids, corn sweeteners, modified starches, and polyol chemicals that can be applied in the production of feed, food and beverage, cosmetics, textiles, pharmaceuticals, and other chemicals. The main polyol chemicals produced from corn starch are 1,2-EDO (1,2-ethanediol), 1,2-PDO (1,2-propanediol), 1,2-BDO, and 2,3-BDO. These polyols are used to produce different resins (A, B, C, E), whose applications include surfactants, emulsifiers, plastics, and plasticizing chemicals.

Biosyncaucho S.L. (www.tecnaliaventures.com) is a Spanish company that develops chemical products with high added value from renewable raw materials. Its biorefinery is called Biokemik and is based on non-food biomass and cheap industrial byproducts, used to produce 2,3-BDO and acetoin by GRAS microorganisms. A pilot plant has been operating since 2015, and a semi-industrial plant with 100 tonnes/year of acetoin (equivalent to 85 tonnes/year of 2,3-BDO) was scheduled to be built by the end of 2019 and put into operation in 2020. The produced bio-based 2,3-BDO is used as a precursor in pesticides, chemicals, pharmaceuticals, plastics, and 1,3-butadiene.

GS Caltex Corporation (www.gscaltex.com) is a South Korean oil refinery that has an eco-friendly method of producing 2,3-BDO based on natural fermentation. It is the only company in the world with a 100% bio-based 2,3-BDO process. The bio-based 2,3-BDO is produced from cassava and sugarcane as feedstocks by microorganisms obtained from an innovative bioengineering technology, and it is recovered from its physical characteristics. The bio-based 2,3-BDO is used as fertilizers, biostimulants, and plant

disease control. GS Caltex Corporation also applies bio-based 2,3-BDO as a natural humectant, dispersant for cosmetical, preservative booster, select extractant, and a skin health product. In these applications, it is called GreenDiol™, being an important selling product of the company. After 10 years of study, GS Caltex has planned to implement a demonstration plant, with an annual capacity of 300 tons of 2.3-BDO. (WOO, JONG, *et al.*, 2019)

Praj Industries Ltd. (www.praj.net) is an Indian project engineering company located in more than 75 countries and recognized worldwide for its engineering solutions for biofuels, biochemicals, and water treatments. The 2,3-BDO bioprocess is based on the use of lignocellulosic biomass (cane bagasse, rice straw, cotton stalk, empty palm fruit bunches, and corn stover) treated by mechanical, hydrothermal, and enzymatic hydrolysis tests to make sugars (C5 and C6) available for fermentation. The microorganism used are genetically modified, which allows a 2,3-BDO yield greater than 80%. The 2,3-BDO recovery is performed by Praj Eco Smart^{TM} distillation technology. The wastewater is treated at the end of the bioprocess.

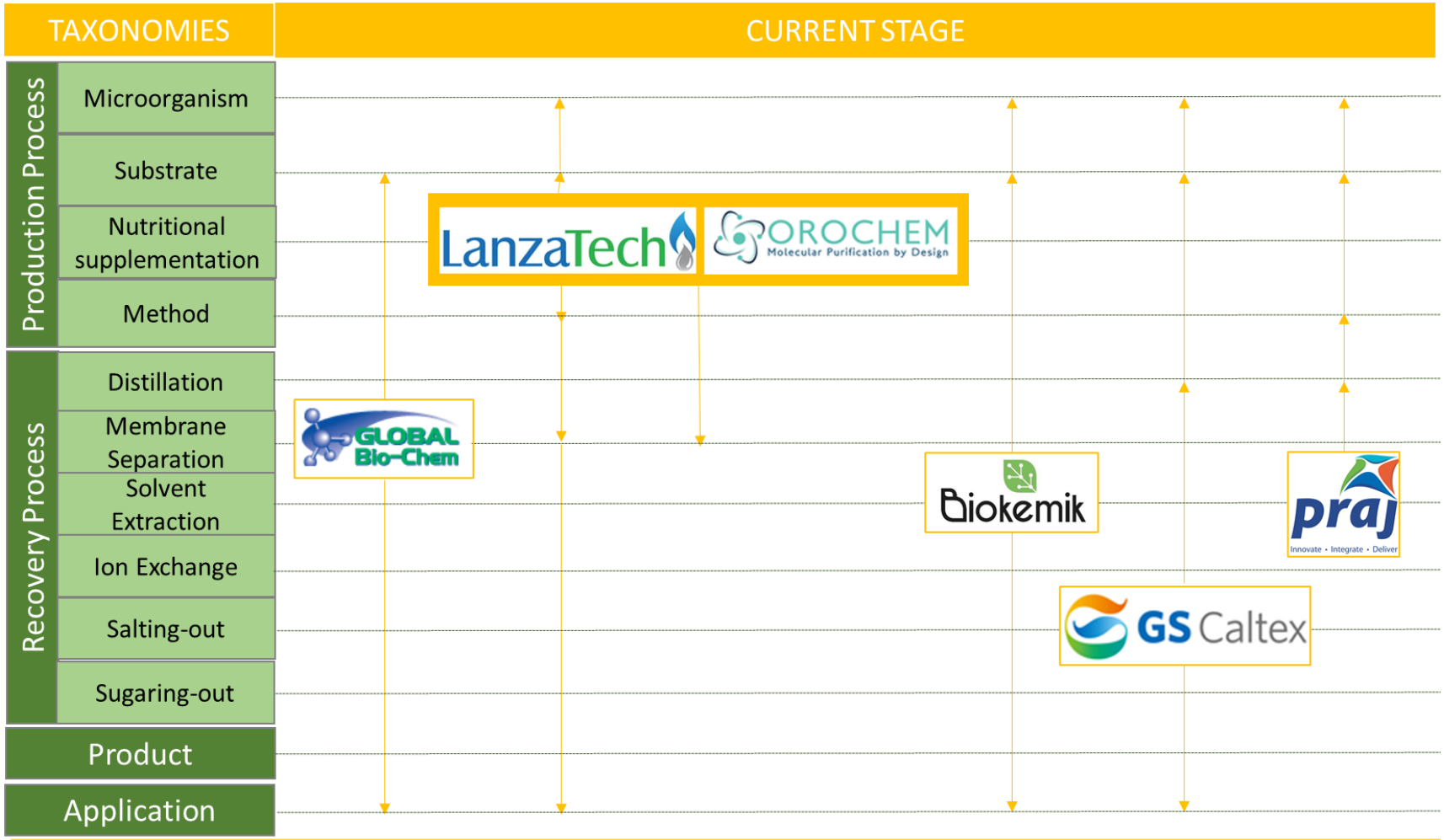
Intrexon, Tokyo Chemical Industry Co. Ltd., and Merck KGaA are 2,3-BDO suppliers only. Technology Roadmap in the current stage showed a greater interest in the Application driver, represented by chiral building blocks, plastics, fuels, pesticides, cosmetics, and pharmaceuticals (Fig. 2.7).

3.4.2 Short term

Technology Roadmap in the short term with a focus on Production Process (use of engineered microorganisms, residual substrates, and efficient fermentation methodologies) was built (Fig. 2.8). About 21.4% of patents were granted to companies and research centers such as China Petroleum & Chemical Corporation (SINOPEC Dalian Research Institute of Petroleum and Petrochemicals), Zhangjiagang Meijingrong Chemical Industry Co. Ltd, and LanzaTech. SINOPEC investigated the metabolism of bio-based 2,3-BDO for the complete glucose assimilation, conducting the bioprocess in microaerobic and aerobic conditions using *K. pneumonia* and *Lactobacillus casei*, respectively, at pH 5-6. The SINOPEC methodology was considered suitable for the mass production of bio-based 2,3-BDO. Its downstream step was carried out by solvent

extraction and other rectification processes (LIN, Z, YACHAO, *et al.*, 2016). Zhangjiagang Meijingrong Chemical Industry Co. Ltd employed an engineered *Klebsiella* strain without the production routes of organic acids, by inactivating its coding genes, which favored the production of 1,3-propanediol, 2,3-BDO, and ethanol. This strain improvement also contributed to a less expensive extracting alcohol (XU, WANG, *et al.*, 2016). LanzaTech used carbohydrates and residual CO as substrates for the 2,3-BDO production by engineered *Clostridium autoethanogenum* in an anaerobic gas fermentation. Positive regulation of a native BDH gene was performed. The 2,3-BDO produced was converted into 2-butanol, a chemical of interest to the solvent, paint, and pharmaceutical industries (SIMPSON, TRAN, *et al.*, 2013).

Approximately 78.6% of patents belonged to academic research institutions linked mainly to Chinese and Korean universities. Industry-Academic Cooperation, represented by the Hanbat National University Industry-Academic Cooperation Foundation, was also identified (PARK, LYM, 2016). Among the academic research institutions, the Chinese universities Yancheng Institute of Technology and Dalian University of Technology stood out in the short term. Yancheng Institute of Technology conducted two studies for the production of bio-based 2,3-BDO involving genetic engineering and solid fermentation, both using *P. polymyxa*. Homologous recombination was used to remove the diacetyl reductase gene, responsible for the synthesis of *meso*-2,3-BDO, which led to a high selectivity of *levo*-2,3-BDO from the Jerusalem artichoke inulin (JIAN, HONG, *et al.*, 2014). In solid fermentation, a low-cost substrate was converted to 2,3-BDO in a device built with a simple structure, easily available and operated, which should contribute to the reduction of capital costs in a possible industrial implementation (LI, Z, CAN, *et al.*, 2016). Dalian University of Technology investigated a new biochemical technology based on the use of Jerusalem artichoke tuber. An inulin hydrolysate was obtained by action of inulase in the SSF and SHF processes. A production of up to 115 g/L of 2,3-BDO and acetoin was achieved in fed-batch fermentations (XIU, SUN, *et al.*, 2008).



Filled clusters: partnerships. Unfilled clusters: same business.

Figure 2.7: Technological Roadmap of the bio-based 2,3-BDO process in the current stage.

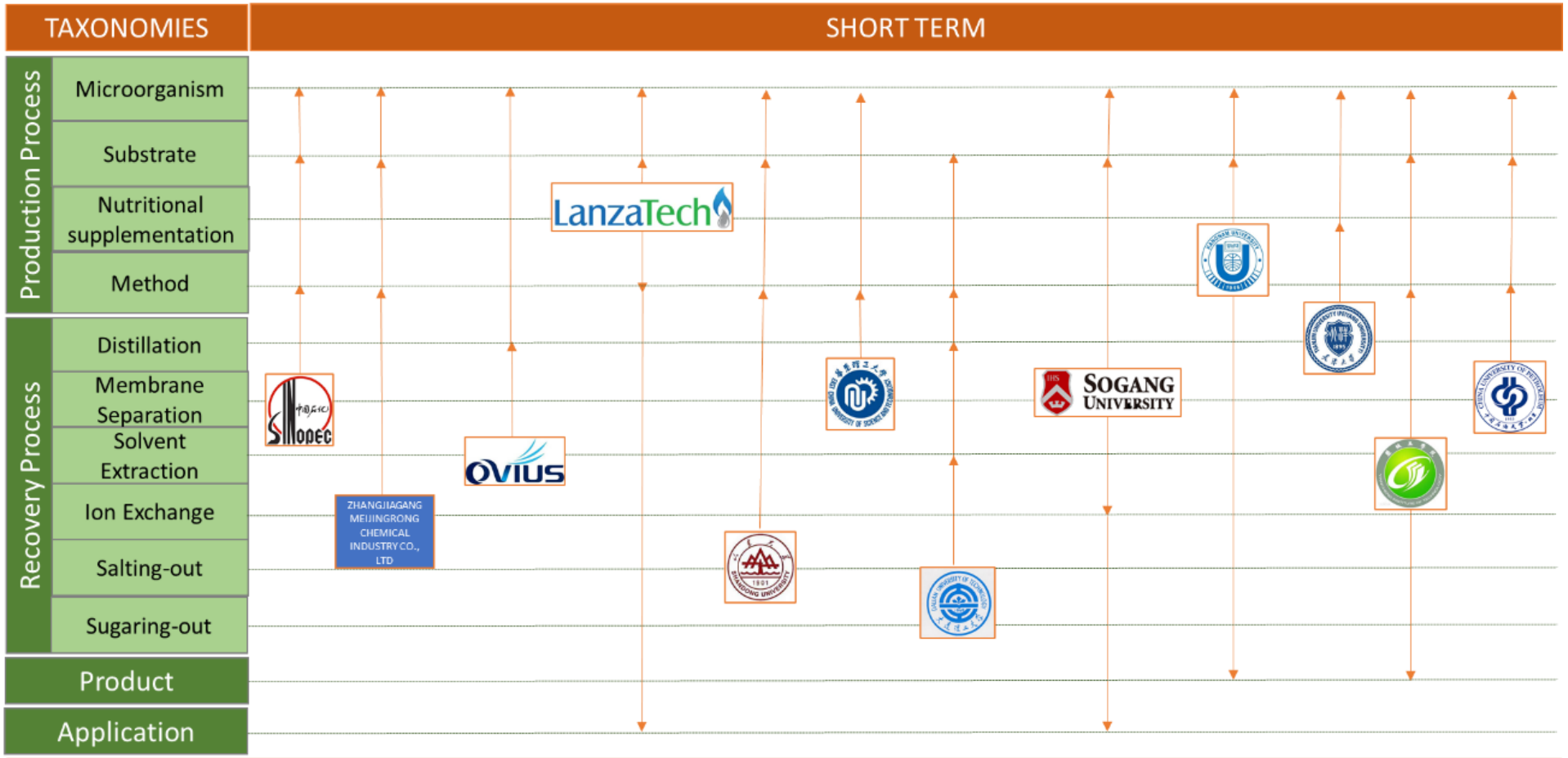


Figure 2.8: Technological Roadmap of the bio-based 2,3-BDO process in the short term.

3.4.3 Medium term

Technology Roadmap in the medium term with a focus on Production Process (use of engineered microorganisms and residual materials) was built (Fig. 2.9). Approximately 53.3% of patents were applied by companies and research centers, while about 46.7% by academic research institutions. Again, LanzaTech (KOEPKE, NAGARAJU, *et al.*, 2013), and SINOPEC (LIAO, WANG, *et al.*, 2018) were identified as 2,3-BDO players, as well as Toray Industries (ISOBE, SAWAI, *et al.*, 2016, KAWAMURA, MORITA, *et al.*, 2014), Mitsubishi Chemicals Corporation, (NAOFUMI, SHUICHI, 2015), Wuhan Junan Biological Technology Co. Ltd, (GAO, L, CHEN, *et al.*, 2015), Biopolis S. L. (BUSTAMANTE, SANCHIS, *et al.*, 2019), S. A. Agricultores de la Vega de Valencia (BUSTAMANTE, SANCHIS, *et al.*, 2019), Nanning Bioclone Biotechnology Co. Ltd. (CHEN, HUANG, *et al.*, 2017), and Guangxi Academy of Sciences (CHEN, HUANG, *et al.*, 2017), SINOPEC proposed a simple, low-cost, and high-yield method for the 2,3-BDO production from microalgae hydrolysate. This method was able to increase the starch amount available for the fermentation process, resulting in higher 2,3-BDO titers, around 76 g/L including acetoin (LIAO, WANG, *et al.*, 2018). Wuhan Junan Biological Technology Co. Ltd employed *B. licheniformis* and *B. subtillis* to co-produce 2,3-BDO and poly-γ-glutamic acid, reaching about 73.7 g/L and 52.2 g/L, respectively. The productivity of 2.10 g/L/h of 2.3-BDO and the glucose conversion of 83.9% were considered suitable for a commercial production of bio-based 2.3-BDO. Furthermore, the use of GRAS bacteria can ensure the process safety, especially on a large scale (GAO, L, CHEN, *et al.*, 2015).

The universities that stood out in the medium term were: the Tsinghua University (ZHANG, WU, *et al.*, 2019, ZHAO, CUI, *et al.*, 2017), and again the Yancheng Institute of Technology (GAO, J, CAO, *et al.*, 2017, XUE, TONG, *et al.*, 2017), both Chinese universities. Tsinghua University has proposed the use of an *E. cloacae* strain with high tolerance to an inhibitor present in non-detoxified lignocellulosic hydrolysate. This bacterium also showed good stability, reuse possibility, and capacity to produce 2,3-BDO and organic acids. As the hydrolysate detox represents an additional unitary operation to the fermentation process, responsible for a higher operating cost, the use of this strain in fermentations with lignocellulosic materials is a promising strategy for the viability of the

process on a large scale (ZHANG, WU, *et al.*, 2019). Yancheng Institute of Technology investigated the *levo*-2,3-BDO production by *P. polymyxa* from vinegar residue. Initially, this low-cost waste was dried, crushed, and submitted to alkaline treatment, followed by enzymatic hydrolysis. No addition of a nitrogen source was necessary, which contributed to an even cheaper fermentation medium. The proposed method had the advantages of adding value to an agro-industrial waste, also overcoming the problem of its environmental disposal. Furthermore, *levo*-2,3-BDO with high selectivity can reduce the downstream step costs, favoring its possible industrial scaling-up (XUE, TONG, *et al.*, 2017).

3.4.4 Long term

Technology Roadmap in the long term with a focus on Recovery Process (distillation and solvent extraction methods) was built (Fig. 2.10). The interest in bio-based 2,3-BDO purification technologies in the long term is due to the influence of the downstream step to the bioprocess economy. Currently, chemically synthesized *meso*-2,3-BDO is sold by Sigma-Aldrich for a price of over 25,000 $/kg, a value considered inappropriate for commercial production (WOO, JONG, *et al.*, 2019). Different recovery technologies have been investigated to reduce the minimum selling price of pure 2,3-BDO. Distillation-based recovery has proved to be a promising technology, especially when energy integration strategies are applied. The use of heat exchangers to preheat the distillers' supply and the latent heat vapor exchanges, responsible for reducing the reboiler and condenser duties for the distillation columns, can reduce a total energy cost and the total annual production cost by up to 67% and 61.2%, respectively (HAIDER, QYYUM, *et al.*, 2018).

Approximately 25.4% of papers were published by professionals from research institutions linked to private and government companies, while about 74.6% of papers were published by professionals from academic research institutions. The main research centers and companies were: Biogas Institute of Ministry of Agriculture (MA, Kedong, HE, *et al.*, 2018) (China); Centre National en Électrochimie et en Technologies Environnementale (FERNÁNDEZ-GUTIERREZ, VEILLETTE, *et al.*, 2020) (France); Indian Agriculture Research Institute (SHARMA, NAIN, *et al.*, 2018), Indian Institute of Toxicology Research (HAZEENA, NAIR SALINI, *et al.*, 2019), National Institute for

Interdisciplinary Science and Technology (HAZEENA, NAIR SALINI, *et al.*, 2019), and Praj Industries Ltd (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015) (India); Changhae Ethanol Co. Ltd. (MOON, KIM, *et al.*, 2018), GS Caltex Corporation (JEON, KIM, *et al.*, 2014, MOON, KIM, *et al.*, 2018), and Korea Institute of Science and Technology (CHO, KIM, WOO, LEE, *et al.*, 2015, KIM, Taeyeon, CHO, *et al.*, 2016) (Republic of Korea); CPC Corporation (WONG, YEN, *et al.*, 2014) (Taiwan); and LanzaTech (USA) (DAVEY, HAVILL, *et al.*, 2016). Continuous countercurrent liquid-liquid extraction (CC-LLE) was used to recover bio-based 2,3-BDO from synthetic solutions (2,3-BDO + water) and fermented broths using n-butanol with the addition of 10 %(w/w) K_2HPO_4. The salt added in CC-LLE increased the recovery efficiency of bio-based 2,3-BDO by 81.8% and 30% for synthetic and fermented broth, respectively. Moreover, a simulation predicted 99% of recovery efficiency of bio-based 2,3-BDO through 6 stages of theoretical equilibrium extraction (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015).

Concerning academic research centers, again Dalian University of Technology (DAI, Jian-Ying, LIU, *et al.*, 2015, DAI, Jianying, WANG, *et al.*, 2018, MA, Kedong, HE, *et al.*, 2018), Nanjing Tech University (CAO, ZHANG, *et al.*, 2017, GAO, J., JIANG, *et al.*, 2019), and Yancheng Institute of Technology (CAO, ZHANG, *et al.*, 2017, GAO, J., JIANG, *et al.*, 2019) (China); and Sogang University (CHO, KIM, WOO, LEE, *et al.*, 2015, KIM, Taeyeon, CHO, *et al.*, 2016), and Yeungnam University (HAIDER, QYYUM, *et al.*, 2018, HARVIANTO, HAIDER, *et al.*, 2018, HONG, VAN DUC LONG, *et al.*, 2019) (Republic of Korea) stood out in the long term. Novel multi-effect-evaporation-assisted distillation (MEED) configurations were used to improve the energy efficiency of the bio-based 2,3-BDO process. There was a synergistic effect of combining the multi-effect evaporator with the distillation processes, responsible for this energy efficiency. Moreover, when MEDD was performed with heat integration, the total annual production cost was significantly reduced by up to 38.9%. Therefore, MEDD methodology can be considered a promising technology comparable to conventional distillation, mainly in large-scale processes (HONG, VAN DUC LONG, *et al.*, 2019).

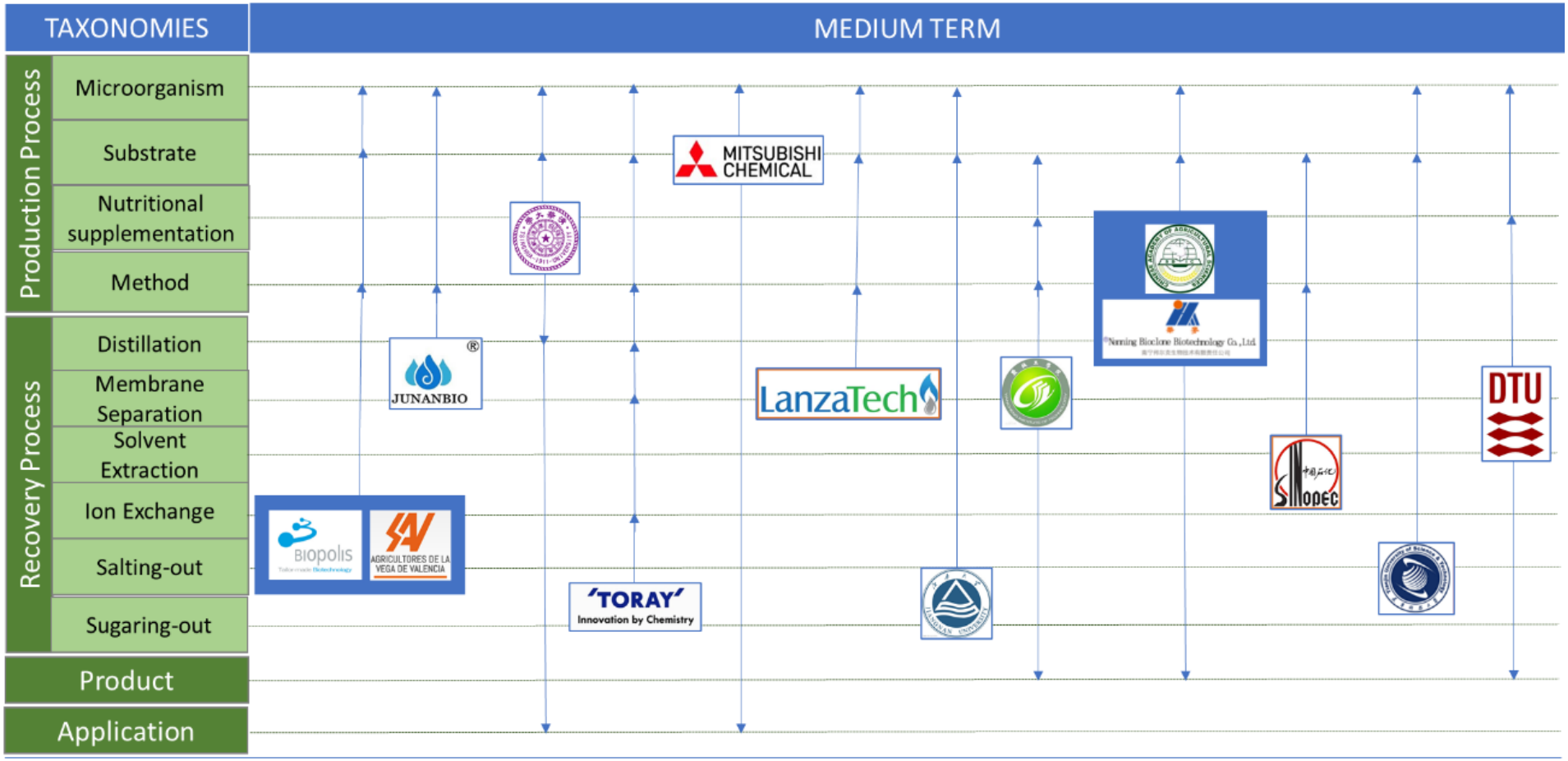


Figure 2. 9: Technological Roadmap of the bio-based 2,3-BDO process in the medium term.

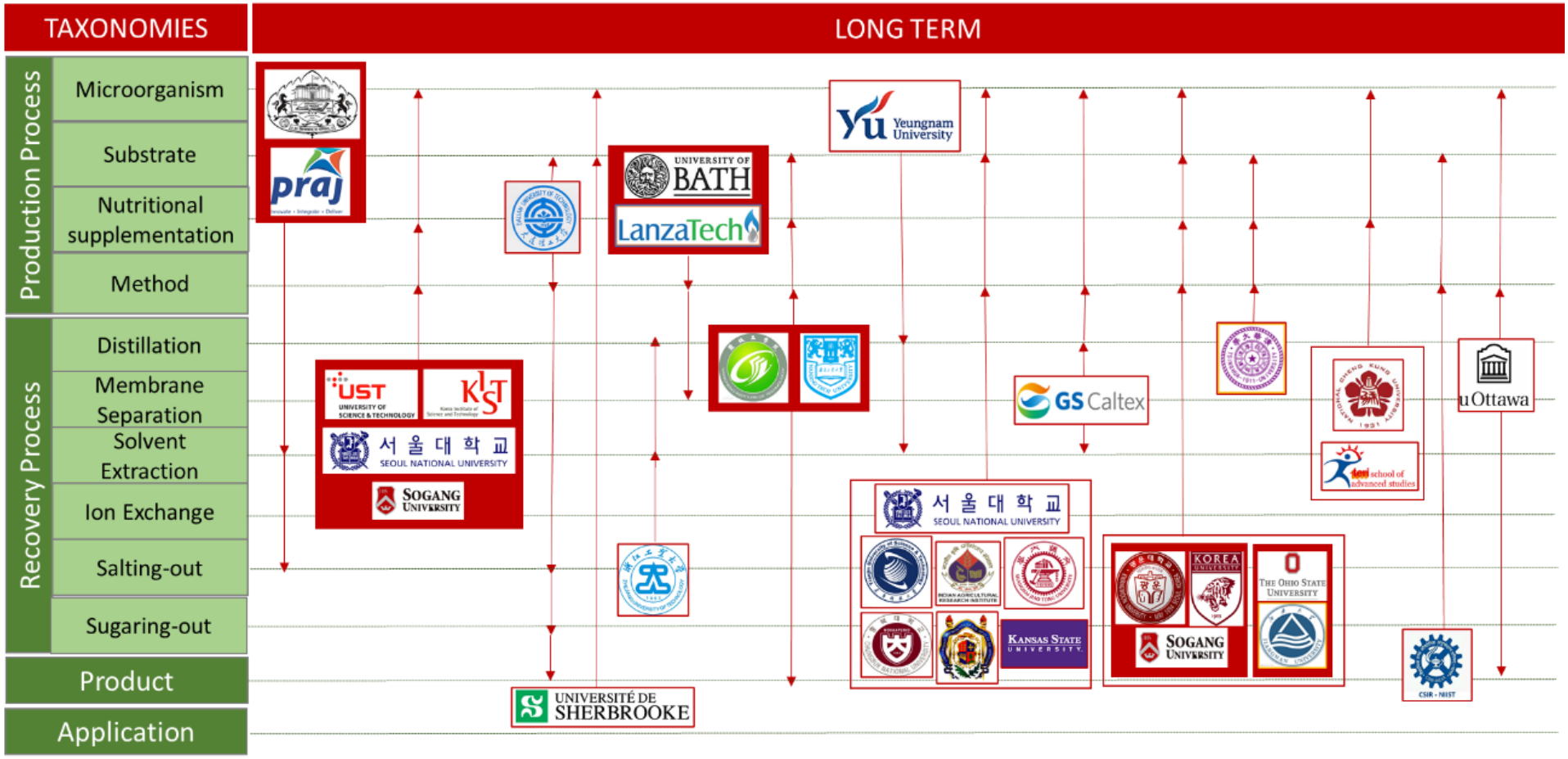


Figure 2.10: Technological Roadmap of the bio-based 2,3-BDO process in the long term.

3.5 Player analysis: Clusters and technological trajectory

Two types of clusters were identified: the same business and partnerships. The same business clusters showed similar technological trends among the main players, while the partnership clusters, also called cooperation networks, showed the existence of combined efforts and resources between independent partners in a joint value creation process (COELHO, BORSCHIVER, 2016). The development of the bio-based 2,3-BDO process was largely marked by partnerships between the players over time: 16.7% (current stage), 12.5% (medium term), and 70% (long term). In the short term, no partnerships were verified in the analyzed studies. These partnerships were formed between: universities of the same nationality (Kwangwoon University, Korea University, and Sogang University) (JOO, LEE, *et al.*, 2016) or from different countries (Jiangnan University and The Ohio State University) (YANG, Tao-Wei, RAO, *et al.*, 2013); companies (LanzaTech and Orochem); research centers and universities (Korea Institute of Science and Technology, Seoul National University, Korea University of Science and Technology, and Sogang University) (CHO, KIM, WOO, LEE, *et al.*, 2015); and universities and industries (University of Bath and LanzaTech) (DAVEY, HAVILL, *et al.*, 2016). Partnerships between public and private players have demonstrated the need to combine scientific knowledge and strategic management for an economically and productively efficient industrial production of bio-based 2,3-BDO.

The technological trajectory of bio-based 2,3-BDO was initiated in the 20th century with the research of Harden and Walpole (1906) and Harden and Norris (1912), followed by the industrial production by Fulmer (1933), during II World War (JI, HUANG, *et al.*, 2011). During this period, the produced 2,3BDO was used for the rubber synthesis (1,3-butadiene) from alternative oil sources (CELIŃSKA, GRAJEK, 2009). After the War, the chemical route was again considered economically advantageous. Only with the oil crisis in 1970, the use of biomass was extensively investigated in large-scale 2,3-BDO production (VOLOCH, JANSEN, *et al.*, 1985). Currently, with the petroleum resources in decline, the environmental risks of greenhouse gas emission, and the fluctuation of crude oil market price, in addition to the biorefineries development and the circular economy concepts (HAZEENA, SINDHU, *et al.*, 2020), the bio-based 2,3-BDO process has been seen as an interesting industrial alternative by different organizations.

Among the identified players, the Yancheng Institute of Technology (YIT - China) and LanzaTech (USA) presented studies on the bio-based 2,3-BDO process in the short, medium, and long terms. Lanzatech has also already industrially performed this bioprocess in the current stage. YIT's studies have been based on the Production Process and Product drivers, with an emphasis on the solid-state fermentation method (short term) (JIAN, HONG, *et al.*, 2014, LI, Z, CAN, *et al.*, 2016), supplemented by amino acids (medium term) (GAO, J, CAO, *et al.*, 2017, XUE, TONG, *et al.*, 2017), and using an engineered *P. polymyxa* strain (long term) (CAO, ZHANG, *et al.*, 2017, GAO, J., JIANG, *et al.*, 2019) to increase the 2,3-BDO selectivity. In turn, LanzaTech has focused on the Production and Recovery Process, and Application drivers, by investigation of the bio-based 2,3-BDO use as a high octane fuel (current stage), produced by *Clostridium* strain (short term) (SIMPSON, TRAN, *et al.*, 2013), from carbon monoxide gas as a substrate (medium term) (KOEPKE, NAGARAJU, *et al.*, 2013), and recovered by membrane separation process (long term) (DAVEY, HAVILL, *et al.*, 2016). The research evolution and the driver focus change have showed the optimization importance of each bioprocess step, so that the bio-based 2,3-BDO production can then be industrially consolidated.

3.6 Economy and market of bio-based 2,3-BDO

The 2,3-BDO market price is currently similar to the 1,4-BDO, around 3.23 $/kg (TAN, SNOWDEN-SWAN, *et al.*, 2017). An economic evaluation of the 2,3-BDO production from glycerol, sucrose, and sugarcane molasses confirmed the minimum selling price (MSP) to be above 3.00 $/kg, considering a discount cash flow evaluation (KOUTINAS, YEPEZ, *et al.*, 2016). For the MSP to be commercially viable, the 2,3-BDO titer in fermentation broth must be at least 80 g/L (MAGEE, KOSARIC, 1987) so that the product recovery step is economically favored (CELIŃSKA, GRAJEK, 2009). MSP of 3.12 $/kg and 2.67 $/kg were achieved for an annual production of 10000 tonnes and 50000 tonnes of 2,3-BDO from very high polarity sugar, respectively, with a titer of 86.8 g/L. This production resulted in about 3.95 g/L/h of 2,3-BDO by *Enterobacter ludwigii* in fed-batch fermentations (MAINA, STYLIANOU, *et al.*, 2019). The substrate nature can also impact the final MSP. In a recent study, a biomass conversion process resulting from the pretreatment of high-load deep eutectic solvent was carried out for the production of 2,3-BDO, furfural, and lignin. The MSP was estimated at 1.703-1.736 $/kg,

about half the current 2,3-BDO market price (ZANG, SHAH, *et al.*, 2020). The MBSP can also base on the utility costs for certain raw materials. In techno-economic analysis on the 2,3-BDO production from glycerol, the estimated utility costs of around 1.30 $/kg were used for the MSP calculations, since crude glycerol had zero cost (0 $/kg) (PRIYA, LAL, 2019). In general, the bio-based 2,3-BDO MSP is greater than 1.00 $/kg, which characterizes it as a chemical platform of industrial interest (KOUTINAS, YEPEZ, *et al.*, 2016).

4. Conclusion

Technology roadmap built here allowed to establish the technological maturity and the main limiting factors for the commercial production of bio-based 2,3-BDO. Production Process driver, investigated in 53.25% of studies, was related to the substrate, engineered microorganisms, efficient production methods, and nutritional supplementation in the current stage, short and medium terms. Low-cost feedstocks such as lignocellulosic materials and biodiesel-derived glycerol have been used to cheaper the bioprocess (32% of studies). Fed-batch mode, solid fermentation, and simultaneous saccharification and fermentation can improve the substrate availability to the biological agent, having been prioritized in industrial fermentations (29% of studies). Highly productive and selective strains to one of the 2,3-BDO isomers have been used to reduce the downstream step costs. These strains have been designed by genetic and metabolic engineering (32.5% of studies). Finally, nutrients such as acetate and nitrogen compounds have been used as supplements to increase the 2,3-BDO yields (6.5% of studies). Product recovery has also been considered a bioprocess critical step, especially in the long term, being investigated in 23.5% of studies. Conventional distillation (50% of studies in the current, short and medium terms) and solvent extraction (36% of long-term studies) have been employed in bio-based 2,3-BDO purification, in which the industrial implementation still depends on future studies and large-scale tests. Few companies like LanzaTech, GS Caltex Corporation, and SINOPEC have been identified as commercial producers of bio-based 2,3-BDO in the current stage and short term, while Asian academic research institutions like Yancheng Institute of Technology and Dalian University of Technology (Chinese universities) dominated long-term studies, showing that the technological maturity still depends on laboratory approaches. Therefore, greater

investments and new partnerships between industry and academia are necessary to consolidate the bio-based 2,3-BDO process at an industrial level, aiming at reaching a production cost of around 1.00 $/kg, equivalent to production costs of conventional platform chemicals.

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CHAPTER 3

Contextualization

In this chapter, a literature review on the bio-based 2,3-BDO production by *Paenibacillus* strains was performed. Microorganisms of the *Paenibacillus* genus are classified as safe and are used as an alternative to traditional microbial 2,3-BDO and acetoin producers. Three main species were investigated: *P. polymyxa*, *P. peoriae*, and *P. brasilensis*. No previous studies have been reported on the 2,3-BDO production by *P. peoriae* strain to date, although this bacterium is phylogenetically related to *P. polymyxa* and *P. brasilensis* and therefore capable of synthesizing 2,3-BDO and acetoin. The 2,3-BDO metabolism by the *P. polymyxa* strain was investigated to confirm this hypothesis. Information on the 2,3-BDO-isomers formation, fermentation conditions, culture medium composition, microbial genetic improvement, and 2,3-BDO separation and purification steps were summarized to be used in future research development involving *P. peoriae* strains.

Therefore, the specific objectives of this chapter were:

- Investigate the 2,3-BDO metabolic pathway: 2,3-BDO isomer and by-products formation;
- Investigate the culture conditions for the bio-based 2,3-BDO production by *Paenibacillus* strains: oxygen supply, pH, temperature, nutritional supplementation, and use of agro-industrial residues;
- Investigate the main microbial enhancement strategies;
- Investigate 2,3-BDO recovery and cell separation and recycling;
- Investigate 2,3-BDO applications, derivatives, and the global market.

Relevance

Through the investigation carried out in this chapter, it was possible to understand:

- The novel 2,3-BDO production degree by *P. peoriae* NRRL BD-62;

- The metabolic potential and fermentation parameters leading to high 2,3-BDO production efficiency, including raw materials, nutritional supplementation, culture conditions, fermentation modes, and isomeric selectivity;
- The biotechnology advances and process improvement involving the upstream, mainstream, and downstream steps;
- The 2,3-BDO global market, its industrial implementation, and applications.

BIOPROCESS DEVELOPMENT FOR 2,3-BUTANEDIOL PRODUCTION BY *Paenibacillus* STRAINS

Abstract

This review focuses on the fermentation potential of GRAS (generally recognized as safe) bacteria that belong to *Paenibacillus* species on the production of 2,3-butanediol (2,3-BDO). *P. polymyxa*, *P. peoriae*, and *P. brasilensis* have been reported as highly efficient microbial producers of 2,3-BDO and acetoin. The factors that contribute to the fermentative production of 2,3-BDO are addressed with particular emphasis on crucial fermentation parameters (i.e. oxygen supply, pH and temperature), precursors that increase fermentation efficiency and the significance of metabolic pathways. Several carbon sources from renewable resources can be consumed by *Paenibacillus* strains, which favors the development of alternative biorefinery concepts including the production of 2,3-BDO within a circular bio-economy approach. Downstream separation and purification processes used for the recovery of bio-based 2,3-BDO are also discussed. The production of 2,3-BDO isomers with high stereoisomeric purity can lead to new applications and markets. The use of *Paenibacillus* strains could be a viable alternative to biosafety level 2 strains that have been widely investigated for the production of 2,3-BDO.

Keywords: Bio-based 2,3-butanediol; GRAS *Paenibacillus* strains; bioprocess efficiency; stereoisomer purity; fermentation parameters.

1. Introduction

The bio-based production of bulk chemicals is challenging and has been motivated by the demand for reduced use of fossil fuels. 2,3-butanediol (2,3-BDO) is an odorless, colorless, and transparent liquid classified as a platform chemical with many potential applications in pharmaceutical, cosmetic, and polymer industries. Table 3.1 presents the physicochemical properties of 2,3-BDO, also called dimethylene glycol, 2,3-dihydroxybutane, and 2,3-butylene glycol.

Table 3.1: Physicochemical properties of 2,3-BDO

Physicochemical properties	**Description**
General classification	Colorless, odorless, and highly viscous alcohol of molecular formula
Molecular formula	$C_4H_{10}O_2$
Molecular mass	90.121 g/mol
Melting Temperature	-60 ºC (*levo* and *dextro*) and 33-34.5 ºC (*meso*)
Boiling Temperature	177-182 ºC
Combustion heat	27.2 kJ/g
Octane rating	High
Biodegradability	Yes
Conversion possibility	High and versatile

It can be found in three different enantiomeric forms: *levo* (2R,3R), *dextro* (2S,3S), and *meso* (2R,3S), whose industrial applications depend on their chirality properties (Fig. 3.1). 2,3-BDO can be used as an intermediate for the production of methyl ethyl ketone, diacetyl, polyurethane, and 1,3-butadiene (MAINA, STYLIANOU, *et al.*, 2019). It can also be used as a drop-in fuel additive due to its high heating value, comparable to other liquid fuels, and low vapor pressure (CELIŃSKA, GRAJEK, 2009, HAIDER, QYYUM, *et al.*, 2018, YANG, ZHANG, 2019). Its significance as a platform chemical lies in the three isomeric forms (*levo*, *dextro*, *meso*) that can provide different properties for versatile applications. Both *levo* and *dextro* isomers have chiral properties and can be used in asymmetric synthesis for fine chemicals, pharmaceuticals, and other applications. The *levo* isomer has a low freezing point (-60 ºC) therefore it can be used as an anti-freezing agent (KOUTINAS, VLYSIDIS, *et al.*, 2014).

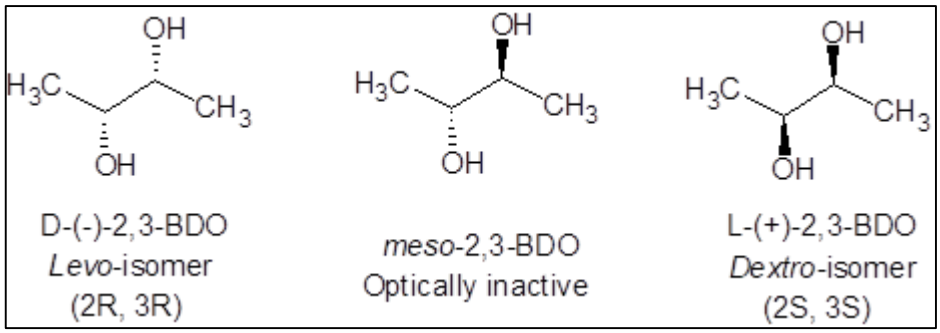

Figure 3.1: Isomeric forms of 2,3-BDO.

Production of 2,3-BDO has been reported by various microorganisms, including *Klebsiella* sp., *Enterobacter* sp., and *Serratia* sp. which are classified as biosafety level 2 microorganisms (KIM, PARK, *et al.*, 2016, PSAKI, MAINA, *et al.*, 2019). The highest 2,3-BDO concentration has been achieved by *Klebsiella pneumoniae*, about 150 g/L (MA, Cuiqing, WANG, *et al.*, 2009). Despite the high fermentation efficiencies, industrial applications would only be feasible using bacteria that lack pathogenity. GRAS microorganisms like *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *Paenibacillus* species can also achieve high 2,3-BDO production efficiency. The highest 2,3-BDO concentration achieved by *Paenibacillus polymyxa* was 111 g/L (HÄßLER, SCHIEDER, *et al.*, 2012). Moreover, these bacteria can produce optically pure *levo*-2,3-BDO (up to 98% purity) (LI, Jinshan, WANG, *et al.*, 2013, NAKASHIMADA, Yutaka, MARWOTO, *et al.*, 2000). Engineering strategies for the production of 2,3-BDO have also been followed in *Enterobacter aerogenes* (THAPA, LEE, *et al.*, 2019) and model microorganisms like *Escherichia coli, Saccharomyces cerevisiae,* and *Lactococcus lactis* (JUNG, JANG, *et al.*, 2013, YANG, ZHANG, 2019).

The 2,3-BDO market price is currently similar to the 1,4-BDO, around 3.23 $/kg (ZANG, SHAH, *et al.*, 2020). The fermentation process could be a sustainable alternative of provided that it generate an economic advantage. The production cost of any chemical should be around 1 $/kg to be considered as a platform chemical (KOUTINAS, YEPEZ, *et al.*, 2016). The production cost of 2,3-BDO is dependent on the cost of carbon source, the use of complex nutrient supplements, and the fermentation efficiency (KOUTINAS, YEPEZ, *et al.*, 2016). The utilization of renewable resources could contribute to the sustainability of the production process. Economic analysis of biological production of 2,3-BDO from very high polarity (VHP) sugar from sugarcane mills in fed-batch cultures

using *Enterobacter ludwigii* at optimum fermentation efficiency (86.8 g/L 2,3-BDO concentration, 0.37 g/g yield, and 3.95 g/L/h productivity) has been estimated. The minimum selling price was $ 3.12 /kg and $ 2.67 /kg for annual production capacities of 10,000 t and 50,000 t, respectively, for the VHP cane sugar market price of $ 0.4 /kg (MAINA, STYLIANOU, *et al.*, 2019).

The economic and environmental sustainability of a biobased process depends on numerous factors that include: 1) the type and pretreatment of the raw material, 2) the optimization of the fermentation, and 3) the downstream separation and purification. Therefore, the selection of a microorganism that can consume a wide range of carbon sources leading to high 2,3-BDO concentration, productivity, and yield is crucial for the development of a bio-based process (DHESKALI, KOUTINAS, *et al.*, 2020). Moreover, the production of pure 2,3-BDO with low concentrations of by-products will lead to a low number of unit operations in the downstream separation and purification stage. Utilization of crude renewable resources, such as industrial by-products or waste streams, contributes to the reduction of production costs (BIAŁKOWSKA, 2016).

Paenibacillus species have gained attention for the production of 2,3-BDO not only due to its biosafety level 1 status but also since certain strains can produce the optically active isomers at high concentrations (NAKASHIMADA, Y., KANAI, *et al.*, 1998). Therefore, this paper aims to review 2,3-BDO production by various *Paenibacillus* strains focusing also on their metabolic potential and the fermentation parameters leading to high 2,3-BDO production efficiency (e.g. raw materials, cultivation conditions, bioreactor operation, isomer selectivity). The global market and the viable 2,3-BDO industrial implementation and application were also approached.

2. The *Paenibacillus* genus

The genus *Paenibacillus* was defined in 1993, after comparative DNA analysis of a group of bacterial species previously classified to the genus *Bacillus* (ASH, PRIEST, *et al.*, 1993). *Paenibacillus* species form rod-shaped cells, with Gram-positive structure (they can stain variable or negative in the laboratory), able to form endospores. Motility occurs with a peritrichous flagella. They are facultative anaerobes or strictly aerobes. Optimum growth occurs at 28–40 °C and pH 7, while some species are alkaliphilic

(WHITMAN, TRUST, 2015). The genus *Paenibacillus* can be found in a variety of environments. The majority of them is found in soil where they are plant growth-promoting rhizobacteria that act via nitrogen fixation, phosphate solubilization, or iron release (DAUD, MOHD DIN, *et al.*, 2019). *Paenibacillus* species can assimilate a wide range of pentoses and hexoses as well as lignocellulosic hydrolysates and produce industrial enzymes (SELDIN, 2011) such as amylases, cellulases, hemicellulases, lipases, pectinases, lignin-modifying enzymes, and mutanases. They can also produce phytohormones (indole-3-acetic acid), exopolysaccharides (EPS), and 2,3-BDO. Moreover, they can produce antimicrobial compounds that offer plant protection against various plant diseases. However, some species can cause a lethal disease of honeybees and can potentially cause human infections (GRADY, MACDONALD, *et al.*, 2016).

2.1 Paenibacillus strains producing 2,3-BDO

Paenibacillus can produce the *levo* isomer with a high degree of purity, accompanied by the *meso* isomer (NAKASHIMADA, Y., KANAI, *et al.*, 1998). A metabolic product at high concentration with low by-product concentration is desirable since the number of unit operations associated with product recovery can be reduced (KOUTINAS, YEPEZ, *et al.*, 2016). The main representative of the *Paenibacillus* genus is *Paenibacillus polymyxa* the wild-type strains of which can produce up to 98% purity of the *levo* isomer (UI, MASUDA, *et al.*, 1986), while engineered strains can reach around 99.99% *levo* isomer purity (ZHANG, Li, CAO, *et al.*, 2018). New species are being investigated regarding their production efficiency and isomer purity level, with emphasis on *Paenibacillus brasilensis* and *Paenibacillus peoriae*.

2.1.1 Paenibacillus polymyxa

Its taxonomical order has been formed as follows: Bacteria; Terrabacteria group; Firmicutes; Bacilli; Bacillales; *Paenibacillaceae*; *Paenibacillus polymyxa*. Cells are gram-positive, neutrophilic, flagellated heterotrophic, rod-shaped of 0.6 to 3.0 μm and spore-forming. *P. polymyxa* is considered to be a facultative anaerobe, capable of producing EPS under certain cultivation conditions. It can assimilate different carbon sources, such as glucose, mannose, xylose, arabinose, mannitol, cellobiose, trehalose, lactose, maltose, sucrose, and glycerol. It can form acetoin, ethanol, organic acids, and

2,3-BDO. Its optimum growth conditions vary at temperatures from 30 to 37 ºC and 4 to 7 pH values (DE MAS, JANSEN, *et al.*, 1988).

The size of *P. polymyxa* genome is 5,775,351 bp (GenBank accession number JMIQ00000000) with a GC content of 45.5%. The genome contains 4,784 protein-coding genes, 6 rRNA genes, and 55 tRNA genes out of 4,964 genes. Annotation showed that 2,245 proteins have KEGG orthologs and 3,779 proteins have COG classifications out of 3,856 proteins that have clear biological functions (XIE, LI, *et al.*, 2015).

2.1.2 Paenibacillus peoriae

P. peoriae cells are gram-positive, occurring individually, in pairs or chains, are rod-shaped, with round ends, from 0.5 to 1.0 µm wide and 3.0 to 6.0 µm long. *P. peoriae* cells are facultative anaerobes. They form butyrous, thin, smooth, circular, translucent colonies in nutrient agar media, with 2 mm diameter. The optimum temperature growth range is 28 to 30 °C. In general, *P. peoriae* cells can produce acids from glucose, xylose, arabinose, fructose, and maltose. Some species can assimilate glycerol as a carbon and energy source (HEYNDRICKX, VANDEMEULEBROECKE, *et al.*, 1996). Some *P. peoriae* cells are phylogenetically related to *P. polymyxa* cells for 2,3-BDO formation (DIAS, LIMA, *et al.*, 2018).

2.1.3 Paenibacillus brasilensis

P. brasilensis cells are facultative anaerobic, gram-positive or gram-variable, rod-shaped, 0.6 to 2.1 µm, motile, and spore-forming. In Brilliant Green agar (BG agar), the colonies are translucent to white, convex, and mucoid. The temperature growth range is 30 to 42 °C and the pH range between 5 and 7. In general, *P. brasilensis* cells can produce acids from glucose, fructose, sucrose, mannose, mannitol, cellobiose, galactose, and lactose. However, it is not able to assimilate glycerol and xylose as sources of carbon and energy (VON DER WEID, DUARTE, *et al.*, 2002). *P. brasilensis* cells are also phylogenetically related to *P. polymyxa* cells for 2,3-BDO formation (ADLAKHA, PFAU, *et al.*, 2015).

3. Microbial production metabolism

3.1 2,3-BDO metabolic pathway

The catabolism of glucose in *P. polymyxa* has been described using genome-based metabolic modeling (ADLAKHA, PFAU, *et al.*, 2015). Catabolism of hexose occurs via Glycolysis using the Embden–Meyerhof–Parnas pathway. A bypass pathway named "Bifid shunt" has been found for the first time in *P. polymyxa*, which allows flexible regulation of redox and energy requirements depending on the metabolic state of the microorganism (ADLAKHA, PFAU, *et al.*, 2015). In turn, catabolism of pentose occurs by the combination between the Pentose-Phosphate Pathway and Glycolysis (JANSEN, TSAO, 1983). *P. polymyxa* can consume a wide range of sugars, such as glucose and xylose in addition to sucrose, maltose, lactose, cellobiose among others. Furthermore, some strains can consume glycerol, which has a specific pathway for the 2,3-BDO formation (Fig. 3.2).

Pyruvate can lead to the production of 2,3-BDO with the reaction of three sequential enzymes. α-Acetolactate synthase (ALS) leads to the production of α-acetolactate, which is converted to acetoin via α-acetolactate decarboxylase (ALDC) and 2,3-BDO via 2,3-butanediol dehydrogenase (BDH) or acetoin reductase (JI, HUANG, *et al.*, 2011). Characterization and gene cloning of 2,3-BDO dehydrogenase by *Paenibacillus polymyxa* ZJ-9 showed that this enzyme is a new type of BDH, significantly different than other reported BDHs. It acts more like a reductase of R-acetoin rather than a dehydrogenase (GAO, Jian, YANG, *et al.*, 2013). R-BDH from *P. polymyxa* ATCC 12321 was functionally characterized and classified in a medium-chain dehydrogenase/reductase superfamily, differently from *meso*- and S-BDH, which belong to the short-chain dehydrogenase/reductase superfamily (YU, SUN, *et al.*, 2011). 2,3-BDO is mostly produced under anaerobic conditions where oxygen is not used as an electron acceptor and alternative electron pools are required. Oxidative phosphorylation is not active, therefore additional ATP must be produced. Genome-scale metabolic modeling resulted in the hypothesis that formate-hydrogen lyase uses protons in the form of NADH as electron acceptors resulting in hydrogen production (ADLAKHA, PFAU, *et al.*, 2015).

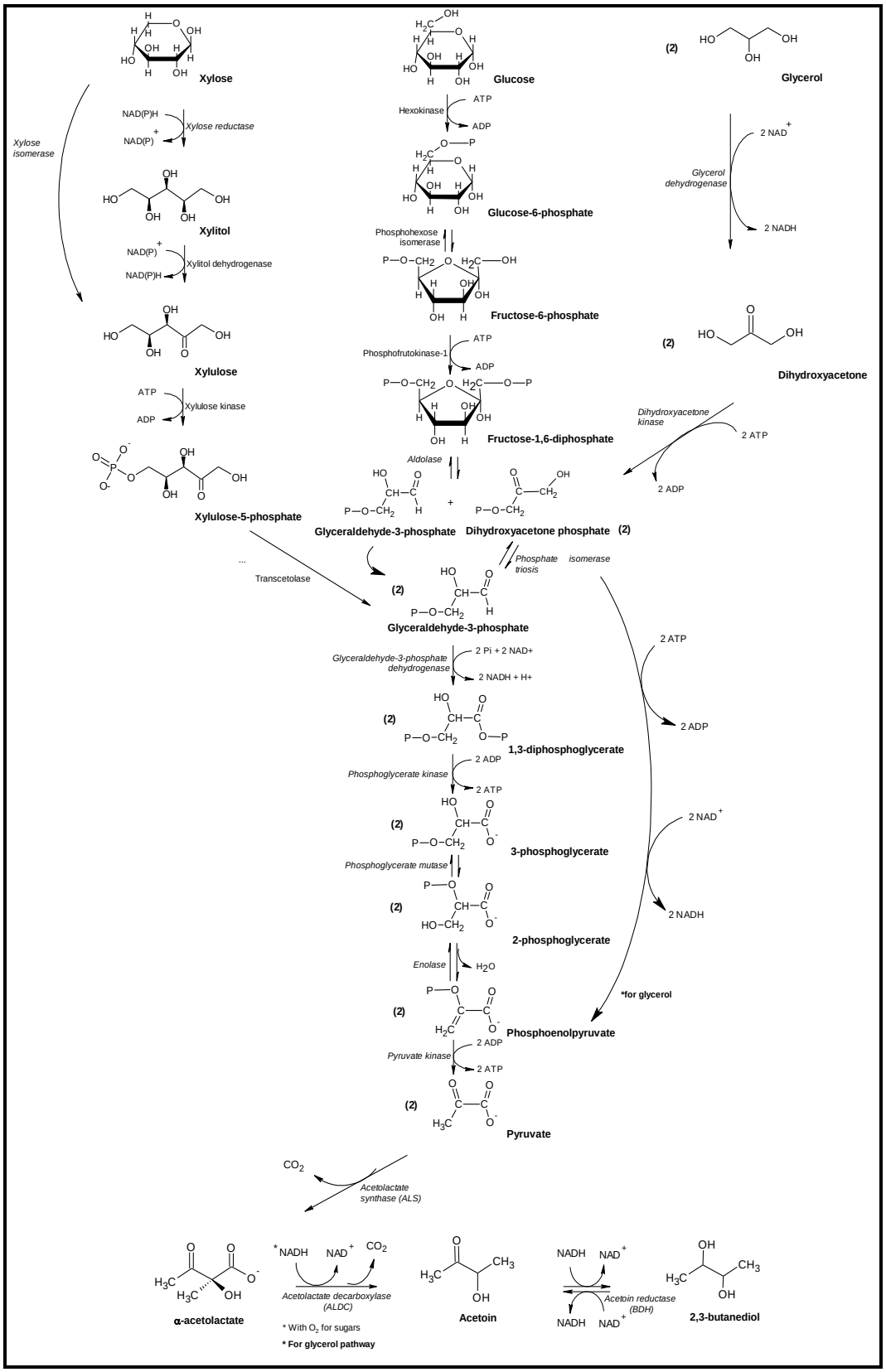

Figure 3.2: Metabolic pathways of 2,3-BDO synthesis from glucose, xylose, and glycerol (based on JIANG, Yudong, LIU, *et al.* (2014), MARWOTO, NAKASHIMADA, *et al.* (2004)).

Production of 2,3-BDO in bacteria is considered a carbon and energy storage mechanism (XIAO, XU, 2007) but it can cause growth inhibition at high concentrations leading to its conversion into acetoin as a defense mechanism (OKONKWO, UJOR, EZEJI, 2017). Specifically, the growth of *P. polymyxa* was completely inhibited when the initial concentration of the *levo* isomer was 60 g/L. The addition of increasing 2,3-BDO concentrations of 20, 40, and 60 g/L at 12, 24, and 36 h, respectively, resulted in the conversion of 2,3-BDO into acetoin when 2,3-BDO reached 60 g/L (OKONKWO, UJOR, EZEJI, 2017).

The theoretical yield (Y_{max}) of 2,3-BDO from glucose, xylose, and glycerol can be calculated according to equations 1, 2 (JANSEN, TSAO, 1983), and 3, respectively (JIANG, Yudong, LIU, *et al.*, 2014). The glycerol pathway requires ATP, while glucose and xylose pathways release ATP. Hence, glycerol has a lower energy value, which justifies the lower 2,3-BDO yield (JIANG, Yudong, LIU, *et al.*, 2014). In both pathways (sugars and glycerol), CO_2 is released and therefore a carbon loss is observed. The CO_2 reintegration to pathways is an important strategy that can be applied to increase the 2,3-BDO yield (BOGORAD, LIN, *et al.*, 2013).

$$C_6H_{12}O_6 + 2ADP + NAD^+ \rightarrow C_4H_{10}O_2 + 2CO_2 + 2ATP + NADH_2 \quad (1)$$

Y_{max}= 0.50 g/g

$$C_5H_{10}O_5 + 5/3\ ADP + 5/6\ NAD^+ \rightarrow 5/6\ C_4H_{10}O_2 + 5/3\ CO_2 + 5/6\ NADH_2 + 5/3\ ATP \quad (2)$$

Y_{max}= 0.50 g/g

$$C_3H_8O_3 + ATP + 2\ NAD^+ \rightarrow 1/2\ C_4H_{10}O_2 + CO_2 + NADH_2 + ADP + H^+ \quad (3)$$

Y_{max}= 0.49 g/g

P. polymyxa also excretes several by-products such as lactate, formate, acetate, acetoin, ethanol, and succinate (Fig. 3). The higher acetate kinase and phosphoketolase activities, besides the lower acetate uptake ability, were found in the xylose-based cultivation of *P. polymyxa* ATCC 12321. Xylose catabolism was found to enhance acetate

production (0.11 g/g) and decrease 2,3-BDO yield (0.25 g/g) compared to glucose-based cultures (yield of 2,3-BDO: 0.28 g/g, yield of acetate: 0.003 g/g) (MARWOTO, NAKASHIMADA, *et al.*, 2004).

3.2 2,3-BDO isomer formation by *Paenibacillus*

The ALS, ALDC, and BDH enzymes are essential for understanding the 2,3-BDO metabolism. However, they are not sufficient to explain the *levo*, *dextro*, and *meso* isomers formation by themselves. The isomeric enzymes existence with interconversion catalytic activities stimulated previous investigations about isomers formation models for *P. polymyxa* (UI, MASUDA, *et al.*, 1986).

The proposed mechanisms for the production of the stereoisomers in *Paenibacillus* involved a set of irreversible reactions from pyruvate to α-acetolactate via ALS or from pyruvate to diacetyl via condensation of acetaldehyde and acetyl-CoA. α-Acetolactate can be converted to R-acetoin via ALDC or diacetyl via spontaneous decarboxylation under O_2 availability. In turn, S-acetoin is formed from diacetyl via NADPH-dependent DAR (UI, MASUDA, *et al.*, 1986). A set of reversible reactions can lead to the production of the three 2,3-BDO isomers: R-2,3-BDO from R-acetoin via R-BDH; S-2,3-BDO from S-acetoin via S-BDH; and *meso*-2,3-BDO from R-acetoin via S-BDH or from S-acetoin via R-BDH (DIAS, LIMA, *et al.*, 2018) (Fig. 3.4).

Three types of genes are related to 2,3-BDO isomers synthesis: *butA*, *butB*, and *butC*. Together with the regulatory gene *butR*, these genes form the *but* operon (CHEN, WEI, *et al.*, 2014). The *butA* and *butB* genes was observed in the genus *Paenibacillus*. The *butA* gene is responsible for coding the S-BDH, while the *butB* gene for coding the R-BDH. *P. polymyxa* and *P. peoriae* present only *butB* gene, which allows the *levo*- and *meso*-2,3-BDO production. In turn, *P. brasilensis* can present both *butA* and *butB* genes, consequently being able to also produce S-2,3-BDO (DIAS, LIMA, *et al.*, 2018). However, the expression of these genes for the production of the 2,3-BDO isomers depends on the fermentative conditions. Although the *but*A gene was found in the genome of *P. brasilensis* PB24, only *levo* and *meso* isomers were formed under the growth conditions of 32 °C and 200 rpm in shake flasks (DIAS, LIMA, *et al.*, 2018).

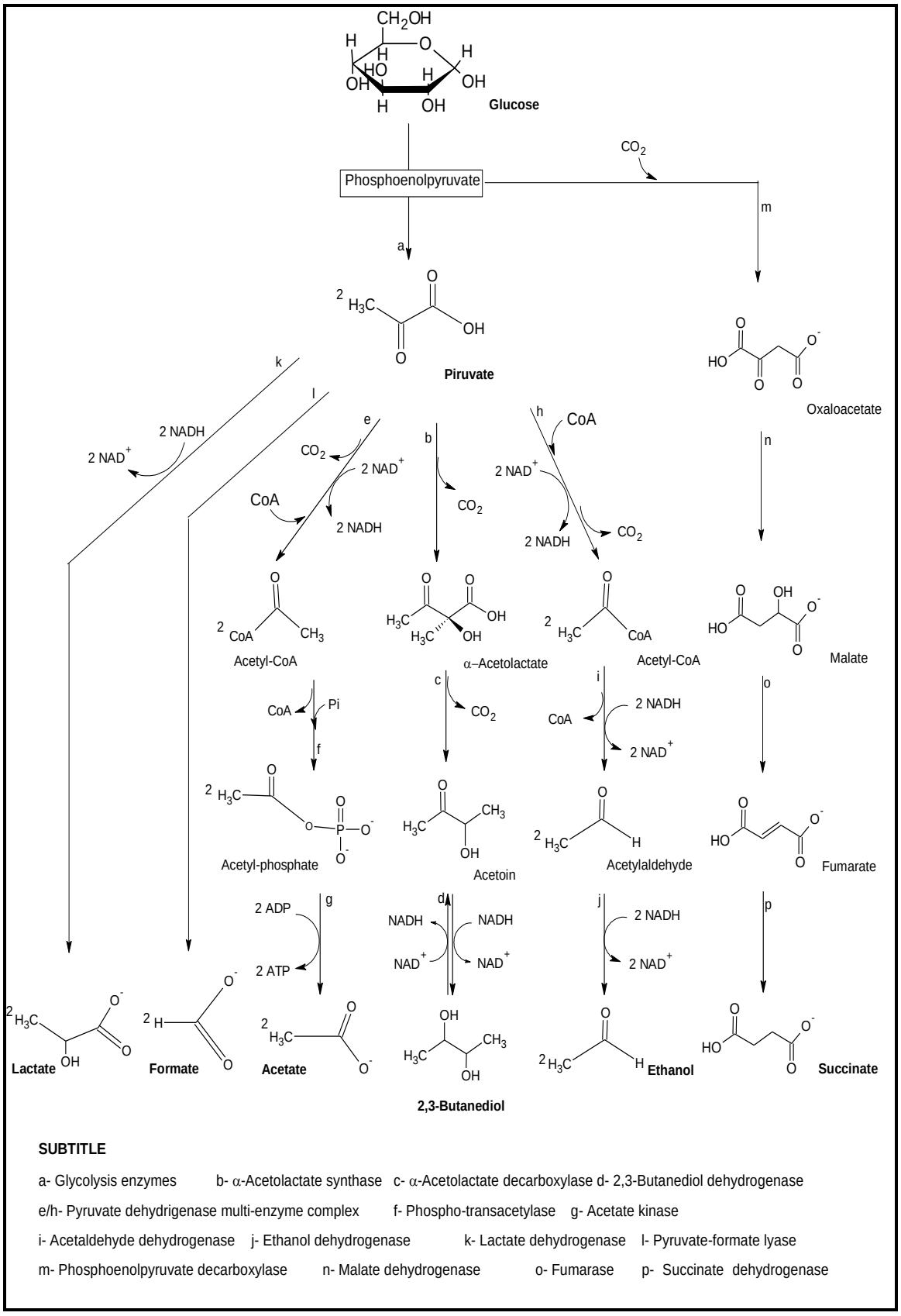

Figure 3.3: Metabolic route for the 2,3-BDO and by-products production (based on BIAŁKOWSKA (2016), CELIŃSKA, GRAJEK (2009)).

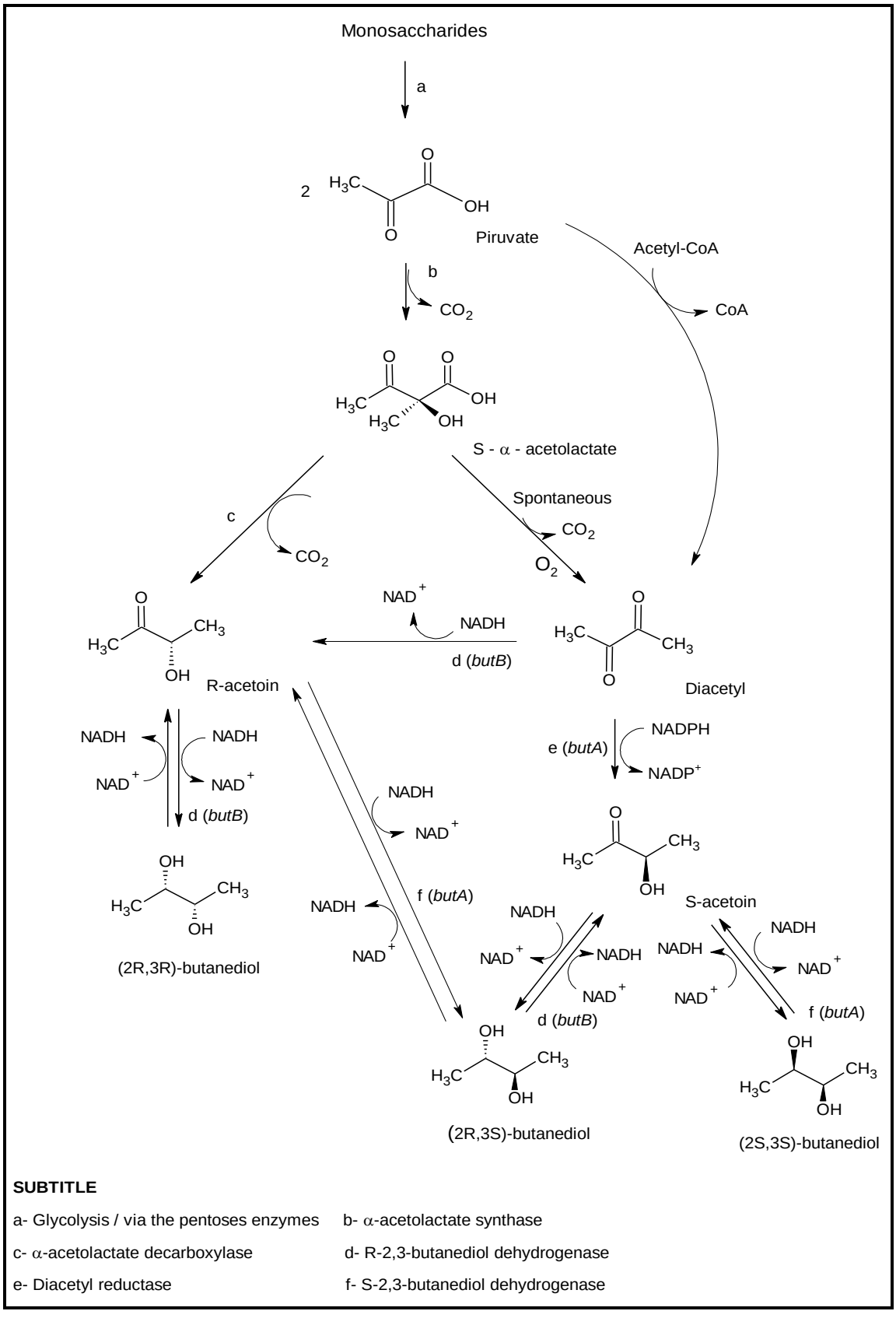

Figure 3.4: Bio-based 2,3-BDO isomers formation mechanism by *Paenibacillus* (based on DIAS, LIMA, *et al.* (2018), UI, MASUDA, *et al.* (1986)).

3.3 Cultivation Conditions

3.3.1 Oxygen Supply

Oxygen supply is one of the most important factors in 2,3-BDO production via fermentation. Facultative anaerobes like *Paenibacillus* species are capable of respiration and/or fermentation metabolic processes. During respiration, oxygen is an electron acceptor, therefore air supply directly affects cell metabolism by regulating the internal redox balance (CONVERTI, PEREGO, *et al.*, 2003). The production of different metabolites depends on the relative oxygen availability. Experiments in *Bacillus polymyxa* showed that acetate and acetoin production was favored at high oxygen availability, 2,3-BDO was favored at intermediate oxygen availability and lactate and ethanol were produced under low oxygen availability (DE MAS, JANSEN, *et al.*, 1988).

Experiments in *B. licheniformis* showed that under aerobic conditions acetoin or 2,3-BDO are not produced, while under oxygen limiting conditions respiration and fermentation processes might occur simultaneously and therefore acetoin, 2,3-BDO, ethanol, and glycerol are being produced. The balance in $NADH/NAD^+$ pool determines which of these fermentation products will prevail. For example, when oxygen availability becomes too critical, ethanol and glycerol productions are favored while acetoin and 2,3-BDO production are limited. Furthermore, the reversible reaction between acetoin and 2,3-BDO is shifted towards the diol under microaerobic conditions (REBECCHI, PINELLI, *et al.*, 2018). Furthermore, under oxygen limiting conditions, the respiration process is limited, which results in an impasse between cell growth and 2,3-BDO production: while the low cell density resulting from the low oxygen supply compromises the 2,3-BDO conversion rate, this same oxygen limitation promotes the NADH reoxidation and consequent NAD^+ regeneration, increasing 2,3-BDO yield (HAKIZIMANA, MATABARO, *et al.*, 2020).

Oxygen availability depends on different parameters including air flow rate, agitation speed, and bioreactor geometry. The oxygen supply can be measured by oxygen uptake rate (OUR), oxygen transfer rate (OTR), and/or the volumetric oxygen transfer coefficient ($k_L\alpha$) (REBECCHI, PINELLI, *et al.*, 2018). Although $k_L\alpha$ is a reliable parameter for identifying oxygen supply, different methodologies for its determination

generate major challenges especially when scale-up is required (ARONIADA, MAINA, *et al.*, 2020). The effect of k_La on 2,3-BDO production has been assessed in limited studies and reported results are contradictory as the oxygen transfer required by different strains to maximize 2,3-BDO production is variable (HEYMAN, LAMM, *et al.*, 2019). The effect of $k_L\alpha$ on 2,3-BDO production by *P. polymyxa* has been investigated using a programmed control mode to keep the respiration and fermentation pathways active on the substrate used. Three different $k_L\alpha$ levels were evaluated throughout cultivation (40 h^{-1} at 0-19 h, 21 h^{-1} at 19-41 h, and 8 h^{-1} at 41-55 h) resulting in 44 g/L of 2,3-BDO concentration with 0.79 g/L/h productivity (FAGES, MULARD, *et al.*, 1986).

Due to this limitation and considering that microaerobic conditions are necessary for 2,3-BDO metabolism, the respiratory quotient (RQ) is used as an effective control parameter, mainly on the large-scale processes (ZENG, A. -P, BYUN, *et al.*, 1994). The RQ corresponds to the ratio between the CO_2 production rate and the O_2 consumption rate for a specific microbial metabolism. An RQ value between 4 and 4.5 for the 2,3-BDO is considered optimum (JI, HUANG, *et al.*, 2011, REBECCHI, PINELLI, *et al.*, 2018). 2,3-BDO production with a RQ control algorithm in fed-batch cultures led to 96 g/L concentration against approximately 78 g/L with an OTR control (ZENG, A. -P, BYUN, *et al.*, 1994). The RQ set combined with constant residual sucrose concentration in fed-batch cultures was performed considering anaerobic and micro-aerobic conditions. The RQ was set to 1-1.5 for the cell growth and 1.8-2 for the 2,3-BDO production (ZHANG, Liaoyuan, YANG, *et al.*, 2010). *P. polymyxa* fermentation has not been reported to apply RQ control but oxygen supply based on agitation and aeration system have been evaluated.

Agitation and aeration are responsible for increasing the exposure of substrates to cell culture and to disseminate oxygen, nutrients, and metabolites formed to the entire culture medium. In previous investigations, the 2,3-BDO fermentations were normally carried out between 200 and 400 rpm (HAKIZIMANA, MATABARO, *et al.*, 2020). At low agitation, a biofilm can be formed, which reduces the 2,3-BDO yield since the cells are unavailable to carry out the metabolic activities (HÄßLER, SCHIEDER, *et al.*, 2012). At high agitation, greater energy demand is required. A two-stage fermentation started with 400 rpm agitation that was subsequently reduced to 300 rpm after 14 h resulted in

an increased NADH/NAD^+ ratio which led to acetoin conversion into 2,3-BDO. Final 2,3-BDO concentration was 21.93 g/L, with a yield of 0.4 g/g and a productivity of 1.46 g/L/h (DAI, Jun-Jun, CHENG, *et al.*, 2014).

The oxygen supply can also alter the level of 2,3-BDO optical purity. An increase in oxygen supply during cell culture led to an increased *meso*-2,3-BDO production by *P. polymyxa* ATCC 12321, which represented an approximately 5% reduction in *levo*-2,3-BDO formed (purity reduced from 98% under anaerobic conditions to 93% at an OTR of 6.7 $mmol^{-1}.h^{-1}$) (NAKASHIMADA, Y., KANAI, *et al.*, 1998). In metabolic terms, this optical change indicates a possible release of greater amounts of NAD(P)H-acetoin dehydrogenase which leads to *meso*-2,3-BDO formation. *Levo*-2,3-BDO selectivity was greater than 90% under limited oxygen supply (from 50% compressed air to 10%) (LI, Jinshan, WANG, *et al.*, 2013).

Another parameter also investigated for the oxygen supply control in microaerobic cultivations is the specific oxygen transfer rate, expressed per cell biomass unit ($mmolO_2/g_{cell}/h$) (CONVERTI, PEREGO, *et al.*, 2003). In a microaerobic condition, the oxygen concentration is negligible in terms of advection in the batches input/output currents, fed-batches, and continuous regimes. Then, this rate is relatively equal to the specific oxygen consumption rate (q_{O2}). The q_{O2} must be maintained between the respiration and fermentation limits so that the 2,3-BDO production process can be favored with maximum efficiency. The best results for the 2,3-BDO production were obtained for a range between 3.5 and 5.0 $mmolO_2/g_{cell}/h$ (REBECCHI, PINELLI, *et al.*, 2018). Again, no studies about q_{O2} control for 2,3-BDO production by *P. polymyxa* strain has been investigated to the moment.

Although kLa is traditionally used as a scale-up parameter, studies show that RQ and q_{O2} are also good criteria for this objective. However, kLa, RQ and q_{O2} parameters are not easily determined (REBECCHI, PINELLI, *et al.*, 2018). Thus, strategies based on agitation speed and aeration rate are often carried out, aiming at scale-up practices (Table 2).

3.3.2 pH and temperature

The pH is an important parameter in the 2,3-BDO production metabolism since it can significantly influence carbon assimilation and the intracellular environment. It has been reported that there is a negative correlation between 2,3-BDO productivity and pH (HAKIZIMANA, MATABARO, *et al.*, 2020). Compared to organic acid production, 2,3-BDO production can be understood as a control intracellular pH metabolic strategy, given its neutral nature. Furthermore, 2,3-BDO is considered to be a less toxic compound when compared to organic acids, which justifies its synthesis throughout the medium acidification process, resulting from the activation of the acid-forming pathway. However, 2,3-BDO production occurs until the pH does not become high enough to promote cellular enzymatic inactivation. Thus, the pH gradient can be seen as responsible for controlling 2,3-BDO metabolism (MADDOX, 1996). In general, 2,3-BDO is produced at a pH range of 5-6.5 (HAKIZIMANA, MATABARO, *et al.*, 2020). Table 2 shows that for *P. polymyxa* strains an optimal pH range for 2,3-BDO production is 5.2-7.0.

The temperature is the fermentative parameter related to bioprocess efficiency, given its influence on enzymatic and cellular activities. In general, 2,3-BDO microbial production occurs between 30 ºC and 39 ºC due to maximum biomass production (PEREGO, CONVERTI, *et al.*, 2003). Below the temperature range, the cell regulation and the metabolic rate can be compromised, while above the temperature range, especially above the optimum temperature, the cells and enzymes can be altered (HAKIZIMANA, MATABARO, *et al.*, 2020). As with pH, the ideal temperature for 2,3-BDO formation is also dependent on the microorganism used. Table 3.2 shows that for *P. polymyxa* strains efficient 2,3-BDO production occurs mainly at 30 °C or 37 °C.

3.3.3 Nutrient supplements

Nitrogen is used in protein synthesis, which is directed to the cellular biomass generation. Vitamins are essential nutrients, not synthesized by microorganisms, favoring cell growth and metabolic activities. Trace metals (Mn, K, Mg, and Fe) and phosphates are used, respectively, as enzymatic cofactors, performing specific actions on cellular metabolism, and for the ATP regeneration together with K^+ ions.

The nitrogen source commonly used in 2,3-BDO production is yeast extract. However, its high cost limits its implementation on a commercial scale, forcing it to be replaced by cheaper nitrogen sources, such as ammonium salts, urea, and corn steep liquor (JI, HUANG, *et al.*, 2011). Fed-batch fermentation for 2,3-BDO production with *P. polymyxa* DMS 365 resulted in 111 g/L of 2,3-BDO when the medium contained 60 g/L of yeast extract (HÄßLER, SCHIEDER, *et al.*, 2012). Experimental design was employed in fermentations using *P. polymyxa* DSM 365 to evaluate the impact of tryptone, yeast extract, ammonium acetate, ammonium sulfate, glycerol, temperature, and inoculum. Batch and fed-batch fermentations using the optimum tryptone (3.5 g/L) and yeast extract (5 g/L) concentrations resulted in 2,3-BDO concentrations of 51.1 g/L (yield: 0.42 g/g, productivity: 1.7 g/L/h) and 68.54 g/L (yield: 0.34 g/g, productivity: 0.7 g/L/h), respectively (OKONKWO, UJOR, MISHRA, *et al.*, 2017).

Amino acid supplementation during fermentation using *P. polymyxa* ZJ-9 in inulin resulted in 24.2, 22.3, 22.03, and 21.04 g/L of *levo*-2,3-BDO at the respective key amino acids Asn, Ser, His, and Arg. Sixteen more amino acids resulted in significantly lower 2,3-BDO production (around 12 g/L). A mixture of four amino acids resulted in 25.1 g/L and 17.5 g/L of *levo*-2,3-BDO at fermentations from glucose and pure inulin, respectively (GAO, J., JIANG, *et al.*, 2019). Urea has been supplied as the only nitrogen source in fed-batch cultures with *Bacillus polymyxa* yielding in 0.35 g/g 2,3-BDO to glucose conversion yield (DZIEWULSKI, HAUGHNEY, *et al.*, 1986). Vitamin C can influence the oxidoreduction potential of the fermentation medium. It is relatively inexpensive, which favors its use as a nutritional supplement in the 2,3-BDO synthesis. The addition of vitamin C in the fermentation medium resulted in 71.1 g/L of 2,3-BDO with a productivity of 1.33 g/L/h by *P. polymyxa*, while 2,3-BDO concentration without vitamin C was 43.66 g/L with a productivity of 0.97 g/L/h. The addition of vitamin C resulted in an increased $NADH/NAD^+$ ratio, therefore 2,3-BDO production was enhanced (DAI, Jun-Jun, CHENG, *et al.*, 2014).

3.3.4 Acetic acid as 2,3-BDO enhancer

Acetate, propionate, pyruvate, and succinate can enhance 2,3-BDO production. When around 9 g/L (150mM) of acetate were added to a batch culture of *P. polymyxa*, 2,3-BDO production was around 22 g/L (248 mM). A fed-batch culture (pH 6.8) with

feeding of acetate and glucose (0.35 mol/mol ratio) resulted in 57.4 g/L (637 mM) of 2,3-BDO with a 2,3-BDO to glucose conversion yield of 0.27 g/g (0.81 mol/mol). When the same fermentation was carried out at pH 6.3, 2,3-BDO was 51 g/L (566 mM) with a yield of 0.27 g/g (0.88 mol/mol) (NAKASHIMADA, Yutaka, MARWOTO, *et al.*, 2000). High concentrations of acetate can be toxic and inhibit cell growth and 2,3-BDO synthesis. Acetate concentrations higher than 200 mM resulted in growth inhibition of *P. polymyxa* and acetate addition reduced ethanol production (NAKASHIMADA, Yutaka, MARWOTO, *et al.*, 2000). In another investigation, acetate concentrations higher than 40mM resulted in growth inhibition of *P. polymyxa* DMS 365 in shake flasks, although in fed-batch this behavior has not been confirmed (HÄßLER, SCHIEDER, *et al.*, 2012). Higher 2,3-BDO production was also observed by the addition of 6 g/L of acetate in fed-batch cultures supplemented with 0.5 g/L yeast extract, resulting in 19.5 g/L 2,3-BDO with a yield of 0.11 g/g (0.22 mol/mol glucose) and 8.5 g/L of ethanol with a yield of 0.19 g/g (a specific rate production ten-fold lower than that of 2,3-BDO) compared to fed-batch cultures supplemented with 0.5 g/L yeast extract, which results in 16.5 g/L of 2,3-BDO with similar glucose to 2,3-BDO conversion yield (LI, Jinshan, WANG, *et al.*, 2013).

The acetate in low pH is normally more toxic to cells than the dissociated form, justifying its lower concentration limit in fermentative processes (JI, HUANG, *et al.*, 2011). It was reported that acetic acid is an effective inducer of ALS, ALDC, and BDH enzymes playing role in formation of 2,3-BD from pyruvate. The addition of acetic acid enabled the production of optically active 2,3-BDO with high yield and high optical purity (>98%) by *P. polymyxa* ATCC 12321 (NAKASHIMADA, Yutaka, MARWOTO, *et al.*, 2000).

Table 3.2: 2,3-BDO production by *Paenibacillus* strains.

Microorganism	Operation Mode	Substrate	Nitrogen Source	pH	Temperature (°C)	Oxygen supply	2,3-BDO (g/L)	Optical purity	Yield (g/g)	Productivity (g/L/h)	References
P. polymyxa DMS 365	Fed-batch	Glucose	Yeast extract and/or dry millet	6	37	500 rpm; 0.2 vvm	111	~98% *levo*; ~2% *meso* and acetoin	-	2.1	HÄßLER, SCHIEDER, *et al.* (2012)
P. polymyxa ATCC 12321	Fed-Batch	Glucose + acetate	Yeast Extract + $(NH4)_2SO_4$ +$Fe(NH_4)_2SO_4 \cdot 6$ H2O	6	37	1 L/min (50% N_2:50% Air 0-7 h → 90% N_2:10% Air 7-65 h)	19.5	>90% *levo*	0.22	-	LI, WANG, *et al.* (2013)
P. polymyxa ATCC 12321	Fed-Batch	Glucose + acetate	Yeast extract; Tryptone; $(NH4)_2SO_4$; $Co(NO_3)_2 \cdot 6$ H2O; $Fe(NH_4)_2SO_4 \cdot 6$ H2O	6.8	30	200 rpm; 0.5 vvm	57.4	>98% *levo*	0.27	-	NAKASHIMADA, Yutaka, MARWOTO, *et al.* (2000)
P. polymyxa ATCC 12321	Continuous – chemostat	Glucose	Yeast Extract + tryptone + $(NH4)_2SO_4$ +$Co(NO_3)_2 \cdot 6$ H2O +$Fe(NH_4)_2SO_4 \cdot 6$ H2O	6.3	30	500 rpm 200 mL/min	7.4	93% *levo*; 7% *meso*	-	-	NAKASHIMADA, Y., KANAI, *et al.* (1998)
P. polymyxa ZJ-9-Δdud (modified)	Fed-batch	Inulin supplemented with glucose	Yeast extract; peptone; NH_4Cl	6	30	300 rpm (22 h) → 200 rpm; 1 vvm	25.9	99,99% *levo*	0.36	0.43	ZHANG, CAO, *et al.* (2018)

P. polymyxa ATCC 12321	Fed-Batch	Glucose	Yeast Extract + $(NH_4)_2SO_4$ + $(NH_4)_2HPO_4$	6.5	30	500-925 rpm 3 L/min	>50	-	-	-	DE MAS, JANSEN, *et al.* (1988)
P. brasilensis PB24	Shake flasks – Batch	Glucose	Yeast extract	Uncontrolled	32	200 rpm	27.1	72.7% *levo*/*dextro*; 27.3% *meso*	0.38	0.41	DIAS, LIMA, *et al.* (2018)
P. polymyxa ATCC 12321	Fed-Batch	Xylose + Glucose	Yeast Extract + peptone	6.3	39	200 rpm	25.1	-	0.28	-	MARWOTO, NAKASHIMADA, *et al.* (2004)
P. polymyxa CJX518	Fed-batch	Glucose; Vitamin C	Yeast extract; $(NH4)_2SO_4$	5.2	37	400 rpm; 0.2 vvm	71.7	92.6% *levo*; 7.4% acetoin	0.39	1.33	DAI, CHENG, *et al.* (2014)
P. polymyxa DMS 365	Fed-batch	Glucose	Yeast extract; Tryptone; $(NH_4)_2SO_4$; $Co(NO_3)_2 \cdot 6$ H2O	6-6.5	35	300 rpm; 0.075 vvm (0.15 L/min)	68.5	-	0.34	0.70	OKONKWO, UJOR, *et al.* (2017)
P. polymyxa ZJ-9	Shake flasks – Batch	Glucose	NH_4Cl; Asn; Ser; His; Arg	6	30	240 rpm (24 h) → 120 rpm	25.1	79.7% *levo*; 20.3% acetoin	0.40	-	GAO, J., JIANG, *et al.* (2019)
		Raw pure inulin					17.5	81.2% *levo*; 18.8% acetoin	0.39	-	
P. polymyxa ATCC 12321	Bioreactor - Continuous with cell recycling	Corn stover hydrolysate	Yeast extract; $(NH_4)_2SO_4$; $(NH_4)_2HPO_4$	6.5	37	500 rpm; 0.2 L/min	18.8	97% *levo*	0.31	1.13	MA, HE, *et al.* (2018)
P. polymyxa ZJ-9	Batch	Jerusalem artichoke tuber	Yeast extract; peptone; NH_4Cl	6	30	240 rpm (12 h) → 120 rpm	36.9	>98% *levo*	-	0.88	GAO, Jian, XU, *et al.* (2010)
P. polymyxa CICC 10010	Shake flasks – Batch	Enzymatic hydrolysate of pretreated cellulose	Peptone; Beef extract	7	30	200 rpm	40 (2,3-BDO + acetoin)	-	0.82	0.70	JIANG, ZHENG, *et al.* (2015)

P. polymyxa XG-1 (modified)	Fed-batch	Inulin supplemented with glucose	Yeast extract; NH_4Cl	6	30	300 rpm (22 h) → 200 rpm 1 vvm	51.3	97.5% *levo*; 2.5% acetoin	-	1.1	ZHANG, XU, *et al.* (2016)
P. polymyxa ZJ-9	Solid-State Fermentation	Jerusalem artichoke pomace (JAP)	Yeast extract peptone + NH4Cl	7	37	110 L/min; Moisture 80%	-	-	67.9 g/kg JAP	-	CAO, ZHANG, *et al.* (2017)
P. polymyxa ICGEB2008	Batch	Glucose	Yeast Extract	6.3	37	Strict anaerobiosis (0.2 mL/min after 12 h)	>16,0	-	0.33	2.01	ADLAKHA, YAZDANI (2015)
P. polymyxa ATCC 12321	Batch	Xylose + Glucose	Yeast Extract + tryptone + $(NH4)_2SO_4$ + $Co(NO_3)_2 \cdot 6$ H2O + $Fe(NH_4)_2SO_4 \cdot 6$ H2O	6.4	39	200 rpm 0,5 vvm	7.4	-	-	-	MARWOTO, NAKASHIMADA, *et al.* (2002)

4. Utilization of agro-industrial residues

The utilization of industrial by-products or agricultural residues could reduce the 2,3-BDO production cost. Moreover, biorefinery development can make a bio-based process sustainable (BONATSOS, DHESKALI, *et al.*, 2016). Examples of residual materials include corn stover and corncob residue, sugar cane bagasse, rice waste and rice straw, oil palm front, kenaf core powder, sugar beet molasses, and soy hull hydrolysate (HAZEENA, SINDHU, *et al.*, 2020). Many of these resources do not contain sugars in easily assimilable form. They contain more complex structures, such as cellulose and hemicellulose, which require a pretreatment step. The lignocellulosic materials are submitted to physical or chemical treatment followed by enzymatic hydrolysis (HAZEENA, SINDHU, *et al.*, 2020). Molasses is another residual carbon source which has gained special attention due to its availability and low cost. Found in the dark syrup form, molasses is obtained from the sucrose extraction process. Molasses can also be used as a nutritional supplement, improving the carbon conversion by a positive effect in the fermentation duration and yield (YANG, ZHANG, 2019).

In addition to sugars, glycerol can be used as a carbon source for 2,3-BDO production among other chemicals (ANITHA, KAMARUDIN, *et al.*, 2016). Glycerol is a co-product of the biodiesel production process, corresponding to 10% of the biodiesel production capacity (HE, MCNUTT, *et al.*, 2017). Its use in biorefinery is therefore environmentally interesting and capable of generating economic benefits with its conversion into commodities with less energy demand (HE, MCNUTT, *et al.*, 2017). Raw glycerol is known as glycerin that has impurities such as methanol, catalysts, salts, free fatty acids, and methyl esters (ALMEIDA, FÁVARO, *et al.*, 2012). For use as a substrate, raw glycerol must be submitted to previous treatment to remove these impurities, responsible for affecting the production yield (YANG, ZHANG, 2019). Some fatty acids with a higher unsaturation degree such as linoleic acid can have a strong inhibitory effect on the glycerol assimilation by cells. In turn, the lower unsaturation degree fatty acids such as oleic and stearic acid can be less inhibitory (VENKATARAMANAN, BOATMAN, *et al.*, 2012).

Paenibacillus species can consume a wide range of carbon sources including glycerol, monosaccharides (glucose, xylose, arabinose), disaccharides (sucrose, maltose,

cellobiose), or polysaccharides (starch) (ADLAKHA, PFAU, *et al.*, 2015). Fed-batch with biomass cell recycle of *Bacillus polymyxa* resulted in higher 2,3-BDO yield (up to 0.35 g/g) than acetate yield compared to batch fermentations without cell recycle (DZIEWULSKI, HAUGHNEY, *et al.*, 1986). An increase in the production from 8.74 g/L to 18.8 g/L, the yield from 0.18 g/g to 0.31 g/g, and the productivity from 0.52 g/L/h to 1.13 g/L/h was observed when using the continuous system with cell recycling (MA, Kedong, HE, *et al.*, 2018). Raw inulin extract from Jerusalem artichoke tubers, without previous hydrolysis, was used for 2,3-BDO production by *P. polymyxa* ZJ-9 resulting in 36.9 g/L *levo*-2,3-BDO with more than 98% optical purity (GAO, Jian, XU, *et al.*, 2010). Corn stover hydrolysate resulted in 18.8 g/L *levo*-2,3-BDO with a yield of 0.31 g/g and a productivity of 1.13 g//L/h, using cell recycle (MA, Kedong, HE, *et al.*, 2018). Enzymatic hydrolysate from ionic liquid pretreated cellulose resulted in acetoin and 2,3-BDO yield of 0.82 g/g and a productivity of 0.7 g/L/h by *P. polymyxa* CICC 10010 (JIANG, Li-qun, FANG, *et al.*, 2015). Crude glycerol and beet molasses were used for the production of 83.3 g/L 2,3-BDO with a yield of 0.42 g/g and a productivity of 0.87 g/L/h by *B. amyloliquefaciens*, overcoming the inhibition of 2,3-BDO production caused by glycerol (ZHANG, Li, XU, *et al.*, 2016).

One major factor of complex substrates with various sugars that can influence the fermentation efficiency is carbon catabolite repression (CCR). This can lead to the production of lower fermentation yield and productivity. In cellulose hydrolysate fermentation by *P. polymyxa* CICC 10010, xylose and cellobiose were consumed after the depletion of glucose (JIANG, Li-qun, FANG, *et al.*, 2015). In xylose-glucose fermentation by *P. polymyxa* ATCC 12321, the xylose was consumed only after glucose depletion (MARWOTO, NAKASHIMADA, *et al.*, 2004). A cell-recycling continuous fermentation system was applied to overcome CCR resulted in 2-fold increased productivity by *P. polymyxa* ATCC 12321. Final *levo*-2,3-BDO was 18.8 g/L with a 1.13 g/L/h productivity and 0.31 g/g yield from corn stover hydrolysates (MA, Kedong, HE, *et al.*, 2018).

5. Biotechnology advances and process improvement

Metabolic engineering is a useful tool for strain improvement that results in improved fermentation efficiencies or target products. It can be used to overcome 2,3-

BDO toxicity and to abolish BDH action in the reverse reaction to acetoin, besides eliminating the *meso*-2,3-BDO biosynthesis, favoring *levo*-2,3-BDO production (OKONKWO, UJOR, EZEJI, 2017). The diacetyl reductase (DAR) gene (*dudA*) of *P. polymyxa* ZJ-9 was knocked out via homologous recombination. This resulted in 25.9 g/L 2,3-BDO in fed-batch fermentation (5 L bioreactor) with increased optical purity of *levo*-2,3-BDO (from about 98% to over 99.99%) (ZHANG, Li, CAO, *et al.*, 2018). Another study introduced a NAD^+-dependent formate dehydrogenase (FDH) gene from *Candida boidinii* to *P. polymyxa* ZJ-9. This increased the intracellular NADH/NAD^+ ratio, therefore, lactic acid and ethanol increased while acetoin and formic acid decreased. The *levo*-2,3-BDO production in batch and fed-batch fermentations were 36.8 g/L and 51.3 g/L (10.2% and 8.0% increase compared to the parental strain) (ZHANG, Li, XU, *et al.*, 2016). Moreover, *Paenibacillus* genes have been introduced to *E. coli*. The gene that encodes for DAR from *P. polymyxa* ZJ-9 was introduced into *E. coli* and subjected to fed-batch fermentation that resulted in 39.4 g/L S-acetoin with higher than 99,9% optical purity from diacetyl (GAO, J., XU, *et al.*, 2013). A co-culture of *P. polymyxa* CJX518, able to produce acetoin and 2,3-BDO, with recombinant *E. coli* LS02T, that produces riboflavin (VB2), was proved to be an alternative way to regulate the distribution of acetoin and 2,3-BDO by regulating the NADH/NAD^+ ratio. Targeting in acetoin production, a low NADH/NAD^+ ratio occurred due to riboflavin production by *E. coli* that affected NADH dehydrogenase activity. The co-culture reached 57.2 g/L of acetoin with 0.4 g/g yield (LIU, XU, *et al.*, 2017).

Metabolic engineering can be also used to prevent the EPS formation, considered a problem for the fermentative process since it increases the viscosity of the cultivation medium, reduces the 2,3-BDO yield, and impacts the 2,3-BDO downstream processing. The levansucrase gene of *P. polymyxa* DMS 365, responsible for producing EPS, was knocked out by homologous recombination. The EPS production was then reduced from 21.7 g/L to 2.5 g/L in sucrose cultures, while the cell growth was increased 1.4-fold compared to the wild strain (OKONKWO, UJOR, *et al.*, 2020).

The Solid-State Fermentation (S-SF) and the Simultaneous Saccharification and Fermentation (SSF) process can also be used as a strategy for improving 2,3-BDO production. The S-SF process was carried out with Jerusalem artichoke pomace as the

raw material for the production of 2,3-BDO by *P. polymyxa* ZJ-9. A novel solid-state bioreactor resulted in a maximum yield of 67.9 g 2,3-BDO per kg Jerusalem artichoke pomace (CAO, ZHANG, *et al.*, 2017). The SSF process was applied to a raw corn cob fermentation using a cell consortium that included *P. polymyxa*. After treatment with a 1.5% NaOH solution, in a 1:10 solid-liquid ratio, at 80 ºC, followed by spraying, the raw corn cob was added to the fermentation medium at 42 ºC. About 34.9 g/L of 2,3-BDO was obtained after 72 h [CN Patent 102643869B].

6. Downstream separation and purification of 2,3-BDO

6.1 2,3-BDO recovery

The bio-based 2,3-BDO recovery is considered the most critical step in the bioprocess, accounting for more than 50% of its total costs (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015). Its main limiting factor is the low 2,3-BDO titer in the fermentation broth and the complexity of the mixture formed, containing metabolites, proteins, salts, sugars, vitamins, and other nutrients used as a growth medium for microorganisms (DAVEY, LEAK, *et al.*, 2016). The presence of these compounds contributes to an energy-intensive downstream separation and purification (DSP) step mainly due to the large amount of water present in the medium (HAIDER, QYYUM, *et al.*, 2018). Although not forming an azeotrope with water, the hydrophilic nature and the high boiling point of 2,3-BDO, around 180 °C, are responsible for a harder and more DSP process (JI, HUANG, *et al.*, 2011). The bio-based 2,3-BDO titer required for an economically viable recovery step is at least 80 g/L. Concentrations below this value result in higher energy expenditure and, consequently, higher operating costs (MAGEE, KOSARIC, 1987). Conventional distillation is the most used technology for the bio-based 2,3-BDO recovery. However, its high energy demand directly affects the bioprocess costs (DAVEY, HAVILL, *et al.*, 2016). Therefore, different low-energy recovery technologies have been extensively investigated as alternatives to conventional distillation, including solvent extraction (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015, JIANYING, CHUBJIAO, *et al.*, 2014, LI, Yanjun, WU, *et al.*, 2016), salting-out (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015, DAI, Jianying, WANG, *et al.*, 2018), sugaring-out (DAI, Jian-Ying, LIU, *et al.*, 2015), and membrane separation (DAVEY, HAVILL, *et al.*, 2016, KAWAMURA, MORITA, *et al.*, 2014).

Solvent extraction is a highly efficient method, especially when solvents with high selectivity and high partition coefficient are employed. Furthermore, the use of green solvents and the reuse of organic extractors contribute to a cheaper and eco-friendly recovery process (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015). Oleyl alcohol was used to extract 2,3-BDO, whose production increased from 17,9 g/L (conventional fermentation) to 23.01 g/L (extractive fermentation), with 68% recovery of 2,3-BDO (ANVARI, KHAYATI, 2009). An environmentally-friendly pre-dispersed solvent extraction (PDSE) system using colloidal liquid aphrons (CLAs) was performed in continuous countercurrent mode, using 0.25% (w/w) of sodium dodecyl sulfate as ionic surfactant and 0.5% (w/w) of Tween 80 as non-ionic surfactant. Compared with conventional liquid-liquid extraction, the PDSE contributed to a 35-85% increase in the mass transfer coefficient and a 30% reduction in the overall solvent load, which allowed for a greater recovery efficiency of 2,3-BDO (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015b). Anionic extraction by reversible esterification with phenylboronates was also investigated for the recovery of bio-based 2,3-BDO, in an organic phase containing phenylboronic acid, Aliquat 336, and 1-octanol as diluent. Up to 72–93% was extracted and up to 80–90% was back-extracted of 2,3-BDO under optimized conditions. The organic phase was reused 4 times during the process (DRABO, TISO, *et al.*, 2017).

The addition of salt (salting-out) and sugar (sugaring-out) can further increase the solvent extraction efficiency leading to 90-99% 2,3-BDO recovery (DAI, Jian-Ying, LIU, *et al.*, 2015). However, the salt recycling can lead to scaling and blockage phenomena (HARVIANTO, HAIDER, *et al.*, 2018), which can be overcome with the use of sugar, since it can be reused as a substrate by microorganisms after the extraction process (DAI, Jian-Ying, LIU, *et al.*, 2015). The addition of 10% (w/w) of K_2HPO_4 to the aqueous feed containing 2.5% (w/w) of 2,3-BDO in a continuous countercurrent liquid-liquid extraction using n-butanol as an extractor contributed to an increase in the extraction efficiency of 81.8% and 30% for a synthetic solution of 2,3-BDO and a fermented broth, respectively (BIRAJDAR, RAJAGOPALAN, *et al.*, 2015a). Extraction using 25% (w/w) of 1-ethyl-3-methylimidazolium trifluoromethanesulfonate [C2mim] [CF3SO3] and 30% (w/w) of K_2HPO_4 allowed a 2,3-BDO recovery of 95.7%, with selectivities of 2.59, 115 and 20.8 in relation to glucose, succinic acid and lactic acid, respectively (DAI, Jianying, WANG, *et al.*, 2018). In turn, the addition of 30% (w/v) of glucose to a fermentation

broth containing 60.3 g/L of 2,3-BDO, 1.8% (w/v) of $(NH_4)_2HPO_4$, and t-butanol led to an 80.4% glucose distribution in the top phase of the extraction solution, which was diluted and reused in the fermentation medium, and 76.3% of 2.3-BDO in the bottom phase, with separation of 78% soluble proteins and 86.8% lactic acid (DAI, Jian-Ying, LIU, *et al.*, 2015).

Membrane separation has been considered a promising technology, capable of providing great savings in processing costs (SHAO, KUMAR, 2009). Pervaporation (SHAO, KUMAR, 2009), nanofiltration and reverse osmosis (DAVEY, HAVILL, *et al.*, 2016) processes have been reported as technologies capable of replacing part of the conventional DSP based on distillation (DAVEY, HAVILL, *et al.*, 2016). However, the membrane separation efficiency tends to decrease with time due to the fouling fibers caused by the increased complexity of the fermentation medium (HAIDER, QYYUM, *et al.*, 2018, HARVIANTO, HAIDER, *et al.*, 2018). In the bio-based 2,3-BDO recovery, there was a greater efficiency of the reverse osmosis membrane compared to the nanofiltration membrane, due to the steric properties of its molecules and to the lower presence of irreversible incrustations (DAVEY, HAVILL, *et al.*, 2016). Mixed matrix membranes have also been investigated. These membranes have combined the low cost and high processability of polymeric membranes with the high selectivity of inorganic membranes in the processes of pervaporation, nanofiltration, and reverse osmosis for efficient 2,3-BDO recovery (DAVEY, LEAK, *et al.*, 2016).

The integration of recovery methodologies such as alcohol precipitation and vacuum distillation (JEON, KIM, *et al.*, 2014), extraction-distillation of hybrids (HARVIANTO, HAIDER, *et al.*, 2018, JEON, KIM, *et al.*, 2014), extraction and salting-out (SUN, JIANG, *et al.*, 2009), solvent extraction and pervaporation (SHAO, KUMAR, 2009), and multi-effect-evaporation-assisted distillation (HONG, VAN DUC LONG, *et al.*, 2019) have also been used to improve the energy efficiency of 2,3-BDO extraction from a fermentation broth and overcome the low purity achieved by the technologies conducted individually. Without these combinations, the bio-based 2,3-BDO recovery may be compromised (FREIDANK, AHRENS, 2014). A concentrated solution of bio-based 2,3-BDO (500 g/L) obtained by vacuum evaporation at 50 °C and 50 mbar, was treated with isopropanol, which led to precipitation removal of 92.5% and 99.8% of

organic acids and inorganic salts, respectively. With the vacuum distillation integration, about 76.2% of 2,3-BDO was recovered, with 96.1% purity (JEON, KIM, *et al.*, 2014). The combination of reactive-extraction and reactive-distillation using n-butylaldehyde allowed a 2,3-BDO recovery greater than 90%, with purity above 99% (LI, Yanjun, WU, *et al.*, 2016). Hybrid extraction-distillation was considered a promising technology in large-scale 2.3-BDO DSP processes, as it reduced duty consumption and total annual cost (TAC) by up to 54.8 and 25.8%, respectively, using oleyl alcohol as a solvent (HARVIANTO, HAIDER, *et al.*, 2018). The salting-out technology based on the use of 34% (w/w) of 2-propanol and 20% (w/w) of $(NH_4)_2SO_4$ contributed to an extraction above 99% of the cells and about 85% of the soluble proteins, with maximum partition and recovery coefficients for 2,3-BDO of 9.9% and 93.7%, respectively (SUN, JIANG, *et al.*, 2009). The association between 1-butanol as an extraction solvent and a polydimethylsiloxane membrane in the pervaporation process contributed to the 2,3-BDO purification as retentate, due to the permeability of water and 1-butanol. Under batch operation, the dehydration process was also carried out with a chitosan membrane. A purity greater than 98% (w/w) of 2,3-BDO has been achieved (SHAO, KUMAR, 2009). Multi-effect-evaporation-assisted distillation (MEED) has been proposed and optimized to create a synergy between multi-effect evaporation and the distillation process, improving the energy efficiency of the 2,3-BDO recovery compared to conventional distillation. The TAC was reduced by up to 21.7% and 38.9% for MEED configurations with 5-fold effect and with heat integration, respectively (HONG, VAN DUC LONG, *et al.*, 2019).

Although these technologies are promising in the efficient bio-based 2,3-BDO recovery, the economic challenges related to capital costs and high energy consumption have limited their industrial implementation (WOO, JONG, *et al.*, 2019). So far, only the American company LanzaTech has investigated the bio-based 2,3-BDO recovery on a commercial scale using the simulated-moving-bed (SMB) method for continuous separation to maximize the industrial value of 2,3-BDO produced by biological route (HARVIANTO, HAIDER, *et al.*, 2018). SMB is a technology designed to implement chromatographic separation of chemical compounds, with continuous flow of solid particles and liquid in countercurrent mode (AZEVEDO, RODRIGUES, 2006). SMB was used to separate acetoin and 2,3-BDO isomers (*levo*-, *dextro*- and *meso*-2,3-BDO).

Considering the 2,3-BDO isomerism effects on the adsorption and mass transfer behavior, more than 99.2% of acetoin was recovered by SMB operated at 60 °C (LEE, Chung Gi, JO, *et al.*, 2019). To overcome these isomerism effects in the separation of acetoin and 2,3-BDO, an adsorbent with less pi bonds was used, which led to a 44% increase in process yield. Furthermore, SMB was operated at room temperature, contributing to greater energy efficiency (LEE, Chung Gi, JO, *et al.*, 2020). SMB was also used to separate the optically active 2,3-BDO isomers and *meso*-2,3-BDO, based on their competitive adsorption behaviors. Purity greater than 99.9% and 97% for *meso*-2,3-BDO and *levo/dextro*-2,3-BDO were obtained, respectively, with losses of less than 1% (LEE, Chung Gi, SONG, *et al.*, 2019).

6.2 Cell separation and recycling

Cell separation and reuse in the fermentation process are also crucial aspects to develop an efficient bio-based 2,3-BDO recovery, which can also increase its production efficiency. *K. pneumonia* NRRL B199 was used to produce 2,3-BDO from 100 g/L of glucose, with complete cell recycling, which led to better production efficiency compared to batch and continuous systems. About 9.84 g/L/h were obtained against 2.02 and 4.25 g/L/h of 2.3-BDO for the batch and continuous fermentations, respectively. For a cell concentration of 40 g/L, the volumetric oxygen transfer coefficient decreased considerably and the fermentation medium viscosity increased since the production of the polysaccharides was preferably favored over the 2,3-BDO synthesis (RAMACHANDRAN, GOMA, 1988). A microfiltration module was used to recover cells, whose 2,3-BDO yield by *E. aerogenes* DSM 30053 was 3-fold higher than that obtained in conventional continuous fermentation. The acetic acid accumulation under a low dilution rate limited the fermentation process, which was prevented by fed-batch fermentation with a pH adaptable to high cell concentrations. About 110 g/L of 2,3-BDO, with a productivity of 5.4 g/L/h and a yield of 97%, were obtained with cell recycling (ZENG, A. P., BIEBL, *et al.*, 1991). A continuous fermentation system with cell recycling was employed to produce *levo*-2,3-BDO by *P. polymyxa* ATCC 12321 from corn stover hydrolysates. The productivity achieved was twice as high compared to the batch, fed-batch, and conventional continuous fermentations (MA, Kedong, HE, *et al.*, 2018b).

7. Applications, derivatives and global market of 2,3-BDO

The 2,3-BDO chirality facilitates its conversion into chemical derivatives. A direct application of this property is directed by asymmetric synthesis using boron esters. *Levo*-2,3-BDO (2R,3R-2,3-BDO) and (s)-pinanediol allyl boronates can be used for the chiral synthesis of (2S,3S)-3-methyl-5-hexen-2-ol (MATTESON, CAMPBELL, 1990). Another application is the chiral chemical groups supply for the synthesis of high-value drugs and liquid crystals. *Levo*-2,3-BDO is capable of forming a spiral nematic *meso*phase in liquid crystals with "fingerprints" texture, accompanied by a decrease in the dielectric anisotropy of the *meso*phase. Consequently, the predominance of trimolecular complexes with hydrogen bonds can be observed (ALEKSANDRIISKII, NOVIKOV, *et al.*, 2019).

The low freezing point of *levo*-2,3-BDO (-60 °C) provides cryoprotective properties, especially in biological tissues of cells subjected to cooling. About 30% of 2,3-BDO containing 97% (w/w) racemic mixture of the *levo* and *dextro* isomers has been used for the cryoprotection of red blood cells, where survival approaches 90%. This racemic mixture can also be a very useful cryoprotectant for organ cryopreservation. Its use must be combined with other cryoprotectants since it is a little more toxic than the traditional cryoprotective solutions, such as glycerol (BOUTRON, 1992).

2,3-BDO can find applications in the cosmetic industry as *meso*-2,3-BDO addition showed improved storage stability due to antibacterial properties compared to chemical preservatives (parabens and phenoxyethanol) that are potential allergens. Therefore, it can be used as an additive in creams, lotions, powder, and hand sanitizers among others replacing the chemically produced 1,3-butanediol (BAEK, WOO, *et al.*, 2016)]. 2,3-BDO could be also used in the agricultural industry as pesticide. *Levo*-2,3-BDO has shown antibacterial properties against bacterial pathogen *Erwinia carotovora* in *Arabidopsis* (RYU, FARAG, *et al.*, 2004), fungal pathogens, *Microdochium nivale*, *Rhizoctonia solani* or *Sclerotinia homoeocarpa* (CORTES-BARCO, HSIANG, *et al.*, 2010), and viral diseases (*Cucumber mosaic virus* and *Tobacco mosaic virus*) (KONG, SHIN, *et al.*, 2018).

The 2,3-BDO versatility is observed in various products that can be obtained through its chemical conversion, from different reactions, such as esterification, dehydration, dehydrogenation, and ketalization (JI, HUANG, *et al.*, 2011). Each of these reactions provides a different product to an industrial segment, e.g. food, pharmaceutical, fine chemicals, cosmetics, solvents, fuels, and medical-hospital products (Fig. 3.5).

1,3-Butadiene (BDE) produced via dehydration of 2,3-BDO is the most well-known derivative as it is the precursor to synthetic rubber, which was widely produced biochemically during World War II, due to the conventional production sources scarcity, especially oil. This situation stimulated research on bioprocess development using renewable resources (JI, HUANG, *et al.*, 2011). However, with the low-cost of oil in the 1950s, the chemical route became more viable. Only in the 1970s, with the oil crisis, the biotechnological process for 2,3-BDO production was used again (VOLOCH, JANSEN, *et al.*, 1985). The bio-based 2,3-BDO production was performed together with the SiO_2-supported CsH_2PO_4 catalyst for the dehydration process, which presented high BDE selectivity (TSUKAMOTO, SAKAMI, *et al.*, 2016). Nowadays, fermentative 2,3-BDO production could be used as a result of environmental concerns and the need for bio-economy development.

2,3-BDO is considered a valuable fuel additive. Its heat of combustion (27.2 kJ/g) is comparable to that of liquid fuels, such as methanol (~22.1 kJ/g) and ethanol (~29.1 kJ/g) (CELIŃSKA, GRAJEK, 2009). The high-octane number of 2,3-BDO is a property that can enhance gasoline's resistance to compression without entering auto-ignition, thereby improving the performance and conservation of engines. 2,3-BDO was selectively dehydrated in a solvent-free process to a dioxolane mixture, that exhibited an antiknock index similar to high-octane gasoline. This dioxolane mixture can also be applied as a sustainable gasoline blending component, diesel oxygenate, and industrial solvent (HARVEY, MERRIMAN, *et al.*, 2016). The fuel-additive property also allows 2,3-BDO to be used as an aviation fuel, mainly after dehydration and formation of methyl ethyl ketone (MEK), whose combustion heat is higher than that of ethanol (ZHAO, CUI, *et al.*, 2017). MEK can be used as a solvent for nitrocellulose adhesives, vinyl resin, and cellulose acetate (LEE, J, KIM, *et al.*, 2018).

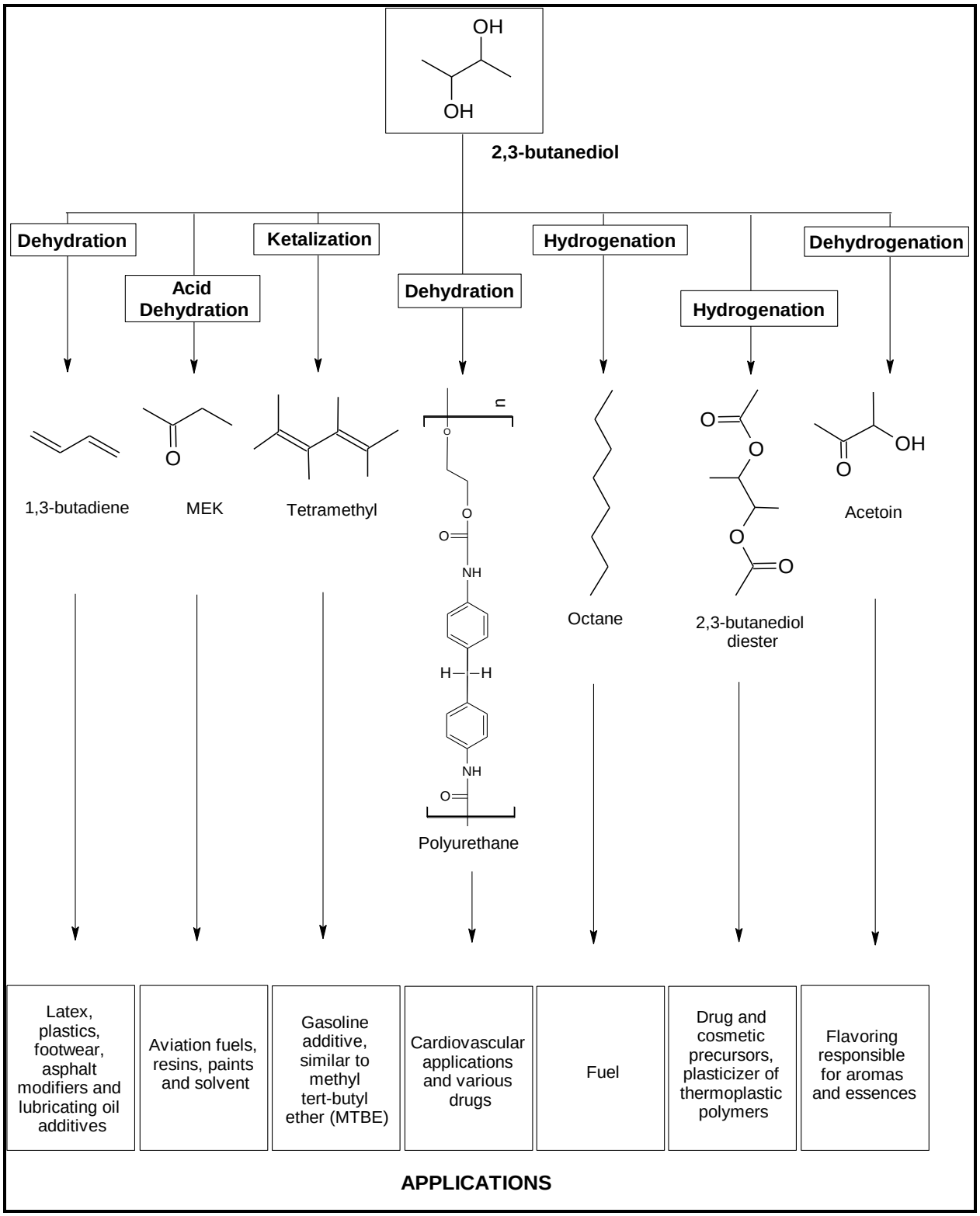

Figure 3.5: Bio-based 2,3-BDO derivatives and its applications (based on BIAŁKOWSKA (2016), JANSEN, TSAO (1983)).

According to Transparency Market Research 2019, a market growth of around 3.5% CAGR (Compound Annual Growth Rate) is predicted for the period 2019-2027. This will bring a market value of approximately US$ 220 million in 2030 for 2,3-BDO (TRANSPARENCY MARKET RESEARCH, 2022). The 2,3-BDO application as a chemical intermediate is another interesting segment in the market since it is related to hydrocarbon fuel production, responsible for approximately 92% of the total expected revenue for 2027. Because of this, the market encourages technology development that contributes to 2,3-BDO production costs reduction (TRANSPARENCY MARKET RESEARCH, 2022). The major players in diol production are BASF, Dairen Chemical, Lyondellbasell, Ashland, Nanya Plastics Corporation, Mitsubishi Chemical Corporation, INVISTA, MarkorChem, Xinjiang Tianye, Changcheng Energy, Shanxi Sanwei Group, Shanxi BidiOu, Sichuan Tianhua, Henan Kaixiang Fine Chemicals, HNEC, TunHe (MARKETWATCH, 2020). The industrial-scale commercialization of 2,3-BDO via biochemical route was first announced in 2012 and completed in 2016 by Lanzatech (www.lanzatech.com). Employing *Clostridium* cells and using residual carbon monoxide gas as a raw material, the company forecasted a 15,000 gallons per year pilot-scale production, in a 1:1 ratio of 2,3-BDO and ethanol, in one of its companies located in New Zealand. Later, the demonstration plant has been integrated with Baosteel, a steel plant located in China, with a 100 thousand gallons per year production capacity, allowing the technology to expand to a commercial scale. The main limitation for large-scale implementation was attributed by the company to the difficulty of separating downstream intermediaries, such as butadiene, MEK, and butene, which ended up leading to the initial extension of the production scheduling deadline from 2014 to 2016 (LANZATECH, 2020). The bio-based 2,3-BDO recovery has been carried out using a simulated moving bed (SMB) (HARVIANTO, HAIDER, *et al.*, 2018).

Koutinas et al. (KOUTINAS, YEPEZ, *et al.*, 2016) have carried out techno-economic evaluation for the production of 2,3-BDO using glycerol, sucrose, and sugarcane molasses as feedstocks. The authors observed the influence of three factors on the bioprocess manufacturing costs: the nutritional supplement type employed, the raw materials market price, and the fermentation efficiency. According to the authors, for example, the use of pure glycerol as a substrate is only justified if its market price is reduced and the bioprocess efficiency is increased. For the raw glycerol, its use is

advantageous if the fermentation efficiency is not compromised by the presence of impurities. The substrate concentration control and the feeding strategies assessment are important aspects to be considered when choosing the best operation mode for higher efficiency of 2,3-BDO production (HAKIZIMANA, MATABARO, *et al.*, 2020). Therefore, the biotechnological 2,3-BDO commercialization depends on the relationship between manufacturing costs, fixed capital investment, and the bioprocess yield, so that its participation in the 2,3-BDO global economy can be justified and, consequently, expanded.

8. Conclusions and future prospects

Efficient bio-based 2,3-BDO production and especially high purity *levo*-2,3-BDO production could be achieved with the GRAS microorganisms belonging to the *Paenibacillus* strains. Their secure nature is a significant advantage over conventional producing strains that may lead to the development of efficient industrial bio-based 2,3-BDO production. These strains can consume a variety of carbon sources leading to improved fermentation efficiency. Future research should focus on reducing the production cost and improving downstream separation and purification of *levo*-2,3-BDO, for its use in different industrial applications and 2,3-BDO global market development.

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CHAPTER 4

Contextualization

In this chapter, the 2,3-BDO production by a newly *P. peoriae* NRRL BD-62 was investigated. Culture conditions (temperature, pH, and oxygen supply) were optimized using a design of experiments (DoE) based on full factorial type with replication at the center point to control 2,3-BDO metabolism. The optimal conditions contributed to a high-yield and high-selectivity 2,3-BDO production by *P. peoriae* NRRL BD-62. The 2,3-BDO yield was higher than 0.40 g/g under limited-oxygen cultures. Furthermore, a *levo*-2,3-BDO optical purity of at least 95% was verified in batch and fed-batch fermentation.

Therefore, the specific objectives of this chapter were:

- Investigate the capacity of *P. peoriae* NRRL BD-62 to produce 2,3-BDO;
- Investigate the culture conditions optimization (temperature, pH, and oxygen supply) for a high-yield and high-selectivity 2,3-BDO production by *P. peoriae* NRRL BD-62;
- Validate the 2,3-BDO production in fed-batch fermentation under optimized culture conditions.

Relevance

Through the investigation carried out in this chapter, it was possible to understand:

- The application of a newly *P. peoriae* NRRL BD-62 for 2,3-BDO production, as yet unreported in the scientific literature;
- The optimized culture condition use as a metabolic control strategy for the high-yield and high-selectivity bio-based 2,3-BDO production;
- The new fermentation system use designed for advanced DoE development in mini bioreactors (low working volume) with controlled temperature, pH, agitation, and aeration rates. Typically, DoE is performed in shake flasks due to the available equipment lack and excessive material expenditure;

- The low volumetric oxygen transfer coefficient ($kLa < 30\ h^{-1}$) effects on the bio-based 2,3-BDO yield and selectivity in fed-batch fermentation, little investigated in the scientific literature.

OPTIMIZATION OF CULTURE CONDITIONS AS A METABOLIC CONTROL STRATEGY FOR A HIGH-YIELD AND HIGH-SELECTIVITY 2,3-BUTANEDIOL PRODUCTION BY A NEWLY *Paenibacillus peoriae* NRRL BD-62

Abstract

The 2,3-butanediol (2,3-BDO) metabolism control of novel microorganisms is critical to achieving high yield and selectivity. This study aimed to investigate the optimal culture conditions for the 2,3-BDO production by a newly *Paenibacillus peoriae* NRRL BD-62. Using a full factorial-type experiment design with replication at the center point, a 2,3-BDO yield of 0.45 g/g and an absolute *levo*-isomer selectivity, without acetoin accumulation, were obtained under the optimal condition reported by the first-order model formed by the significant variables, in this order, pH, aeration, and agitation. The high 2,3-BDO yield was validated in fed-batch culture, although it has been limited at high initial glucose concentrations. Because of this, the bioprocess's economics and technical-operational aspects were considered for choosing the optimal culture conditions, equal to 32 °C, pH=5, 400 rpm, and 0.1 vvm. About 39.4 g/L 2,3-BDO, with a yield of 0.43 g/g, and a *levo-:meso*-2,3-BDO ratio of 1.9:1, was achieved in fed-batch fermentation without acetoin accumulation. Furthermore, the 2,3-BDO production occurred at the cell growth and byproduct production expense, such as lactic acid and ethanol. The volumetric oxygen transfer coefficient of 7.5 h^{-1} established from these conditions was one of the lowest values investigated in a bioreactor system so far, contributing to a highly oxygen-limited environment for the 2,3-BDO synthesis. To the best of our knowledge, this is the first time that a wild-type *P. peoriae* strain is used for high-yield 2,3-BDO production without acetoin accumulation in a highly limited-oxygen fed-batch fermentation.

Keywords: Low oxygen supply, kLa, acetoin, GRAS bacteria, 2,3-BDO metabolism.

1. Introduction

2,3-Butanediol (2,3-BDO) is an important platform chemical used in different industrial applications, mainly in pharmaceutical, cosmetic, food, polymer, fuel, and energy segments (KOU, CUI, *et al.*, 2022, NARISETTY, ZHANG, *et al.*, 2022, WANG, HU, *et al.*, 2016). Several studies have attempted to understand the main limiting factors of bio-based 2,3-BDO metabolism and their effects on isomeric selectivity, acetoin accumulation, and byproduct formation (MITREA, VODNAR, 2019, NAKASHIMADA, Y., KANAI, *et al.*, 1998, WOO, JONG, *et al.*, 2019). Various microorganisms producing 2,3-BDO from a mixed acid fermentation in anaerobic or microaerobic conditions have been investigated (ZENG, SABRA, 2011), including the *Klebsiella*, *Enterobacter*, *Serratia*, *Bacillus*, and *Paenibacillus* genera (JI, HUANG, *et al.*, 2011).

Although many microorganisms can efficiently produce 2,3-BDO, few can produce it with biosafety, low energy cost, and good performance (GE, LI, *et al.*, 2016). *Paenibacillus* is an example of GRAS (generally recognized as safe) bacteria widely used to produce 2,3-BDO. Its leading representative is the *P. polymyxa* strain, which produces *levo*-2,3-BDO with high optical purity (NAKASHIMADA, Yutaka, MARWOTO, *et al.*, 2000, TINÔCO, PATERAKI, *et al.*, 2021). Preferential 2,3-BDO isomer production (*levo*-, *dextro*-, or *meso*-2,3-BDO) contributes to bioprocess economics as a less costly downstream step can be achieved (GAO, J., JIANG, *et al.*, 2019, TINÔCO, BORSCHIVER, *et al.*, 2020).

Other *Paenibacillus* species can also produce 2,3-BDO, such as *P. brasilensis* and *P. peoriae* strains (TINÔCO, PATERAKI, *et al.*, 2021). The 2,3-BDO production by a *P. brasilensis* strain was first reported by DIAS, LIMA, et al. (2018). The authors sequenced the *P. brasilensis* PB 24 genome and identified at least 6 genes related to 2,3-BDO synthesis and a substantial similarity with *P. polymyxa*, *P. terrae*, and *P. peoriae*. About 27 g/L of 2,3-BDO from glucose in shake flasks was produced in 72 h. On the other hand, *P. peoriae* strain has not been investigated for 2,3-BDO production. This bacterium is known to be facultatively anaerobic, able to grow at temperatures in the range of 28-45 °C (the optimum temperature at 28-30 °C), pH 5-6.5, and produce organic acids and alcohols by the butylene glycol route (Voges-Proskauer test) from different substrates, such as glucose, xylose, sucrose, and lactose (MONTEFUSCO, NAKAMURA, *et al.*,

1993). Therefore, *P. peoriae* strain can be considered a promising new microbial 2,3-BDO producer whose metabolism needs to be investigated.

Metabolic engineering has enhanced the microbial ability to produce bio-based 2,3-BDO. The intermediate accumulation, such as organic acids and acetoin, has been reduced, and the isomer selectivity increased (YANG, Taowei, RAO, *et al.*, 2017, YANG, Zhiliang, ZHANG, 2019). Inactivation and overexpression of key genes have allowed controlling the activity of the main enzymes involved in 2,3-BDO metabolism: α-acetolactate synthase (ALS), α-acetolactate decarboxylase (ALDC), and 2,3-butanediol dehydrogenase (BDH) (ZHANG, Li, CAO, *et al.*, 2018). However, these techniques' high 2,3-BDO metabolic fluxes are still costly and challenging (KAY, JEWETT, 2015). Cell viability is sometimes opposed to the overproduction and release of a single bio-based interest product (KAY, JEWETT, 2020). Furthermore, the target chemical reaction control depends on overcoming the physical barrier established by the microbial cell wall (KAY, JEWETT, 2015). On the other hand, the 2,3-BDO metabolism control by optimized fermentation conditions can be performed relatively simply, preserving cell integrity and avoiding additional bioprocess costs (TINÔCO, PATERAKI, *et al.*, 2021).

The 2,3-BDO metabolism is based on redox balance, in which the $NADH/NAD^+$ pool control is used to direct the carbon flux to the 2,3-BDO synthesis rather than its byproducts (CHOI, JIAO, *et al.*, 2020). The primary way to regulate the $NADH/NAD^+$ ratio is by control of dissolved oxygen (DO) (DAI, Jun-Jun, CHENG, *et al.*, 2014). Under limited oxygen conditions, the respiration and fermentation processes are activated simultaneously, and cell energy can be obtained by both glycolysis and biosynthesis pathways (CHU, JIANG, *et al.*, 2021). For 2,3-BDO production to be favored, a high $NADH/NAD^+$ ratio must be established, achieved by a low oxygen supply responsible for activating the ALS enzyme. However, this condition hinders cell biomass formation (BAO, ZHANG, *et al.*, 2015). Since bio-based 2,3-BDO is considered a metabolite semi-associated with microbial growth, its synthesis depends on cell biomass (TINÔCO, DE CASTRO, *et al.*, 2021). Therefore, one of the challenges in bio-based 2,3-BDO production has been determining the optimal oxygen supply condition that allows a balance between fermentation and cell respiration (JI, HUANG, *et al.*, 2011).

Temperature and pH also affect 2,3-BDO metabolism (BIAŁKOWSKA, 2016). Both act on cell maintenance and key-enzymes activity (CELIŃSKA, GRAJEK, 2009). The pH directly affects the metabolites' distribution and concentration, altering pathways according to fermentation conditions adopted (LEE, CHOI, *et al.*, 2017, REHMAN, KHAIRUL ISLAM, *et al.*, 2021). Under alkaline pH at 7.1-8, the ALS enzyme inactivation favors organic acid production, such as lactic acid. In contrast, 2,3-BDO yield tends to increase from 3-7 fold, accompanied by lower cell biomass formation, under acidic pH at 5-6.5 (CELIŃSKA, GRAJEK, 2009). Since these conditions depend on the microorganism and substrate used, their optimal values should be determined individually for each 2,3-BDO-producing species.

Therefore, this study aimed to optimize fermentation conditions (DO, temperature, and pH) as a metabolic control strategy for a high-yield and high-selectivity 2,3-BDO production by a newly *P. peoriae* NRRL BD-62. A 2,3-BDO yield higher than 0.40 g/g, typical for non-GRAS microorganisms, and a high 2,3-BDO selectivity of at least 95% *levo*-isomer were achieved at 32 °C and pH=5 in batch and fed-batch fermentation at volumetric oxygen transfer coefficient (kLa) at about 5-7.5 h^{-1}. To the best of our knowledge, this was the first time a wild-type *P. peoriae* strain has been used for high-yield and high-selectivity 2,3-BDO production in a strongly oxygen-limited environment.

2. Materials and methods

2.1 Microorganism and inoculum preparation

Paenibacillus peoriae NRRL BD-62 has been isolated from the maize rhizosphere (CSM36 genotype), planted in Cerrado soil (EMBRAPA-CNPMS, Sete Lagoas, MG), and deposited in the culture collection of the Microbial Genetics Laboratory of the Microbiology Institute of the Federal University of Rio de Janeiro (VON DER WEID, DUARTE, *et al.*, 2002). It was maintained in Tryptic Soy Broth – TSB medium (17 g/L tryptone, 3 g/L soytone, 5 g/L NaCl, 2.5 g/L K_2HPO_4, and 2.5 g/L glucose) supplemented with 25% (v/v) glycerol, at -80 ºC.

The inoculum was prepared in two steps. First, the cells were reactivated by transferring 0.2 mL glycerol stock solution to a 250 mL shaken flask containing 50 mL TSB medium. Then, 1% (v/v) cell reactivation medium was transferred to 1 L shaken flasks containing 350 mL YPD medium (20 g/L glucose, 10 g/L yeast extract, and 20 g/L peptone), thus characterizing the seed medium. The following conditions were used: 32 °C, 200 rpm, for 24 and 16 h, respectively. The media were sterilized by autoclaving at 121 °C for 15 min.

2.2 Fermentation procedure

The fermentation assays were carried out in the DASbox® mini bioreactor – 300 mL (Eppendorf, Germany), designed as a 4-fold system with 16 parallel bioreactors (H/D~1.5). About 10% (v/v) seed medium was inoculated into the fermentation medium containing: 15 g/L yeast extract, 0.5 g/L KH_2PO_4, 2.0 g/L K_2HPO_4, 0.0225 g/L $MnSO_4$, and 0.3 g/L KCl (ADLAKHA, YAZDANI, 2015). Glucose was used as a carbon source at C/N= 8.5 g/g. The fermentation assays were carried out for 24 h, with an initial working volume of 200 mL. The temperature was controlled by a liquid-free heating and cooling system (Peltier). The pH was controlled by adding 2 M NaOH or 1 M H_2SO_4. A submerged gas supply via an L-sparger (4 mm compression fitting) through which air was injected into a sterile filter (0.22 μm), and an agitation system consisted of two Rushton-type impellers 3 cm apart, rotating clockwise, which allowed both radial and axial flows, controlled the oxygen supply. The fermentation assays were sterilized by autoclaving at 121 °C for 15 min. Antifoam 204 (Sigma-Aldrich, USA) was used when needed.

Two fed-batch fermentations (initial glucose of 30 g/L or 50 g/L at C/N=8.5 g/g) were carried out in duplicate to validate the optimal culture conditions obtained by the design of experiments (DoE). Again, the DASbox® system was used due to its efficient scalability and reproducibility in microbial culture applications, requiring less working volume, thus reducing costs with the cell material amount, culture medium, and other nutritional supplements (www.eppendorf.com). A concentrated glucose solution, around 500 g/L, was used as a feeding medium. One or two pulses were fed when the glucose concentration was 0–10 g/L. Fermentation assays were carried out until glucose

exhaustion. Again, the fermentation assays were sterilized by autoclaving at 121 °C for 15 min, and antifoam 204 (Sigma-Aldrich, USA) was used when needed.

2.3 Desing of experiments (DoE)

A two-level full factorial experimental design with three replicates at the center point was used to optimize the cultures conditions for an efficient 2,3-BDO production by *P. peoriae* NRRL BD-62 (BOX, HUNTER, *et al.*, 2005). The experimental matrix was formed by a 19-run design with 4 independent variables: (X_1) temperature – °C; (X_2) pH; (X_3) agitation rate – rpm; and (X_4) aeration rate - vvm. The variables' levels and the corresponding value are presented in Table 4.1.

Table 4.1: Coded factors levels and real values used in the (2^4 + 3) design to optimize the culture conditions for the 2,3-BDO production by *P. peoriae* NRRL BD-62

Factors (variables)	**Experimental levels**		
	-1	**0**	**+1**
Temperature - °C (X_1)	32	34.5	37
pH (X_2)	5	6	7
Agitation – rpm (X_3)	200	300	400
Aeration rate – vvm [L_{air}/L_{medium}/min] (X_4)	0.1	0.2	0.3

Response surfaces were constructed for the 2,3-BDO yield. A mathematical regression model, minimizing the difference between the predicted and experimental values, was represented by a first-order polynomial equation added by an interaction term indicating the curvature presence in the response variable (Equation 1). The significant variables were identified and used in the effective 2,3-BDO yield prediction.

$$Y = \beta o + \Sigma \beta_i X_i + \Sigma \beta_{ij} X_i X_j \qquad (1)$$

Where Y is the predicted response, βo is the intercept parameter, β_i is the linear effect parameter, β_{ij} is the interaction effect coefficient, and X_i and X_j are coded variables.

2.4 Fermentative parameters

The bioprocess yield was determined based on three parameters: $Y_{P/S}$ - the ratio between 2,3-BDO production and glucose consumption (Equation 2), $Y_{X/S}$ - the ratio

between cell biomass and substrate consumption (Equation 3), and $Y_{P/X}$ - the ratio between 2,3-BDO production and cell biomass (Equation 4). The specific cell growth rate (μ) was calculated using the cell optical density (OD) within the linear step of the microbial culture logarithmic growth (Equation 5). Substrate consumption (SC) was calculated by the percentage change in the glucose titer (Equation 6). Moreover, the C4-compounds selectivity was calculated from the percentage composition between *levo*-2,3-BDO, *meso*-2,3-BDO, and acetoin (Equation 7). All parameters were calculated after the fermentation end.

$$Y_{P/S} = \frac{[2,3BDO]_f - [2,3\text{-}BDO]_i}{[Glu]_i - [Glu]_f} \qquad (2)$$

Where $Y_{P/S}$ is the 2,3-BDO yield from glucose (g/g), [2,3-BDO] is the 2,3-BDO titer (g/L) at the final (f) and initial (i) times, and [Glu] is the glucose titer (g/L) at the final (f) and initial (i) times.

$$Y_{X/S} = \frac{[X]_f - [X]_i}{[Glu]_i - [Glu]_f} \qquad (3)$$

Where $Y_{X/S}$ is the cell biomass yield from glucose (g/g), [X] is the cell biomass concentration (g/L) at the final (f) and initial (i) times, and [Glu] is the glucose titer (g/L) at the final (f) and initial (i) times.

$$Y_{P/X} = \frac{[2,3BDO]_f - [2,3\text{-}BDO]_i}{[X]_f - [X]_i} \qquad (4)$$

Where $Y_{P/X}$ is the ratio between 2,3-BDO production and cell biomass concentration (g/g), [2,3-BDO] is the 2,3-BDO titer (g/L) at the final (f) and initial (i) times, and [X] is the cell biomass concentration (g/L) at the final (f) and initial (i) times.

$$\mu = \frac{\ln OD_f - \ln OD_i}{t_f - t_i} \qquad (5)$$

Where μ is the cell growth specific rate (h^{-1}), and OD is the cell optical density at 600 nm at the final (f) and initial (i) times (h).

$$SC\ (\%) = \frac{[Glu]_i - [Glu]_f}{[Glu]_i} * 100 \qquad (6)$$

Where SC is the substrate consumption in the fermentation process (%), and [Glu] is the glucose titer (g/L) at the final (f) and initial (i) times.

$$\text{Selectivity } (\%) = \frac{[C4]}{[2,3\text{-BDO}]_{levo} + [2,3\text{-BDO}]_{meso} + [\text{acetoin}]} * 100 \qquad (7)$$

Where [C4] is the titer of one of three C4-compounds (*levo*-isomer, *meso*-isomer, or acetoin) (g/L), $[2,3\text{-BDO}]_{levo}$ is the *levo*-2,3-BDO titer (g/L), $[2,3\text{-BDO}]_{meso}$ is the *meso*-2,3-BDO titer (g/L), and [acetoin] is the acetoin titer (g/L).

2.5 Volumetric oxygen transfer coefficient (kLa)

The kLa is an important parameter for measuring the culture medium's dissolved oxygen (DO) concentration. It can be calculated by different methodologies, such as physical methods based on the absorption or desorption of oxygen in the culture medium, widely used for oxygen transfer estimates. The dynamic method was used in this study. It consisted of analyzing the dynamic change in oxygen concentration after a gradual change in the inlet gas. For this, nitrogen gas (N_2) was bubbled in the culture medium with no cell until DO was equal to zero. Then, the culture medium was submitted to aeration and agitation until complete DO saturation. DO was measured by an amperometric oxygen sensor (Metter Toledo - InPro 6800, USA) with a PTFE-coated membrane. The DO values were collected every 15 s, and the linear interval of the generated curve was used to calculate the kLa (the slope coefficient), according to Equations 8 and 9 (GARCIA-OCHOA, GOMEZ, 2009).

$$\frac{dC}{dt} = kLa\ (Cs - C) \qquad (8)$$

$$\ln \frac{(Cs - C_1)}{(Cs - C_0)} = -kLa\ (t_1 - t_0) \qquad (9)$$

Where kLa is the volumetric oxygen transfer coefficient (h^{-1}), C is the DO concentration in the culture medium at two instants: initial (t_0) and final (t_1) times within the linear region of the DO curve (%), and C_S is the saturated DO concentration (100%).

2.6 Analytical methods

Cell biomass was determined by dry cell weight (DCW) analysis. Optical density at 600 nm ($OD_{600\ nm}$) was measured in a UV–visible spectrophotometer (Biospectro SP-22, Brazil), and cell concentration was determined by filtering cell biomass samples through a 0.22 μm cellulose acetate membrane (Sartorius, Germany), followed by weighing after drying in an oven at 60 °C until constant weight. Thus, 1 $OD_{600\ nm}$ = 0.392 ± 0.016 g/L DCW in the fermentation medium.

Glucose and fermentation products (*levo*- and *meso*-2,3-BDO, acetoin, and lactic and acetic acids) were analyzed on a high-performance liquid chromatography system (HPLC; Agilent, USA), equipped with an HPX-87H Aminex column (300 × 7.8 mm, Bio-Rad, USA). The mobile phase consisted of 5 mM H_2SO_4 at 0.6 mL/min, and the column temperature was controlled at 45 ºC. After sample treatment, glucose and fermentation products were detected in a Refractive Index Detector (RID; Agilent, USA). First, samples were collected every 2 h (experimental design) or 24 h (fed-batch fermentation) and centrifuged at 10,000 x *g*, at 25 ºC for 10 min, in a microtube centrifuge (MiniSpin®, Eppendorf, Germany). The supernatant was diluted 10-fold with Milli-Q water and filtered through a 0.22 μm cellulose acetate membrane (Minisart® NML Syringe Filters, Sartorius, Germany). Then, the supernatant was analyzed by the HPLC system. The concentrations of glucose and fermentation products were determined by calibration curves of standard substances (Merck KgaA, Darmstadt, Germany).

Statistic treatments such as variance analysis (ANOVA) and response surface analysis were performed using the STATISTICA® 7.0 software (StatSoft, Inc., USA - www.statsoft.com). The error estimate was calculated based on the standard deviation (std. dev.). Coefficient of variation (CV) was used to estimate the error range for the experimental design assays, which were not repeated (HÄßLER, SCHIEDER, *et al.*, 2012) based on the triplicate central point. Fermentation parameters were analyzed by the *t*-test, with a 95% confidence level (α=0.05).

3. Results and discussion

3.1 Production capacity of 2,3-BDO by *P. peoriae* NRRL BD-62

The 2,3-BDO production by *P. peoriae* NRRL BD-62 was preliminarily evaluated in shake flasks at 35 °C and 225 rpm, as presented in Figure 4.1. These conditions were based on previous information about *P. peoriae* strains (MONTEFUSCO, NAKAMURA, *et al.*, 1993) and the conditions required for bio-based 2,3-BDO production, like low oxygen supply (JI, HUANG, *et al.*, 2011). The fermentation kLa was approximately 37 h^{-1}, favoring a 2,3-BDO titer and yield of 6.33 g/L and 0.33 g/g in 24 h, respectively.

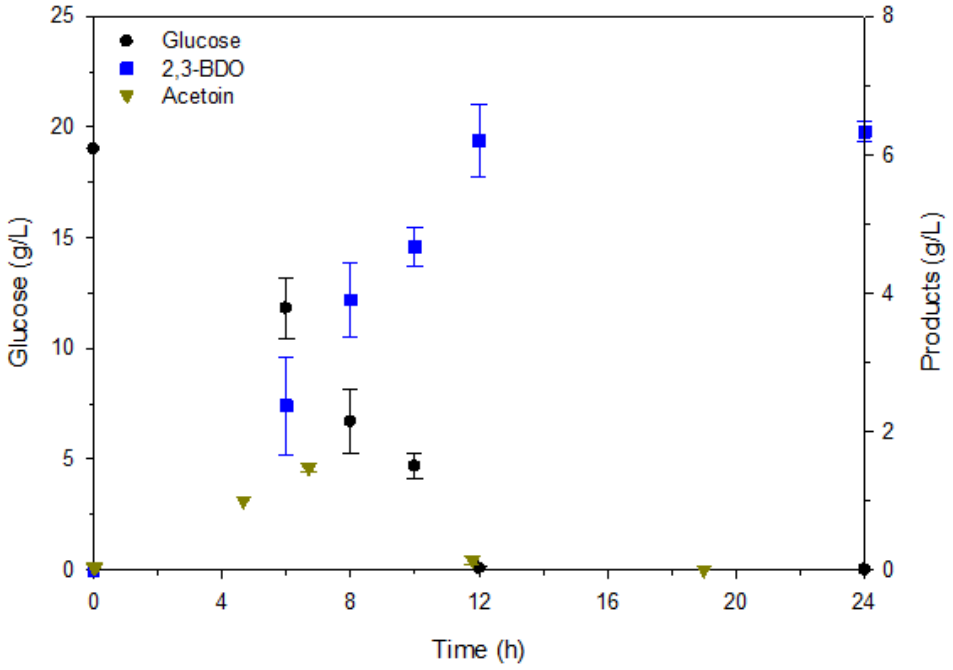

Figure 4.1: Time course of the 2,3-BDO production by *P. peoriae* NRRL BD-62 at 35 °C and 225 rpm in shaken flasks.

Acetoin production varied throughout the fermentation, reaching a maximum titer of 1.47 g/L in 8 h. As the extended fermentation, acetoin appears to have been converted almost entirely to *levo*-2,3-BDO, showing a purity of 99.5% in 24 h. The increased culture medium viscosity at late fermentation steps possibly contributed to reduced kLa (LI, FAN, *et al.*, 2017), favoring the acetoin conversion to 2,3-BDO. *Meso*-2,3-BDO was not detected under these conditions.

3.2 Design of experiments

The culture condition ranges were defined based on the information about *P. polymyxa* and *P. brasilensis* strains (temperature and pH) and bio-based 2,3-BDO metabolism (oxygen supply) (Table 4.1) since no previous studies reporting the 2,3-BDO production by *P. peoriae* strain were found (TINÔCO, PATERAKI, *et al.*, 2021). Based on these studies, there was a consensus that microaerobic conditions are required for efficient bio-based 2,3-BDO production (CHU, JIANG, *et al.*, 2021) and were therefore replicated in this study.

A full factorial design of experiments (DoE) with replication at the center point was chosen to determine the optimal temperature, pH, and oxygen supply for a high 2,3-BDO yield by *P. peoriae* NRRL BD-62. The center points represented the variables' interaction in the first-order factorial model. As a result, the curvature effects on 2,3-BDO yield could be determined (RODRIGUES, IEMMA, 2009). Furthermore, $Y_{P/S}$ was defined as a response variable for normalized DoE and fed-batch fermentation results and future comparisons. Initial glucose concentration was around 10 g/L to avoid any possible excess substrate inhibition and accurately estimate $Y_{P/S}$ in 24 h.

ANOVA analysis demonstrated the linear model efficiency with the interaction of culture conditions in predicting $Y_{P/S}$, proven by the p-values of the curvature ($p< 0.05$) and lack of fit ($p> 0.05$) effect, as presented in Table 4.2. Furthermore, the model with all variables analyzed showed a good capacity to predict $Y_{P/S}$, with high variation coefficients ($R^2= 0.99$ and $R^2_{adj}= 0.97$). However, regarding the temperature variable (X_1) and its interaction with the other variables, the effect was not statistically significant ($p> 0.05$). The pH-N and N-Q_{ar} interactions also showed no statistically significant effects, although these variables were individually significant in the prediction model.

3.2.1 Temperature effects

Although the temperature is closely related to the 2,3-BDO efficiency, due to the dependence on enzyme activity and cell maintenance and growth (RIPOLL, DE VICENTE, *et al.*, 2016), it did not significantly influence the $Y_{P/S}$ within the investigated range (32-37 °C). The 2,3-BDO yields at 32 and 37 °C were statistically similar for the

assays with equal and constant culture conditions (pH, N, and Q_{ar}), as observed in Figure 4.2.

Table 4.2: ANOVA of the experimental design (2^4+3) to optimize the cultivation conditions for the 2,3-BDO production by *P. peoriae* NRRL BD-62

Factors	SS	df	MS	F-value	p-value
Curvature	0.0096	1	0.0096	26.2595	0.0360*
(X_1) T (°C)	0.0019	1	0.0019	5.2543	0.1489
(X_2) pH	0.1315	1	0.1315	358.8316	0.0028*
(X_3) N (rpm)	0.0117	1	0.0117	31.9947	0.0299*
(X_4) Q_{ar} (vvm)	0.0332	1	0.0332	90.6978	0.0108*
X_1X_2	0.0013	1	0.0013	3.5090	0.2019
X_1X_3	0.0032	1	0.0032	8.8500	0.0969
X_1X_4	0.0007	1	0.0007	1.9299	0.2992
X_2X_3	0.0061	1	0.0061	16.7454	0.0549
X_2X_4	0.0178	1	0.0178	48.5012	0.0200*
X_3X_4	0.0028	1	0.0028	7.7688	0.1082
Lack of Fit	0.0018	5	0.0004	0.9767	0.5761
Pure Error	0.0007	2	0.0004		
Total	0.2225	18			

T= temperature; N= agitation rate; Q_{ar}= aeration rate (vvm= L_{air}/L_{medium}/min); SS= sum of squares; df= degrees of freedom; MS= mean square; R^2= 0.99; R^2_{adj}= 0.97. * Statistical significance ($p<0.05$).

Studies using *P. polymyxa* strains were mainly carried out at 30 or 37 °C, as summarized by TINÔCO, PATERAKI, *et al.* (2021). MARWOTO, NAKASHIMADA, *et al.* (2002) investigated the temperature effect on the glucose and xylose conversion to *levo*-2,3-BDO by *P. polymyxa* ATCC 12321 in anaerobic batch and continuous cultures, respectively. As in this study, the authors observed no significant temperature influence at 30-39 °C on the 2,3-BDO yield from glucose, showing statistically similar consumption throughout the range. Regarding xylose assays, the BDH enzyme activity did not change at 30-39 °C, while the alcohol dehydrogenase enzyme activity, responsible for ethanol synthesis, was lower at 30 °C. GAO, Jian, YANG, *et al.* (2013) also investigated the temperature influence on the BDH enzyme activity of *P. polymyxa* ZJ-9. The authors observed high activity at 30 °C, which increased *levo*-2,3-BDO production. In assays using *K. pneumoniae*, it was observed that decreasing the temperature from 35 to 30 °C favored the 2,3-BDO synthesis over byproducts such as ethanol (HAKIZIMANA, MATABARO, *et al.*, 2020). In this study, ethanol production at 32 °C was low (< 0.10 g/L); therefore, 32 °C was the optimal temperature for 2,3-BDO production by *P. peoriae*

NRRL BD-62. Under this condition, lower energy expenditure, lower operating costs, and higher 2,3-BDO yield with reduced byproducts were expected.

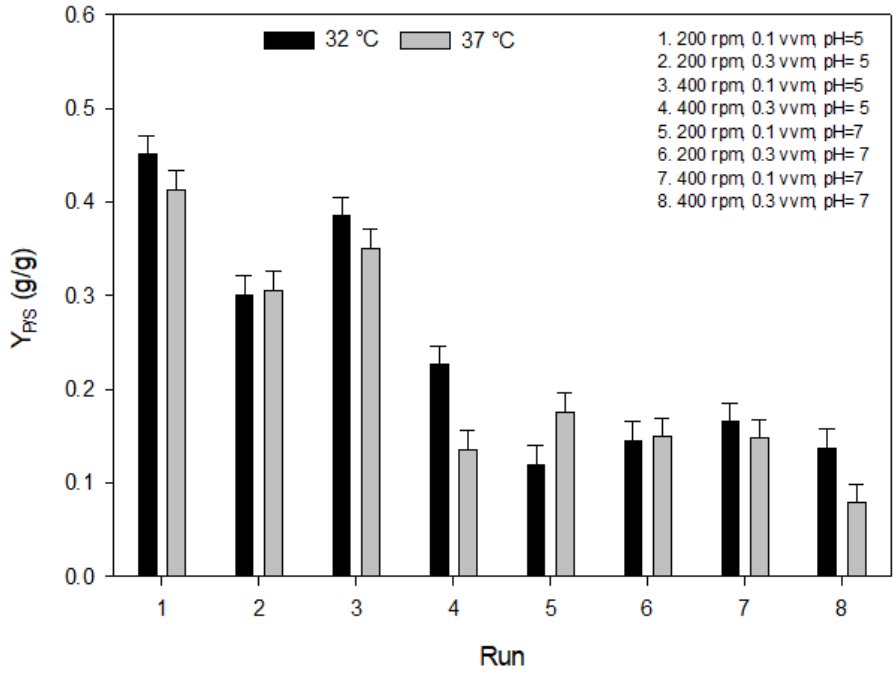

Figure 4.2: Effect of temperature on 2,3-BDO yield ($Y_{P/S}$) by *P. peoriae* NRRL BD-62 at constant conditions: pH, agitation rate (rpm), and aeration rate (vvm= L_{air}/L_{medium}/min).

3.2.2 pH and oxygen supply effects

The proposed $Y_{P/S}$ prediction model was reparameterized, considering only the significant variables and interactions. The new model coefficients were determined and analyzed based on the t-test and p-value, which indicated the interaction strength between each independent variable (Table 4.3). A large t-value and a small p-value indicated more significant effects on the $Y_{P/S}$ (PSAKI, MAINA, *et al.*, 2019). Therefore, in that order, the statistically most important variables were pH and oxygen supply, represented by N and Q_{ar}.

SHARMA, NAIN, *et al.* (2018) optimized fermentation conditions for 2,3-BDO and ethanol coproduction by *K. oxytoca* XF7 from hemicellulosic hydrolysate and found that pH was one of the most critical factors in 2,3-BDO metabolism. YANG, Zhiliang, ZHANG (2018) investigated the 2,3-BDO production by the engineered *Pichia pastoris*

X33 under pH control (4-6) and no pH control. The authors observed no significant differences in the final 2,3-BDO titer, around 10.8 g/L. However, cell growth was compromised at pH=4, while the highest acetoin titer was obtained at pH=6. Based on previous studies in which fermentation acidification is responsible for 2,3-BDO production, as an intracellular strategy to counteract the culture medium acidity, the authors set pH=5 as the optimal condition for 2,3-BDO production by *P. pastoris* X33. The reducing pH effect on 2,3-BDO production was also observed by PRIYA, DUREJA, *et al.* (2016). The authors investigated a two-step pH control strategy: at constant pH=7.5; at two-step pH (initial 10 h at pH=7.5, and later at pH=6.5). By reducing the pH to slightly acidic conditions in the second assay, the 2,3-BDO production by *E. cloacae* TERI BD18 increased by approximately 15%, while cell biomass formation was reduced by about 27%. So, the yield decreased from 0.44 to 0.46 g/g, and the byproduct production, such as ethanol and acetoin, also decreased. PETROV, PETROVA (2009) observed a 4-fold lower organic acid production at pH=5 than at pH=7 when they investigated the 2,3-BDO production by *K. pneumoniae* G31 from glycerol. However, the 2,3-BDO yield was relatively small at both pH conditions, equal to 0.13 g/g (pH=5) and 0.02 g/g (pH=7). In contrast, the experimental planning assays performed here at pH=5 showed high and statistically superior $Y_{P/S}$ to those performed at pH=7, other conditions being held constant, as presented in Figure 4.3. Although not discussed by the authors, DAI, CHENG, *et al.* (2014) also maintained pH near 5 in their studies about the extracellular oxireduction potential effects on 2,3-BDO production by *P. polymyxa* CJX518 in fed-batch fermentation.

Table 4.3: Significant parameters estimation of the reparameterized prediction model of 2,3-BDO yield by *P. peoriae* NRRL BD-62.

Factor	Regression coefficient	Standard error	t-value	p-value
Intercept	1.3467	0.0666	20.2038	0.0024
(X_2) pH	-0.1573	0.0107	-14.7005	0.0046
(X_3) N (rpm)	-0.0003	4.7855×10^{-5}	-5.6564	0.0299
(X_4) Q_{ar} (vvm)	-2.4554	0.2911	-8.4352	0.0138
X_2X_4	0.3333	0.0479	6.9643	0.0200

N= agitation rate; Q_{ar}= aeration rate (vvm= L_{air}/L_{medium}/min); R^2= 0.92; R^2_{adj}= 0.88.

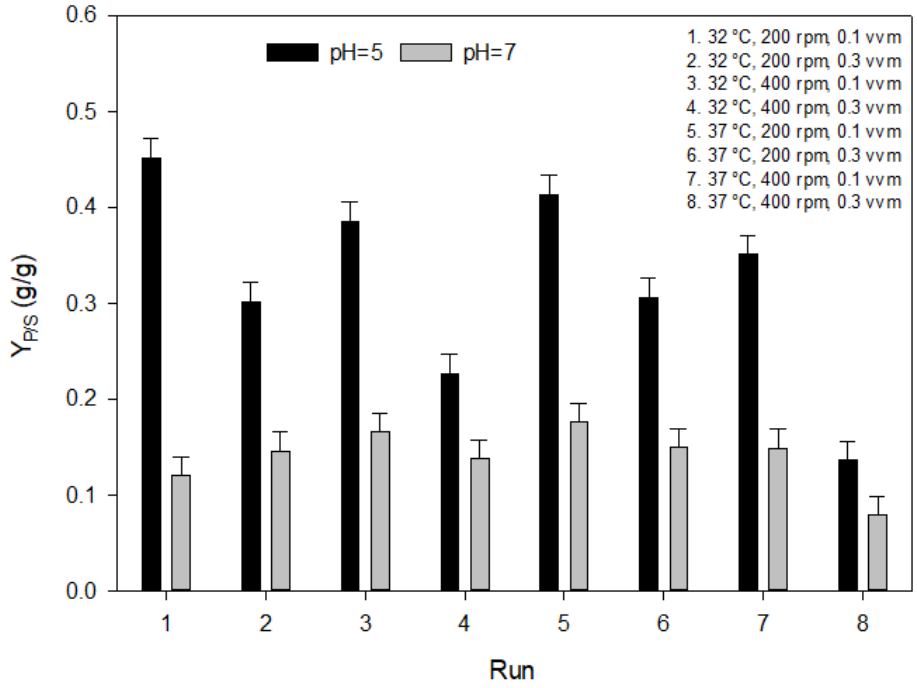

Figure 4.3: Effect of pH on 2,3-BDO yield ($Y_{P/S}$) by *P. peoriae* NRRL BD-62 at constant conditions: temperature (°C), agitation rate (rpm), and aeration rate (vvm= L_{air}/L_{medium}/min).

Regarding oxygen supply, DAI, CHENG, *et al.* (2014) investigated the agitation and aeration effects on *levo*-2,3-BDO production by *P. polymyxa* CJX518 in batch fermentation. The authors implemented a two-step fermentation strategy: 400 rpm and 0.4 vvm in 0-6 h; 300 rpm and 0.2 vvm in 6-24 h, in which it was possible to maximize the 2,3-BDO titer and productivity simultaneously. A higher 2,3-BDO productivity was obtained in the first step due to higher cell growth and glucose consumption, while a higher 2,3-BDO was obtained in the second step since, under a lower oxygen supply, the acetoin conversion accumulated in the first step was favored. HÄßLER, SCHIEDER, *et al.* (2012) investigated various agitation/aeration setups at 300-800 rpm and 0-1.67 vvm for the efficient 2,3-BDO production by *P. polymyxa* DSM 365 from sucrose in fed-batch fermentation. The authors found that under sufficient oxygen conditions, the lactate/2,3-BDO ratio can be reduced below 0.01 g/g, while its production tends to be higher under microaerobic conditions. The same was observed for ethanol under low dissolved oxygen conditions. There was almost no acetate accumulation (<0.5 g/L) under aerobic conditions. In contrast, acetoin reached its maximum production at 800 rpm and 0.2 vvm. The authors then defined 500 rpm and 0.2 vvm as the best bioprocess condition. CHU,

JIANG, *et al.* (2021) investigated the optimized oxygen supply effects on the 2,3-BDO yield by an engineered *Klebsiella* sp. The authors evaluated a range of 0-70% oxygen. At 70% oxygen, the 2,3-BDO yield was increased by more than 50%, while at 0-30% oxygen, the 2,3-BDO yield was below 25 mM. Higher acetoin, formate, and acetate were obtained at 70% oxygen, while lactate and ethanol production was decreased. REHMAN, KHAIRUL ISLAM, *et al.* (2021) investigated the agitation effects at 100-300 rpm on 2,3-BDO production by *K. pneumoniae* PM2 in batch fermentation. The authors observed a higher 2,3-BDO production at 150-200 rpm in 24 h. Outside this range, the 2,3-BDO yield and productivity were compromised. Cell growth was sequentially promoted by increasing the agitation from 250 to 300 rpm, and byproducts were generated at the 2,3-BDO expense.

A first-order polynomial equation represented the new proposed model with interaction effects (Equation 10), in which the variables pH and Q_{ar} showed stronger effects (individual and interaction terms) than the N effects (individual term) in predicting $Y_{P/S}$.

$$Y_{2,3\text{-BDO/glucose}} = 1.3467 - 0.1573*pH - 0.0003*N - 2.4554*Q_{ar} + 0.3333*pH*Q_{ar} \quad (10)$$

Where: $Y_{2,3\text{-BDO/glucose}}$ is the 2,3-BDO yield from glucose, N is the agitation rate (rpm), and Q_{ar} is the aeration rate (vvm= L_{air}/L_{medium}/min).

Synergism between pH and aeration was previously investigated by PETROV, PETROVA (2010) in the 2,3-BDO production by *K. pneumoniae* G31 from glycerol. The authors implemented a forced pH fluctuation strategy combined with an optimized aeration regime, which resulted in increased 2,3-BDO yield and productivity. Fed-batch long fermentations at 2.2 vvm and pH variation equal to 1 (ΔpH=1 every 12 h) were adequate, as they did not cause cell lysis and resulted in maximum 2,3-BDO production. A similar strategy was performed by AMRAOUI, PRABHU, *et al.* (2022). The authors reported maximum 2,3-BDO production by a mutant *E. ludwigii* strain when fed-batch fermentations were carried out with forced pH fluctuation (ΔpH= 2) and high aeration (2 vvm). Unlike this study, CHAN, JANTAMA, *et al.* (2016) reported agitation as the most critical parameter for 2,3-BDO production by engineered *K. oxytoca* KMS005 from maltodextrin. The authors performed a central composite rotational (DCCR) type DoE of

the culture conditions and found a strong agitation influence when correlated to aeration and substrate concentration. Batch fermentations below 200 rpm showed ethanol, succinate, and acetate accumulation, with a reduced 2,3-BDO yield, while the highest 2,3-BDO production was obtained in the assays at 300-400 rpm.

ANOVA of the reparameterized model again indicated that the linear model with curvature effect was suitable for $Y_{P/S}$ prediction, as the lack of fit showed $p > 0.05$, and no further statistical assay was required (Table 4.4). The model explained 92% of the variation in response, and 88% was explained only by the independent variables that affected $Y_{P/S}$ ($R^2 = 0.92$ and $R^2_{adj} = 0.88$, respectively). Both $R^2 > 0.75$ indicated the model fitness (YANG, Taowei, ZHANG, *et al.*, 2012). The model significance was further proven by the F-test, with $F_{(5,13,\alpha=0.05)} = 28.3925$ greater than the tabulated F-value ($F_{(5,13,\alpha=0.05)} = 3.025$), and $p < 0.00001$.

Table 4.4: ANOVA of the reparameterized prediction model of 2,3-BDO yield by *P. peoriae* NRRL BD-62.

Source	SS	df	MS	F-value	p-value
Model	0.2038	5	0.0408	28.3925	< 0.00001
Error	0.0187	13	0.0014		
Lack of Fit	0.0179	11	0.0016	4.4492	0.1977
Pure Error	0.0007	2	0.0004		
Total	0.2225	18			

SS= sum of squares; df= degrees of freedom; MS= mean square; $R^2 = 0.92$; $R^2_{adj} = 0.88$.

The response surfaces and contour curves for the proposed model significant variables (pH, N, and Q_{ar}) were constructed, as presented in Figure 4.4. It was possible to verify the interaction between the independent variables and their optimal values for a maximum 2,3-BDO yield response. The highest $Y_{P/S}$ were obtained when the culture conditions were equal to pH=5, N=200 rpm, and Q_{ar}=0.1 vvm, higher than 0.40 g/g. About 90% of the theoretical 2,3-BDO yield from glucose was achieved at 32 °C, pH=5, N=200 rpm, and Q_{ar}=0.1 vvm, corresponding to 0.45 g/g. High 2,3-BDO yields above 0.40 g/g have been reported mainly by wild and engineered biosafety level 2 strains: *E. aerogenes* (JOO, LEE, *et al.*, 2016, KIM, Duck Gyun, YOO, *et al.*, 2020), *E. cloacae* (PRIYA, DUREJA, *et al.*, 2016, PRIYA, LAL, 2019, ZHANG, LI, *et al.*, 2016), *Klebsiella* sp. and *K. oxytoca* (GURAGAIN, VADLANI, 2017, KHUNNONKWAO,

JANTAMA, *et al.*, 2021, KIM, Duk Ki, PARK, *et al.*, 2016), *K. pneumoniae* (DAI, Jian Ying, GUAN, *et al.*, 2020, REHMAN, KHAIRUL ISLAM, *et al.*, 2021), and *Serratia sp*. and *S. marcescens* (HUANG, JIANG, *et al.*, 2013, SHI, GAO, *et al.*, 2014, ZHANG, Liaoyuan, YANG, *et al.*, 2010).

PASAYE-ANAYA, VARGAS-TAH, *et al.* (2019) selected a wild-type *K. oxytoc*a UM2-17 capable of efficiently producing 2,3-BDO using a synthetic mineral medium. A 2,3-BDO yield of 0.46 g/g was obtained from 150 g/L glucose and 15 g/L xylose in shake flasks at 30 °C, 100 rpm, for 120 h. MENG, ZHANG, *et al.* (2020) constructed a recombinant *K. oxytoca* PDL-K5 by gene deletion responsible for the acetate, succinate, lactate, and formate synthesis. The authors obtained 2,3-BDO yields of 0.43 and 0.44 g/g from lactose and whey powder in fed-batch fermentation at 37 °C, 400 rpm, 1 vvm, initial pH=7 followed by controlled pH=6, respectively.

On the other hand, wild-type *P. polymyxa* strains (biosafety level 1) have shown 2,3-BDO yields of around 0.22-0.42 g/g (TINÔCO, PATERAKI, *et al.*, 2021). GAO, J., JIANG, *et al.* (2019) reported a 2,3-BDO yield of 0.40 g/g by *P. polymyxa* ZJ-9 from glucose supplemented with 4 amino acids (asparagine, serine, histidine, and arginine). The authors conducted the fermentation assays in stirred flasks at 30 °C, 240 rpm (0-24 h) - 120 rpm (24-48 h), and pH=6. OKONKWO, UJOR, *et al.* (2017) performed optimization of culture medium, temperature, and inoculum size for 2,3-BDO production by *P. polymyxa* DSM 365. Glucose batch fermentation containing 7 g/L glycerol contributed to a 2,3-BDO yield of 0.42 g/g at 35 °C, 300 rpm, 0.075 vvm, and pH=6.5.

Regarding engineered *P. polymyxa* strains, SCHILLING, CICCONE, *et al.* (2020) reported a 2,3-BDO yield of 0.43 g/g by mutant *P. polymyxa* DSM 365 from glucose. The authors performed a lactate dehydrogenase enzyme deletion and uncoupling of the BDH enzyme from its natural regulation via constitutive episomal expression, optimizing the carbon flux for 2,3-BDO production. The assays were conducted at 35 °C, 300 rpm, 0.075 vvm, and pH=6 in batch fermentation. OKONKWO, UJOR, *et al.* (2020) inactivated the levansucrase gene of *P. polymyxa* DMS 365 by double-crossover homologous recombination, resulting the reduced exopolysaccharide (EPS) synthesis. EPS can affect the medium viscosity and 2,3-BDO recovery costs. The authors reported a 2,3-BDO yield of 0.42 g/g from uncontrolled pH glucose fermentation and low EPS production at 35 °C,

300 rpm, and 0.075 vvm. To the best of our knowledge, the 2,3-BDO yield of 0.45 g/g was the highest reported for a wild-type *Paenibacillus* strain from glucose at low oxygen supply and low pH.

3.2.3 Optimized culture conditions selection

The experimental results of (2^4+3) design and $Y_{P/S}$ predictions from the proposed model are presented in Table 4.5. Besides $Y_{P/S}$, the other fermentation parameters were calculated to the optimal condition selection for 2,3-BDO production by *P. peoriae* NRRL BD-62. The time courses of the DoE runs (2^4+3) were presented in the supplemental material (Fig. S1, S2, and S3 – Supplementary material).

As observed in Figures S1, S2, and S3 (Supplementary material), glucose consumption, 2,3-BDO production, and cell biomass formation showed a non-exponential profile in the initial 8 h. The linear behavior revealed that the kLa range corresponded to microaerobic conditions, in which cell growth and 2,3-BDO synthesis occurred to a limited extent. Similar observations were verified by REBECCHI, PINELLI, *et al.* (2018) when evaluating the oxygen mass transfer rate effects on 2,3-BDO production by *B. licheniformis* ATCC9789 from glucose. The authors conducted a DCCR and concluded that for kLa at 6.5-81 h-1, the 2,3-BDO metabolism was represented by linear time courses. As observed in this study, the 2,3-BDO started to be produced around 2-3 h after the microaerobic condition establishment. Therefore, the results were satisfactory and consistent with the theory involved in 2,3-BDO synthesis regarding oxygen supply.

The highest $Y_{P/S}$ were obtained at kLa equal to 5-9.8 h^{-1} (runs 1, 2, 3, 9, 10, and 11) (Table 4.5). Increasing kLa beyond this range, $Y_{P/S}$ was reduced at the cell growth expense, reaching values less than 0.23 g/g. Furthermore, the μ and cell biomass increased by an average of 64 and 68% (runs 4 and 12), with $Y_{X/S}$ of 0.12-0.13 g/g, relative to the lowest kLa (runs 1 and 9), respectively.

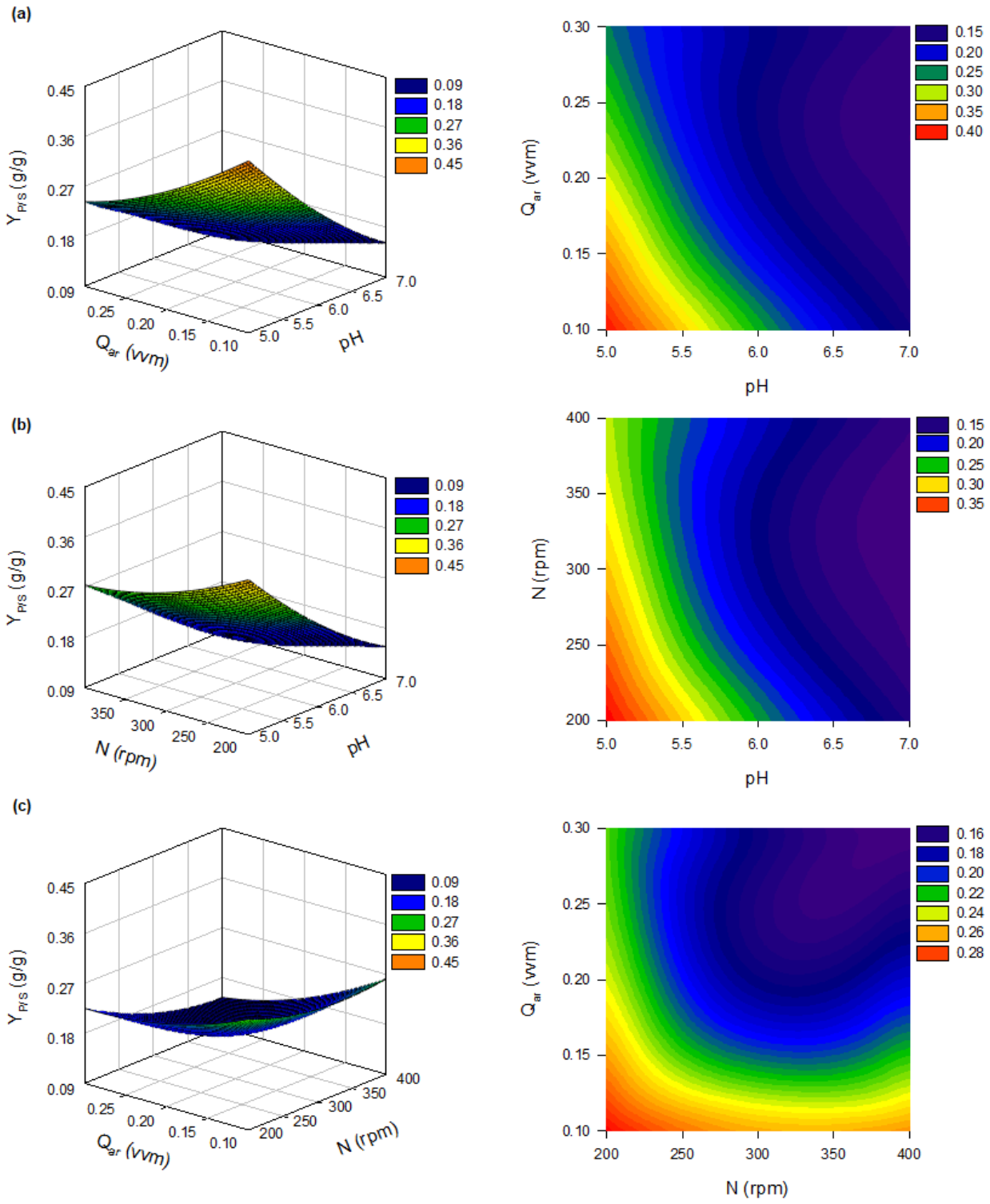

Figure 4.4: Response surface and contour graphs of 2,3-BDO yield ($Y_{P/S}$) by *P. peoriae* NRRL BD-62 at 24 h fermentation: (a) effects of pH and aeration rate (Q_{ar} - vvm= L_{air}/L_{medium}/min); (b) effects of pH and agitation rate (N - rpm); (c) effects of agitation rate (N - rpm) and aeration rate (Q_{ar} - vvm= L_{air}/L_{medium}/min).

The lowest $Y_{P/S}$ of 0.08 g/g was obtained at the highest kLa of 33.4 h^{-1}, where μ was equal to 0.32 h^{-1} and DCW equal to 0.63 g/L (run 16) (Table 4.5). Similar kLa behavior was observed by MAINA, STYLIANOU, *et al.* (2019) when investigating four kLa values between 17 and 76 h^{-1} for 2,3-BDO production by an *E. ludwigii* strain from a complex medium containing very high polarity (VHP) cane sugar (>99% w/v sucrose) as a carbon source in fed-batch fermentation. The 2,3-BDO yield reduced from 0.43 g/g (kLa=17 h^{-1}) to 0.12 g/g (kLa=76 h^{-1}) while μ and DCW increased by approximately 46 and 71%, respectively.

RODRIGUEZ, RIPOLL, *et al.* (2017) evaluated the effects of fluid-dynamic conditions resulting from oxygen transfer and uptake rates (OTR and OUR) for 2,3-BDO production by *Raoultella terrigena* CECT 4519 from glycerol in batch fermentation. The 2,3-BDO synthesis was favored over cell growth at kLa of approximately 0.05 s^{-1}. However, with increasing kLa to a range of approximately 0.1-0.7 s^{-1}, the metabolic flux shifted towards cell biomass production, while the glycerol consumption rate remained unchanged. An increased μ was also observed with increasing agitation. For high kLa, both 2,3-BDO production and cell biomass formation were negatively affected due to the culture medium hydrodynamic stress.

A dependence between kLa and pH was also observed (Table 4.5). Holding the other conditions constant, increasing pH from 5 to 7 led to an increased kLa from 5-13.8 h^{-1} (pH=5) to 18.6-33.4 h^{-1} (pH>5), which reduced $Y_{P/S}$ to below 0.18 g/g. LI, FAN, *et al.* (2017) investigated the pH effects on the oxygen mass transfer bubble swarms in non-Newtonian fluids. The authors used a xanthan solution at different concentrations and correlated the solution pH with the kLa. The NaOH addition increased pH (pH>7) and kLa. This behavior was even more pronounced with increased xanthan solution concentration. The increased kLa was attributed to the change in the xanthan molecular structure, resulting from the reaction between the hydroxyl and acetyl group, decreasing the solution viscosity. The pH adjustment with NaOH was investigated because it is one of the most widely used alkalis in biological processes for optimal pH control, capable of efficiently promoting gas-liquid mass transfer. Moreover, the Na^+ released is responsible for substrate permeation across the transmembrane gradient, increasing its uptake into cells (CHO, KIM, *et al.*, 2015).

Table 4.5: Experimental data (2^4+3 design) and model predictions for the 2,3-BDO yield by *P. peoriae* NRRL BD-62 at 24 h fermentation.

Run	T (°C)	pH	N (rpm)	Q_{ar} (vvm)	2,3-BDO (g/L)	X (g/L)	μ (h^{-1})	$Y_{P/S}$ (g/g)		$Y_{X/S}$ (g/g)	$Y_{P/X}$ (g/g)	SC (%)	kLa (h^{-1})
	Real values				Exp. values			Exp. values	Pred. values	Exp. values			
1	32	5	200	0.1	5.05	0.77	0.16	0.45	0.43	0.07	6.57	80.8	5.0
2	32	5	200	0.3	3.31	0.98	0.22	0.30	0.27	0.09	3.37	87.5	8.7
3	32	5	400	0.1	4.79	0.92	0.22	0.39	0.37	0.07	5.18	100.0	7.1
4	32	5	400	0.3	2.52	1.42	0.26	0.23	0.22	0.13	1.77	99.9	12.2
5	32	7	200	0.1	1.37	0.30	0.30	0.12	0.18	0.03	4.63	100.0	26.5
6	32	7	200	0.3	1.52	0.32	0.29	0.15	0.15	0.03	4.80	100.0	25.1
7	32	7	400	0.1	1.59	0.18	0.29	0.17	0.13	0.02	8.72	100.0	23.1
8	32	7	400	0.3	1.51	0.17	0.35	0.14	0.10	0.02	8.64	100.0	28.3
9	37	5	200	0.1	4.63	1.07	0.17	0.41	0.43	0.10	4.33	82.2	5.7
10	37	5	200	0.3	3.46	1.19	0.21	0.31	0.27	0.10	2.92	87.5	9.8
11	37	5	400	0.1	4.37	1.45	0.25	0.35	0.37	0.12	3.02	100.0	8.0
12	37	5	400	0.3	1.89	1.61	0.28	0.14	0.22	0.12	1.17	100.0	13.8
13	37	7	200	0.1	2.10	0.63	0.25	0.18	0.18	0.05	3.34	99.9	18.6
14	37	7	200	0.3	1.71	0.66	0.27	0.15	0.15	0.06	2.57	100.0	28.1
15	37	7	400	0.1	1.56	0.67	0.27	0.15	0.13	0.06	2.32	100.0	26.5
16	37	7	400	0.3	0.85	0.63	0.32	0.08	0.10	0.06	1.34	100.0	33.4
17	34.5	6	300	0.2	2.78	0.82	0.29	0.17	0.17	0.05	3.39	100.0	21.5
18	34.5	6	300	0.2	2.87	0.84	0.27	0.18	0.17	0.05	3.42	100.0	20.4
19	34.5	6	300	0.2	2.27	0.88	0.29	0.15	0.17	0.05	2.58	100.0	25.5
Std. dev.					0.32	0.03	0.01	0.02		0.00	0.48	0.0	2.7
CV (%)					12.16	3.70	3.14	11.34		2.66	15.25	0.00	11.95

T= temperature; N= agitation rate; Q_{ar}= aeration rate (vvm= L_{air}/L_{medium}/min); 2,3-BDO= 2,3-BDO titer (*meso-* + *levo*-isomer titers); X= cell biomass; μ= cell growth specific rate; $Y_{P/S}$= 2,3-BDO/glucose yield; $Y_{X/S}$= cell biomass/glucose yield; $Y_{P/X}$= 2,3-BDO/cell biomass yield; SC= substrate consumption; kLa= volumetric oxygen transfer coefficient; Exp. Values= experimental values; Pred. values= predicted values; Std. dev.= standard deviation from triplicate central point; CV = coefficient of variation; R^2= 0.92; R^2_{adj}= 0.88.

Except for runs 1, 2, 9, and 10, glucose was depleted in all conditions investigated (Table 4.5). The low kLa values given by N=200 rpm seem to have hindered glucose consumption (~80.8-87.5%), although they favored 2,3-BDO production. In contrast, total glucose consumption was achieved at 400 rpm (runs 3, 4, 11, and 12) and pH 7 (runs 5, 6, 13, and 14) while keeping the other conditions constant. Increased kLa also favored cell plasticity, represented by μ. This parameter was chosen because of the different fermentation times for DCW_{max}, equal to 8 h (runs 5, 6, 13, and 14) and 24 h (runs 3, 4, 11, and 12) (Fig. S3 - a and b). DAI, Jian Ying, GUAN, *et al.* (2020) observed a similar substrate consumption pattern. Under low oxygen supply conditions (200 rpm and 0.2 vvm) and pH=6.5, a maximum inulin consumption of 87% was achieved. By keeping agitation and aeration constant and reducing pH to 6, the inulin consumption decreased to about 83%. However, the 2,3-BDO + acetoin production was favored at the cell growth expense, with an increase of approximately 28%. According to the authors, the pH effects on inulin consumption were associated with its partial hydrolysis and polymerization degree.

To confirm the results found, $Y_{P/X}$ was calculated for all conditions investigated. This yield is an important parameter that can correlate the fermentation and microbial respiration processes and characterize the product type based on cell growth (LUEDEKING, PIRET, 2000). The highest $Y_{P/X}$ values were obtained in runs 1, 3, 7, and 8. Runs 7 and 8 showed $Y_{P/X}$ equal to 8.72 g/g and 8.64 ± 0.48 g/g in 24 h, respectively, despite the 2,3-BDO low production, around 1.5 g/L. These $Y_{P/X}$ results showed inconsistencies since glucose depletion occurred in 8 h (Fig. S1-a), being therefore disregarded. Thus, run 1 showed the highest $Y_{P/X}$, equal to 6.57 g/g, consistent with the results observed at low oxygen supply, in which 2,3-BDO is produced at the cell growth expense.

The selectivity of 4C-compounds (*levo*-2,3-BDO, *meso*-2,3-BDO, and acetoin) was also determined. Acetoin accumulation is usually associated with two culture conditions: intolerance to excess 2,3-BDO (OKONKWO, UJOR, EZEJI, 2017) and oxygen supply (HÄßLER, SCHIEDER, *et al.*, 2012). Since the maximum 2,3-BDO production was 5.05 g/L (run 1), the effects caused by excess 2,3-BDO were not

significant. Therefore, acetoin accumulation was attributed to the increased kLa, as presented in Figure 4.5.

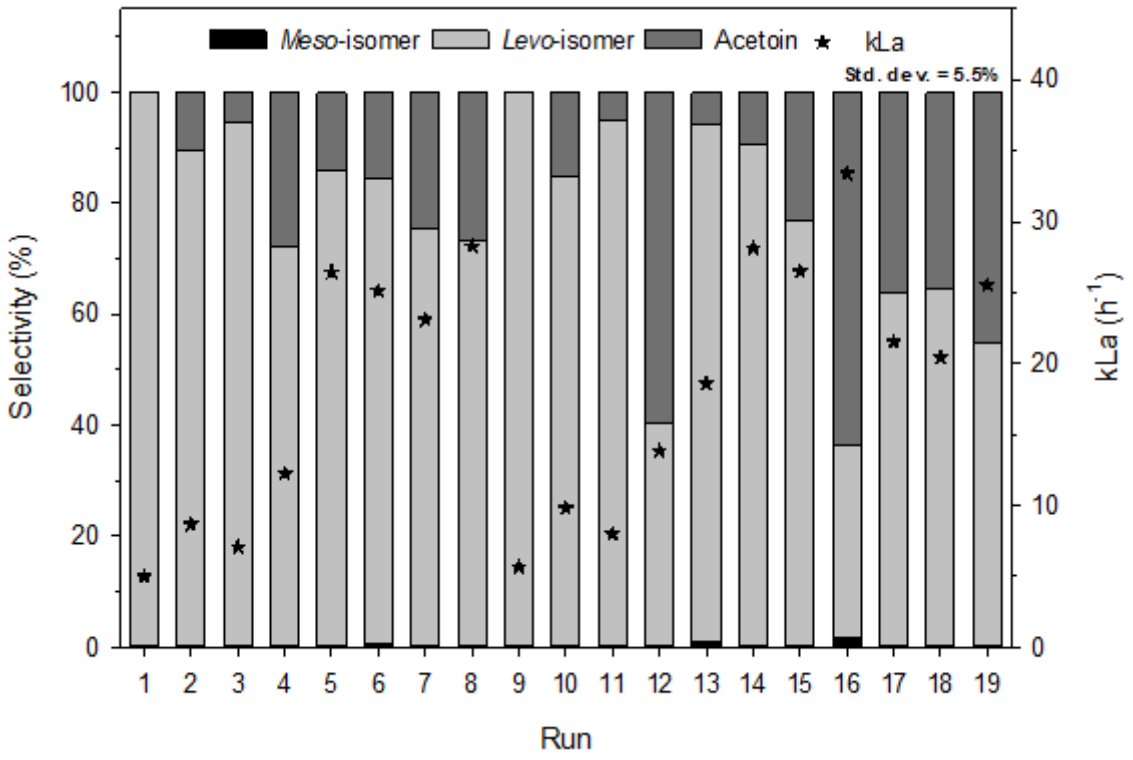

Figure 4.5: Selectivity of the C4-compounds (*meso*-/*levo*-2,3-BDO and acetoin) produced by *P. peoriae* NRRL BD-62 at 24 h fermentation and kLa parameter for the experimental design (2^4+3). Std. dev.= selectivity standard deviation.

For kLa of 5.0-8.0 h^{-1} (runs 1, 3, 9, and 11), acetoin production corresponded to 0.0-5.3% of total 4C-compounds, statistically indicating the acetoin absence in fermentations with low oxygen supply. In contrast, for the highest kLa, equal to 33.4 h^{-1} (run 16), acetoin corresponded to 63.6% of total 4C-compounds, the highest amount reported in this DoE. MAINA, SCHNEIDER, *et al.* (2021) reported a similar acetoin accumulation pattern when investigating the acetoin and *levo*-2,3-BDO production by *B. amyloliquefaciens* 18,025 (BCCM-LMG) from bakery waste. The authors evaluated the kLa effects on acetoin and 2,3-BDO production. For low kLa values (kLa=26 h^{-1}), the 2,3-BDO production was favored, while for high kLa values (kLa=203 h^{-1}), acetoin was the predominant metabolic product.

Despite having statistically similar kLa values, runs 4 and 12, and 14 and 15 showed differences in temperature and oxygen supply, respectively. It was observed that for higher temperatures (runs 4 and 12) and higher aeration and agitation rates (runs 14 and 15), keeping all other conditions constant, acetoin accumulation was favored at the *levo*-isomer production expense. HÄßLER, SCHIEDER, *et al.* (2012) also observed different acetoin production patterns for similar kLa values, resulting from high surface gassing effects caused by the agitation at later fermentation steps. For small-scale fermentations, oxygen transfer of up to 50% of the total available oxygen can be accomplished through surface aeration. Therefore, surface gassing should be considered during the kLa measurement to avoid possible inconsistencies in its values. As reported by the authors, surface aeration was not considered in this study, ultimately leading to the differences in acetoin production, as mentioned above.

While *meso*-isomer production was almost zero, *levo*-isomer was verified in all investigated DoE conditions, with a selectivity of 94.6-100 % in runs at the lowest kLa (runs 1, 3, 9, and 11). The 2,3-BDO-isomers accumulation is dependent on the BDH stereospecificity produced by wild-type and engineered strains (GE, LI, *et al.*, 2016). According to DIAS, LIMA, *et al.* (2018), the S-BDH and R-BDH enzymes, encoded by *but*A and *but*B genes, respectively, may be present in *Paenibacillus* strains genotypes linked to 2,3-BDO metabolism, responsible for the *meso*-, *levo*- and *dextro*-2,3-BDO production. For *P. peoriae* strain, the *but*A absence makes the *dextro*-2,3-BDO production impossible. Furthermore, the interconversion between R-acetoin and *meso*-2,3-BDO also ends up not occurring, leading to the conclusion that *meso*-2,3-BDO is produced only via spontaneous S-α-acetolactate decarboxylation in the oxygen presence.

In this study, the low oxygen supply and low initial glucose concentration contributed to the preferential *levo*-2,3-BDO production. The kLa less than 30 h^{-1} favored low dissolved oxygen, and the initial glucose concentration of approximately 10 g/L did not allow toxic *levo*-2,3-BDO levels to be reached, preventing its conversion to *meso*-2,3-BDO (OKONKWO, UJOR, EZEJI, 2017). High *levo*-2,3-BDO selectivity by wild-type *P. polymyxa* strain around 98% under suitable micro-aerobic conditions was reported by HÄßLER, SCHIEDER, *et al.* (2012), and NAKASHIMADA, Yutaka, MARWOTO, *et al.* (2000). The metabolism control by exclusive optimized culture conditions for

Paenibacillus strain is an alternative to the metabolic engineering for increased 2,3-BDO isomer purity since selectivities higher than 98% *levo*-isomer are comparable to those obtained by engineered *P. polymyxa* strains, whose value can exceed 99.99% (ZHANG, Li, CAO, *et al.*, 2018).

Therefore, the optimized culture conditions for *P. peoriae* NRRL BD-62 were obtained by run 1, equal to 32 °C, pH=5, and kLa~5 h^{-1} (corresponding to 200 rpm and 0.1 vvm). The $Y_{P/S}$ prediction error under these conditions was less than 4.5%.

3.3 Culture conditions validation

Figure 4.6 presents the time courses of the fed-batch fermentation assays at 32 °C, pH=5, 200 rpm, and 0.1 vvm. Besides 2,3-BDO production, the oxygen-limited conditions favored ethanol and lactic acid production throughout the fermentation (DE MAS, JANSEN, *et al.*, 1988). However, the ethanol/2,3-BDO and lactic acid/2,3-BDO ratios were low, equal to 0.100 g/g and 0.056 g/g, respectively. These results suggested a high ALS enzyme activity at the 2,3-BDO byproducts expense. Again, HÄßLER, SCHIEDER, *et al.* (2012) observed a similar ethanol and lactic acid production pattern by *P. polymyxa* DMS 365 in fed-batch fermentation at low kLa (20-30 h^{-1}). The authors reported ethanol and lactate productions of 3.5-4.5 g/L versus about 26-52 g/L of *levo*-/*meso*-2,3-BDO and acetoin.

As in the DoE assays, acetoin production was not detected. The optimized culture conditions seem to have favored the $NADH/NAD^+$ ratio and thus the total acetoin conversion to 2,3-BDO without applying metabolic engineering. Differently, YANG, Taowei, RAO, *et al.* (2015) reported a decreased acetoin production upon adequate $NADH/NAD^+$ ratio using an engineered *B. amyloliquefaciens* strain. The authors overexpressed the genes responsible for the activity of the glycerol dehydrogenase (GDH), ALS, and BDH enzymes, which directed the metabolic flux to the 2,3-BDO synthesis from glycerol. Acetoin production was reduced by 61.5% using a three-step oxygen control, and the 2,3-BDO yield exceeded 0.40 g/g.

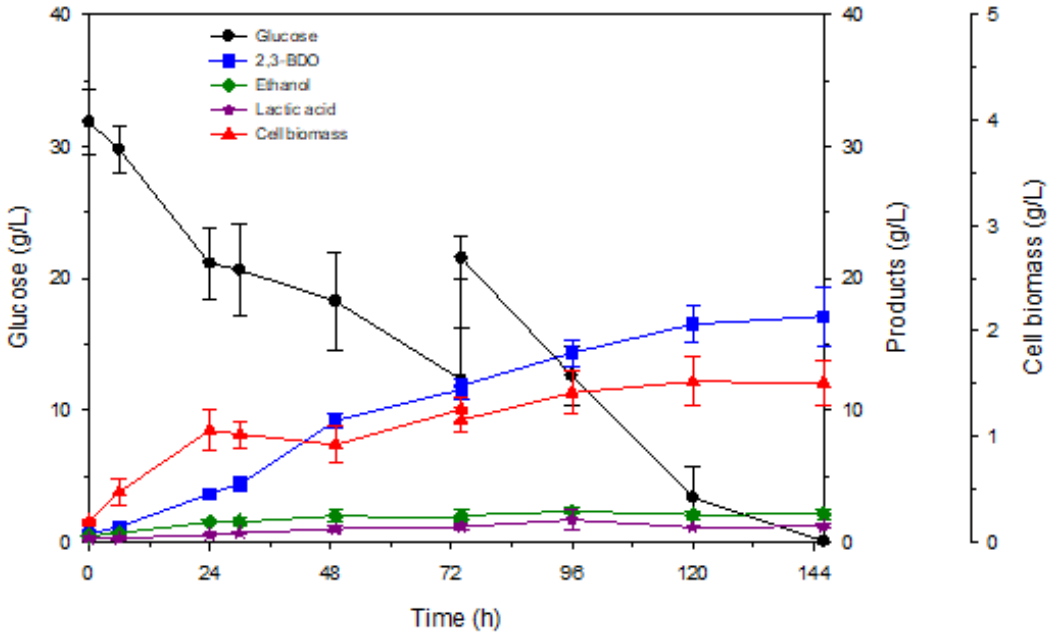

Figure 4.6: Time course of the fed-batch 2,3-BDO production by *P. peoriae* NRRL BD-62 at 32 °C, pH=5, 200 rpm, and 0.1 vvm (L_{air}/L_{medium}/min).

A maximum 2,3-BDO yield and cell biomass of 16.5 g/L, and 1.3 g/L, respectively, were obtained after 146 h, when glucose was exhausted. Again, 2,3-BDO production appeared to have been favored at the cell growth expense since low $Y_{X/S}$ of 0.03 g/g and μ f 0.065 h^{-1} were verified. A high $Y_{P/X}$ of approximately 12.6 g/g was also achieved (Table 4.6). The 2,3-BDO yield of 0.40 g/g was similar to the value predicted by the proposed model, considering the statistical deviations. The optimized cultivation conditions were efficiently validated for an initial glucose concentration of approximately 30 g/L, added to 10 g/L pulse-fed glucose.

However, for initial glucose concentrations higher than 50 g/L, the 2,3-BDO production by *P. peoriae* NRRL BD-2 was strongly compromised under low agitation and aeration conditions (data not shown). To overcome the limited operational production, two strategies could be employed: decrease the initial glucose concentration and adopt multiple feeding strategies throughout the fermentation (MEARS, STOCKS, *et al.*, 2017), and select other culture conditions within the optimal range indicated by the DoE (RODRIGUES, IEMMA, 2009). Even under subinhibitory conditions, repeated and successive glucose feedings could alter the culture medium C/N ratio (SAINI, OSORIO-GONZALEZ, *et al.*, 2021), especially at late fermentation steps, when nutritional

limitation caused by non-replacement of the nitrogen source would be more pronounced. As a result, lower 2,3-BDO yields would be obtained, even though its production could be improved. In contrast, choosing a new culture condition within the DoE optimized range would increase the possibility of using higher initial glucose titers by enhanced mass and energy transfer without decreased 2,3-BDO yield.

Table 4.6: Fed-batch fermentation parameters of validation assays at 32 °C and pH=5 for 2,3-BDO production by *P. peoriae* NRRL BD-62.

Cultivation conditions	**Fermentation parameters**	
	200 rpm + 0.1 vvm	**400 rpm + 0.1 vvm**
kLa (h^{-1})	5.0 ± 2.7[a]	7.1 ± 2.7[a]
2,3-BDO (g/L)	16.5 ± 2.0	39.4 ± 1.0
X (g/L)	1.3 ± 0.2	2.4 ± 0.6
μ (h^{-1})	0.065 ± 0.014[a]	0.056 ± 0.004[a]
$Y_{P/S}$ (g/g)	0.40 ± 0.05[a]	0.43 ± 0.03[a]
$Y_{X/S}$ (g/g)	0.03 ± 0.01[a]	0.03 ± 0.01[a]
$Y_{P/X}$ (g/g)	12.6 ± 0.6[a]	16.9 ± 3.8[a]
SC (%)	99.9 ± 0.0	98.2 ± 0.2
Selectivity (%)	73.1 ± 2.7 (*levo*-2,3-BDO) 26.9 ± 2.7 (*meso*-2,3-BDO) 0.0 ± 0.0 (acetoin)	65.3 ± 0.8 (*levo*-2,3-BDO) 34.7 ± 0.8 (*meso*-2,3-BDO) 0.0 ± 0.0 (acetoin)
Time (h)	146	216

N= agitation rate; Q_{ar}= aeration rate (vvm= L_{air}/L_{medium}/min); kLa= volumetric oxygen transfer coefficient; 2,3-BDO= 2,3-BDO titer (*meso*- + *levo*-isomer titers); μ= cell growth specific rate; X= cell biomass; $Y_{P/S}$= 2,3-BDO/glucose yield; $Y_{X/S}$= cell biomass/glucose yield; $Y_{P/X}$= 2,3-BDO/cell biomass yield; SC= substrate consumption. The means followed by the same letter do not show significant differences by t-test (α = 0.05).

According to RODRIGUES, IEMMA (2009), the optimized conditions selection in a DoE should not be based exclusively on the designed model mathematical response but also on the physical meaning and economy and technical-operational effects on the bioprocess. Therefore, the 2,3-BDO production by *P. peoriae* NRRL BD-62 at 32 °C, pH=5, and kLa=7.1 h^{-1} (400 rpm and 0.1 vvm) was investigated in new fed-batch fermentation assays (Figure 4.7). Similar to this study, DAI, Jian Ying, GUAN, *et al.* (2020) reported limited metabolic activity of *K. pneumoniae* H3 for 2,3-BDO production under low oxygen supply and high initial raw inulin concentration, even under optimized agitation and aeration conditions. Enhanced raw inulin consumption was achieved by the agitation increasing from 200 to 250 rpm while maintaining the aeration rate at 0.2 vvm.

Despite increasing agitation from 200 to 400 rpm, kLa did not change much, being statistically similar to the value initially investigated (Table 4.6). Glucose consumption

and 2,3-BDO formation showed a nearly linear profile, indicating that an oxygen-limited environment was reached. The increased dissolved oxygen reduced the ethanol lactic acid production, whose ratios with 2,3-BDO were 0.017 g/g and 0.010 g/g, respectively. Therefore, an average reduction of more than 80% in 2,3-BDO metabolism byproducts was observed with increased oxygen supply. Similar behavior was reported by DAI, Jian Ying, GUAN, *et al.* (2020).

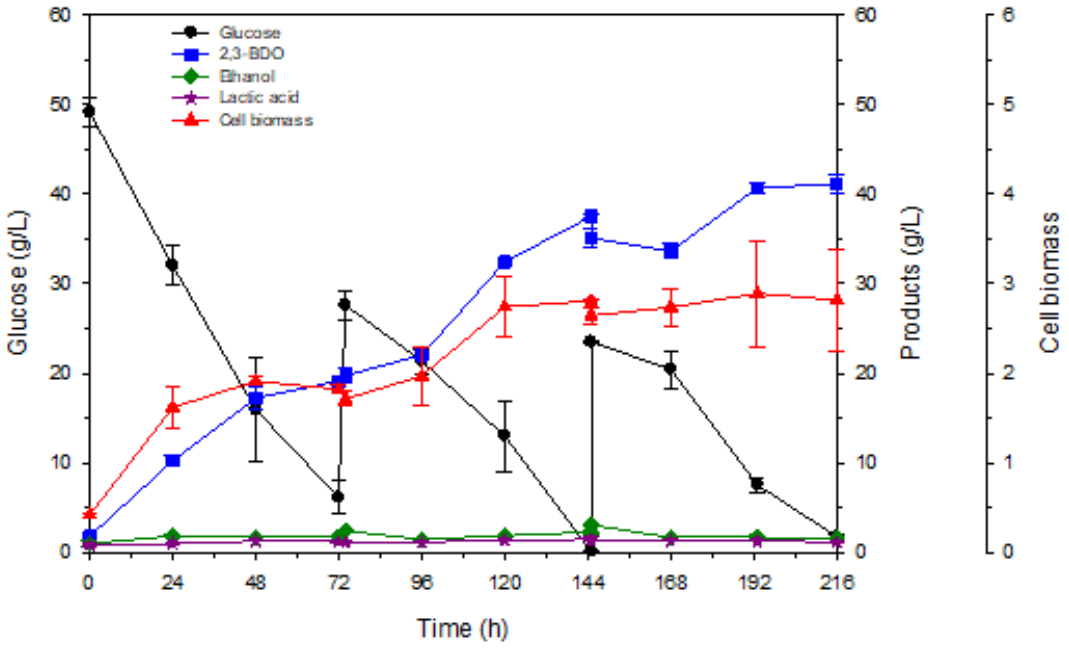

Figure 4.7: Time course of the fed-batch 2,3-BDO production by *P. peoriae* NRRL BD-62 at 32 °C, pH=5, 400 rpm, and 0.1 vvm (L_{air}/L_{medium}/min).

Again, acetoin was not detected. However, the optical isomer distribution was modified. About 35% of the total 2,3-BDO corresponded to the *meso*-isomer, whose yield increased in the presence of at least 15 g/L *levo*-isomer. For *levo*-2,3-BDO titers higher than 25 g/L in the final fermentation step, a maximum *meso*-isomer accumulation was observed (Figure 4.8). The *meso*-2,3-BDO formation can be attributed to the spontaneous S-α-acetolactate decarboxylation in the oxygen presence, as already discussed in this study, and to the stress mitigation mechanism reported by OKONKWO, UJOR, EZEJI (2017) for *P. polymyxa* strains. The authors observed a decrease in the *levo*-/*meso*-2,3-BDO ratio with increasing *levo*-2,3-BDO supplementation (20, 40, and 60 g/L) to the culture medium, especially at late fermentation steps, which was attributed to the lower *meso*-2,3-BDO toxicity compared to *levo*-2,3-BDO.

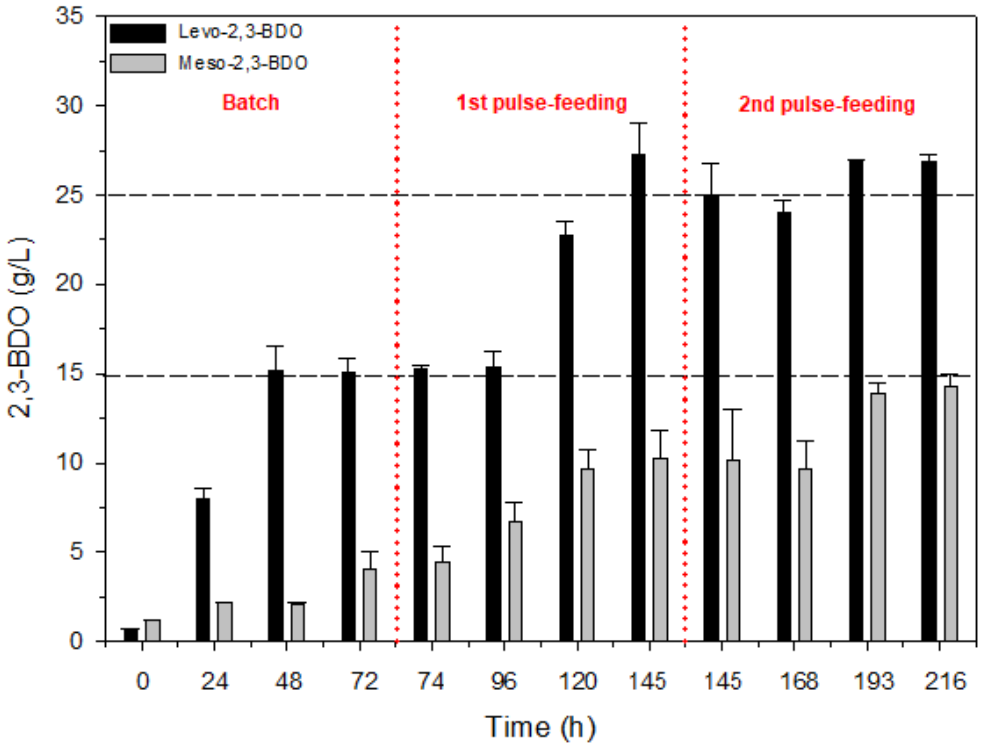

Figure 4.8: 2,3-Butanediol isomers ratio in fed-batch fermentation at 32 °C, pH=5, 400 rpm, and 0.1 vvm (L_{air}/L_{medium}/min). The region between the dashed horizontal lines corresponds to the *levo*-2,3-BDO titer from which the *meso*-2,3-BDO accumulation increases.

Despite the changes in the 2,3-BDO isomer percentage distribution, its production followed a similar behavior to that observed in the previous validation assays, showing no statistical differences in the fermentative parameters (Table 4.6). The 2,3-BDO metabolism in this new condition seems to have been further favored at the cell growth expense, as verified by the high $Y_{P/X}$ of 16.9 g/g and low $Y_{X/S}$ and μ values equal to 0.03 g/g and 0.056 h^{-1}, respectively. The 2,3-BDO yield of 0.43 g/g exceeded the value predicted by the proposed model, validating the optimized culture conditions.

The $Y_{P/S}$ values within the range predicted by the model at kLa of 5 and 7 h^{-1} indicated a high-optimized culture conditions reproducibility. Furthermore, fed-batch fermentation under optimized conditions showed lower yields than batch fermentation, despite a higher final 2,3-BDO production. This behavior was also observed by OKONKWO, UJOR, MISHRA, *et al.* (2017), who reported a lower 2,3-BDO yield of 0.34 g/g in fed-batch fermentation compared to 0.42 g/g in batch fermentation, both under

optimized conditions. A 34% increase in 2,3-BDO production by *P. polymyxa* DSM 365, equal to 68.5 g/L, was also verified.

In this study, the feeding strategy was based on two pulses in 72 h and 144 h. After the second feeding, a plateau profile was observed for the cell biomass, whose growth was low. The culture medium nutritional limitation, especially the nitrogen source, seems to have contributed to reduced cell activity, which ultimately compromised the 2,3-BDO production, as observed after 192 h (Figure 4.6). Furthermore, the final 2,3-BDO titer approached the toxic threshold of approximately 47 g/L reported by OKONKWO, UJOR, EZEJI (2017) for *P. polymyxa* DSM 365, contributing to reduced 2,3-BDO metabolism. Therefore, no additional feeding was performed after 216 h, and the final 2,3-BDO production by *P. peoriae* NRRL BD-62 under optimized culture conditions was equal to 39.4 g/L.

Table 4.7 presents a comparative analysis of the results obtained here with those reported by previous studies using *Paenibacillus* strains under different culture conditions. The production achieved by *P. peoriae* NRRL BD-62 is comparable to or higher than the 2,3-BDO production presented by engineered *Paenibacillus* strains, confirming its production capacity and promising application in large-scale processes.

Table 4.7: Comparative analysis of bio-based 2,3-BDO production by *Paenibacillus* strains under different culture conditions.

Strain	Fermentation mode	Substrate	Temperature (°C)	pH	Oxygen supply	2,3-BDO (g/L)	$Y_{P/S}$ (g/g)	Reference
P. brasilensis PB 24	Shake flasks	Glucose	32	Uncontrolled	200 rpm	27.1	0.38	DIAS, LIMA, *et al.* (2018)
P. polymyxa ATCC 12321	Continuous with cell recycling	Corn stover hydrolysate	37	6.5	500 rpm; 0.2 L/min	18.8	0.31	MA, HE, *et al.* (2018)
P. polymyxa ZJ-9-Δdud (modified)	Fed-batch	Inulin supplemented with glucose	30	6	300 rpm (22 h) → 200 rpm; 1 vvm	25.9	0.36	ZHANG, CAO, *et al.* (2018)
P. polymyxa ZJ-9	Shake flasks	Raw pure inulin	30	6	240 rpm (24 h) → 120 rpm	17.5	0.40	GAO, JIANG, *et al.* (2019)
P. polymyxa DSM 365 Lev null mutant (modified)	Batch	Sucrose + 0.4 g/L $CaCl_2$	35	-	300 rpm; 50 mL/min	35.4	0.37	OKONKWO, UJOR, *et al.* (2020)
P. polymyxa DSM 365 Δldh1 pHEiP_Ppbdh (modified)	Batch	Glucose	35	6	300 rpm; 0.075 vvm	43.8	0.43	SCHILLING, CICCONE, *et al.* (2020)
P. polymyxa DSM 365	Shake flasks	60% (v/v) non-detoxified wheat straw hydrolysate	35	Uncontrolled	200 rpm	32.5	0.33	OKONKWO, UJOR, *et al.* (2021)
P. polymyxa PM 3605	Shake flasks	Crude glycerol supplemented with molasses	37	Uncontrolled	200 rpm	19.0	0.35	TINÔCO, DE CASTRO, *et al.* (2021)
P. peoriae NRRL BD-62	Fed-batch	Glucose	32	5	400 rpm; 0.1 vvm	39.4	0.43	This study

2,3-BDO= 2,3-BDO titer (*meso*- + *levo*-isomer titers); $Y_{P/S}$= 2,3-BDO/glucose yield; vvm= L_{air}/L_{medium}/min.

4. Conclusion

Culture conditions optimization was used as a metabolic strategy for controlling 2,3-BDO yield and selectivity by a newly *P. peoriae* NRRL BD-62. In this order, pH, $Q_{ar,}$ and N were the most relevant bioprocess variables, whose individual and interaction effects on kLa contributed to the microaerobic environment establishment, responsible for directing the carbon flux to the 2,3-BDO synthesis rather than its byproducts and cell biomass. The low oxygen supply and pH made acetoin accumulation difficult and favored the high *levo*-2,3-BDO optical purity. While the available oxygen is responsible for regulating the redox balance represented by $NADH/NAD^+$ ratio, the pH acts on the activity of 2,3-BDO metabolism key enzymes, mainly BDH. This enzyme can preferentially activate the *levo*-2,3-BDO production pathway in the NADH presence, resulting in its higher isomeric yield. The 2,3-BDO production in fed-batch fermentation was also made possible by the optimized culture conditions selection based on the physical significance and effects on the economics and technical-operational bioprocess aspects. As a result, the highest 2,3-BDO titer was reported for a wild-type *P. peoriae* strain. Therefore, the optimized culture conditions investigation can be considered an important strategy capable of improving the 2,3-BDO yield and selectivity, allowing to control the cell metabolism without applying genetic engineering, only microbiological knowledge.

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CHAPTER 5

Contextualization

In this chapter, different nitrogen sources were investigated to replace the yeast extract traditionally used for 2,3-BDO production by *P. peoriae* NRRL BD-62, thereby reducing the culture medium costs. The effects on 2,3-BDO yield and selectivity at low oxygen supply were considered when choosing the most efficient alternative nitrogen source. Nutritional supplementation by amino acid and microbial pH autoregulation were also investigated. The results found were validated in batch fermentation. Furthermore, an initial attempt of 2,3-BDO scale-up assay was performed in fed-batch fermentation. Overall, an in-depth study of the nitrogen source and pH effects on 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 was carried out here.

Therefore, the specific objectives of this chapter were:

- Determine the carbon/nitrogen (C/N) ratio responsible for the best 2,3-BDO yield and selectivity;
- Investigate inexpensive alternative organic and inorganic nitrogen sources (commercial and residual nature);
- Evaluate the amino acid supplementation effects on 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 using the selected alternative nitrogen source;
- Evaluate the microbial pH autoregulation effects on 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 using the selected alternative nitrogen source;
- Validate the 2,3-BDO production by *P. peoriae* NRRL BD-62 using the new culture medium in batch fermentation;
- Investigate the 2,3-BDO production scale-up using the new culture medium in fed-batch fermentation;
- Determine the new culture medium costs.

Relevance

Through the investigation carried out in this chapter, it was possible to understand:

- The C/N ratio effects on the bio-based 2,3-BDO production and selectivity (little discussed in the literature);
- The effects of replacing yeast extract with alternative nitrogen sources (organic and inorganic) on yield, selectivity, and economy of 2,3-BDO by *P. peoriae* NRRL BD-62;
- The amino acid supplementation and microbial pH autoregulation effects on 2,3-BDO metabolism by *P. peoriae* NRRL BD-62;
- The metabolic behavior of bio-based 2,3-BDO production in scale-up assays;
- The relevance of low-cost culture medium for a preliminarily feasible gross economic margin for large-scale 2,3-BDO production, considering the raw material costs in the international market adopted by Mercosur (free on board prices) and international input sales prices.

EFFECTS OF THE NITROGEN SOURCE AND pH ON THE 2,3-BUTANEDIOL METABOLISM AND THE CULTURE MEDIUM COSTS BY *Paenibacillus peoriae* NRRL BD-62 UNDER MICROAEROBIC CONDITIONS

Abstract

Nitrogen source is one of the main limiting factors for large-scale bio-based 2,3-butanediol (2,3-BDO) production due to the costs and downstream processing complications. In this study, eight organic and inorganic nitrogen sources were investigated to replace the yeast extract traditionally used for the 2,3-BDO production by a newly *Paenibacillus peoriae* NRRL BD-62. Yield, selectivity, and culture medium costs of 2,3-BDO were used to evaluate the efficiency of the investigated nitrogen sources. Assays were carried out at C/N=8.5 g/g and kLa~5 h^{-1}. The Arg and Asn supplementation and microbial pH autoregulation effects on 2,3-BDO metabolism at low oxygen supply were also investigated. The highest 2,3-BDO yields were obtained using corn steep liquor (0.43 g/g) and ammonium chloride (0.45 g/g) with a pH control of around 5. The NH_4Cl-based medium was selected, which reduced raw material costs by 67%. High *levo*-2,3-BDO selectivity of 85% was achieved with no acetoin accumulation. Amino acid supplementation did not contribute to a statistically improved 2,3-BDO production and was disregarded. A 2,3-BDO yield of 0.38 g/g and a *levo*-isomer selectivity of 87% were obtained in batch fermentation without external pH control, contributing to a higher bioprocess economy. To the best of our knowledge, this is the first time that a high 2,3-BDO yield and selectivity have been reported by a safe and wild-type *P. peoriae* strain using a low-cost NH_4Cl-based medium under microaerobic conditions.

Keywords: Ammonium chloride, corn steep liquor, optical purity, low-cost medium, large-scale.

1. Introduction

Bio-based 2,3-butanediol (2,3-BDO) is a green platform chemical used in different industrial segments, recently investigated as a virus resistance inducer in plants (KONG, SHIN, *et al.*, 2018), a biostimulant for inducing drought tolerance in the agriculture industry (LEE, Jae Won, BHAGWAT, *et al.*, 2023), and with potential application in the COVID-19 pathogen-killing plastic films production (TRANSPARENCY MARKET RESEARCH, 2022). With a growing market expected to reach around U$ 300 million by 2030, large-scale bio-based 2,3-BDO production has been encouraged, although only a few companies have implemented it in practice (TINÔCO, BORSCHIVER, *et al.*, 2020, TRANSPARENCY MARKET RESEARCH, 2022).

The culture medium is one of the main limiting factors for the bio-based 2,3-BDO production scale-up, as it accounts for a significant production costs portion of around 50% of the total spent in the bioprocess (DAS, PRAKASH, *et al.*, 2021, HAZEENA, SINDHU, *et al.*, 2020). Usually, the culture medium is composed of vitamins, amino acids, mineral salts, and carbon and nitrogen sources considered the greater productive importance and cost components (JI, Xiao-jun, HUANG, *et al.*, 2011). The C/N ratio is responsible for the nutritional balance used in the synthesis, growth, and cell metabolism (LI, Ruixue, ZHI, *et al.*, 2014), being one of the fermentation conditions to be kept constant for an efficient scale-up process (DUTTA, DAS, 2017).

In particular, the nitrogen source is considered an ionic growth factor used in the proteins and nucleic acids synthesis destined for cell plasticity processes (THAPA, LEE, *et al.*, 2019), whose deficient supply may cause the slowing down and growth fermentation failure (YU, O'HAIR, *et al.*, 2022). Furthermore, the nitrogen source prices are decisive for the culture medium selection because they influence the direct production costs (CARDOSO, CAMPANI, *et al.*, 2020). Traditionally, complex nitrogen sources such as yeast extract, peptone, and tryptone have been used for the 2,3-BDO production (NARISETTY, ZHANG, *et al.*, 2022). These sources may contain free amino acids and metal ions such as $Mn2^{+}$ responsible for regulating the carbon flux from α-acetolactate to acetoin rather than branched-chain amino acid synthesis by stimulating α-acetolactate decarboxylase (ALDC) activity, thus contributing to improved specific 2,3-BDO

production (CHO, KIM, *et al.*, 2015, THAPA, LEE, *et al.*, 2019). However, high concentrations of these compounds are often required, which compromises the techno-economic feasibility of large-scale 2,3-BDO production since they increase the culture medium costs and downstream processing complexity (LI, Jinshan, WANG, *et al.*, 2013, NARISETTY, ZHANG, *et al.*, 2022, YANG, Taowei, ZHANG, *et al.*, 2012).

Several previous studies have investigated alternative nitrogen sources to reduce the culture medium costs and downstream step complexity. The organic nitrogen sources recently investigated have been corn steep liquor (HAZEENA, BINOD, *et al.*, 2019), soy-based hydrolysates (DAS, PRAKASH, *et al.*, 2021), and casamino acid (KIM, Bora, PARK, *et al.*, 2022), while inorganic nitrogen sources have been mainly ammonium salts (AMRAOUI, PRABHU, *et al.*, 2022, ERIAN, GIBISCH, *et al.*, 2018, FERNÁNDEZ-GUTIÉRREZ, VEILLETTE, *et al.*, 2022). Despite a large number of studies on the nitrogen source effects on 2,3-BDO production and economy, few studies have attempted to understand the relationship between nitrogen source, oxygen supply, and pH, and their synergistic effects on 2,3-BDO yield and selectivity, considered important production factors that can affect final bioprocess costs.

The 2,3-BDO metabolism is characterized by the activity of four key enzymes, including butanediol dehydrogenase (BDH) and diacetyl reductase (DAR), responsible for the synthesis and distribution of acetoin and 2,3-BDO-isomers (ZHANG, Xian, BAO, *et al.*, 2014). The activity of these enzymes depends on the redox balance established by the $NADH/NAD^+$ ratio, which in turn is regulated by the oxygen supply and the pH (DAI, Jun-Jun, CHENG, *et al.*, 2014, ZHANG, Xian, BAO, *et al.*, 2014). Under microaerobic conditions, NADH formation is favored, which maintains adequate redox potential for the 2,3-BDO synthesis (FU, WANG, *et al.*, 2014, KOU, CUI, *et al.*, 2022, ZHANG, Yanjie, LI, *et al.*, 2013). The pH controls the oxidation-reduction reactions, promoting the metabolic flow partition between acetoin and 2,3-BDO. The cofactor specificity of BDH and DAR implies the strict NADH use for reduction reactions and NAD^+ for oxidation reactions. In an acidic or slightly acidic environment, reduction reactions are favored, shifting the equilibrium from acetoin to 2,3-BDO (ZHANG, Xian, BAO, *et al.*, 2014).

The $NADH/NAD^+$ balance can also be affected by the nitrogen source nature due to the limited oxygen mass transfer caused by the fermentation broth viscosity (HÄßLER, SCHIEDER, *et al.*, 2012). Furthermore, the pH gradient established between the intracellular and extracellular compartments from the nitrogen source can alter the ATP amount required to transport protons (H^+) (THAPA, LEE, *et al.*, 2019). As a result, substrate consumption is regulated, and carbon flux is directed toward the by-products synthesis, such as acetic acid and cell respiration, rather than 2,3-BDO (TINÔCO, PATERAKI, *et al.*, 2021). Therefore, the redox potential established by the synergism between dissolved oxygen (DO), pH, and nitrogen source is an essential factor for the 2,3-BDO metabolism control (BAO, ZHANG, *et al.*, 2015), which should be considered for the success of its large-scale production economy.

This study aimed to investigate alternative low-cost nitrogen sources to replace yeast extract for efficient and low-cost 2,3-BDO production by a newly GRAS (generally recognized as safe) *P. peoriae* NRRL BD-62 on a large scale. The nitrogen source was defined based on its effects on 2,3-BDO metabolism, considering the yield and *levo*-isomer optical purity from the synergism between DO, pH, and nitrogen supply. The C/N ratio and amino acid supplementation effects on redox balance and 2,3-BDO production economy were also evaluated. A high 2,3-BDO yield similar to that observed for wild non-GRAS microbial producers was achieved in the NH_4Cl-based medium at C/N= 8.5 g/g, whose raw material costs were reduced by 67%. Under microaerobic conditions, no acetoin accumulation was detected, while a *levo*-2,3-BDO optical purity of at least 85% was achieved. To the best of our knowledge, this is the first time that a 2,3-BDO yield of 90% of theoretical value without acetoin accumulation has been reported by a safe and wild-type *P. peoriae* strain using a low-cost NH_4Cl-based medium without nutritional supplementation in microaerobic batch fermentation.

2. Materials and methods

2.1 Bacterial strain, inoculum, and media

Paenibacillus peoriae NRRL BD-62 was obtained from the culture collection of the Microbial Genetics Laboratory of the Microbiology Institute of the Federal University of Rio de Janeiro (Brazil), maintained at -80 °C in Tryptic Soy Broth – TSB medium (17

g/L tryptone, 3 g/L soytone, 5 g/L NaCl, 2.5 g/L K_2HPO_4, and 2.5 g/L glucose) and supplemented with 25% (v/v) glycerol. Initially, 0.2 mL glycerol stock solution was transferred to 250 mL shake flasks containing 50 mL TSB medium for cell reactivation at 32 °C, 200 rpm, for 24 h. Then, 1% (v/v) reactivated cells were transferred to 1 L shake flasks containing 350 mL YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) for inoculum growth at 32 °C, 200 rpm, for 16 h. Subsequently, 10% (v/v) inoculum was used for fermentation assays in medium proposed by ADLAKHA, YAZDANI (2015) containing 0.5 g/L KH_2PO_4, 2.0 g/L K_2HPO_4, 0.0225 g/L $MnSO_4$, and 0.3 g/L KCl. Commercial glucose was used as a substrate, while different nitrogen sources were investigated to replace the yeast extract (YE) in the original fermentation medium. Culture conditions were previously optimized for *P. peoriae* NRRL BD-62 in a medium containing glucose, as presented in the next section.

2.2 Fermentation procedure

Batch fermentations were carried out in DASbox® Mini Bioreactor – 300 mL (Eppendorf, Germany), designed as a 4-fold system with 16 parallel bioreactors, using a starting volume of 200 mL. The temperature was maintained at 32 °C by a liquid-free heating and cooling system (Peltier). The oxygen supply was represented by the volumetric oxygen transfer coefficient (kLa), initially maintained at around 5 h^{-1} by a submerged gas supply via an L-sparger (4 mm compression fitting) through which air was injected into a sterile filter (0.22 μm) at 0.1 vvm. The agitation system consisted of two Rushton-type impellers 3 cm apart, rotating clockwise at 200 rpm, that provided turbulent mixing of the culture broth. Solutions of 2 M NaOH or 1 M H_2SO_4 were used to keep the pH equal to 5 during the whole fermentation. Glucose and the remaining nutrients were separately sterilized by autoclaving at 121 °C for 15 min. Antifoam 204 (Sigma-Aldrich, USA) was used when needed. Fermentation assays were performed in biological duplicate.

2.2.1 Carbon/nitrogen (C/N) ratio

The C/N ratio (g/g) effects on 2,3-BDO metabolism were evaluated under four conditions using initially 10 g/L glucose and varying the YE concentration: 8.5 (7.5 g/L YE), 11 (5.0 g/L YE), 18.5 (2.5 g/L YE), and 40.7 (1.0 g/L YE). A C/N ratio of 8.5 g/g

was used as a control, based on the results obtained by ADLAKHA, YAZDANI (2015) for the 2,3-BDO production by *P. polymyxa* ICGEB2008.

2.2.2 Alternative nitrogen sources

Eight low-cost commercial and residual nitrogen sources were investigated to replace commercial YE. Four organic compounds (YE Senai, YE brewer, corn steep liquor – CSL, and urea) and four inorganic compounds ($NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$) were evaluated. Each nitrogen source was used sufficiently to maintain the C/N ratio at the previously selected condition, considering an initial glucose concentration of 10 g/L. YE Senai and CSL were kindly provided by the SENAI Innovation Institute for Biosynthetic - ISI Biossintéticos (Brazil) and the Microbial Biotechnology Laboratory - LaBiM - of the Chemistry Institute of the Federal University of Rio de Janeiro (Brazil), respectively. Elemental analysis of the nitrogen sources used in this study is presented in Table S1 (Supplementary material).

2.2.3 Amino acid supplementation

Arginine (Arg) and asparagine (Asn) were investigated as nutritional supplements to enhance 2,3-BDO metabolism. These amino acids were added at the beginning of fermentation using a syringe with a sterile filter (0.22 μm). The initial concentration in the culture medium was adjusted to 1.5 g/L (GAO, J., JIANG, *et al.*, 2019).

2.2.4 pH control

The pH effects on 2,3-BDO metabolism were investigated by removing its external control around 5 mediated by alkali and acid in a medium containing the nitrogen source selected under the most suitable conditions of C/N ratio and nutritional supplementation for 2,3-BDO production by *P. peoriae* NRRL BD-62. This strategy evaluated pH microbial autoregulation to control cell homeostasis and the effects on 2,3-BDO metabolism and culture medium costs. The initial pH of each culture medium was equal to the nitrogen source pH in the salt medium supplemented or not with Arg and Asp, whose value ranged from 5-7.

2.2.5 Validation assays

After defining the nutritional culture medium composition and the most suitable fermentation conditions for large-scale 2,3-BDO production, validation assays were carried out in batch cultures using higher initial glucose concentrations (~30 g/L). A scale-up attempt was performed as fed-batch fermentation in 3 L-vessels (Bioflo®/CelliGen® 310, New Brunswick Scientific-Eppendorf, USA) using a starting volume of 1 L. The bioreactor was equipped with temperature, dissolved oxygen (DO), and pH sensors. The temperature was maintained at 32°C. The oxygen supply was controlled by the initial kLa of 5 h^{-1} through aeration of 0.4 vvm by injecting filtered air (0.22 μm) through a ring sparger at the bottom of the vessel, and an agitation of 200 rpm promoted by one Rushton 6-plate-impeller. Validation assays were performed without external pH control. The fermentation started with approximately 10 g/L glucose, and after 28 h, a 500 g/L solution was pulsed to restore its initial concentration. Assays were carried out until substrate exhaustion in biological duplicate. Again, the culture media sterilization was performed by glucose autoclaving separated from the other nutrients at 121 °C for 15 min. Antifoam 204 (Sigma-Aldrich, USA) was used when needed.

2.3 Analytical methods

Samples were taken throughout the batch and fed-batch fermentations and analyzed for dry cell weight (DCW) determination, glucose consumption, and metabolite production. Cell biomass samples were filtered through a 0.22 μm cellulose acetate membrane (Sartorius, Germany), followed by weighing after drying in an oven at 60 °C until constant weight. The DCW values were correlated to the optical density at 600 nm ($OD_{600\,nm}$), measured in a UV–visible spectrophotometer (Biospectro SP-22, Brazil). The following relationship was established in the fermentation media: 1 $OD_{600\,nm}$ = 0.392 ± 0.016 g/L DCW. Glucose and fermentation products (*levo*- and *meso*-isomers, acetoin, ethanol, and lactic and acetic acids) were analyzed on a high-performance liquid chromatography system (HPLC; Agilent, USA). HPLC system was equipped with an HPX-87H Aminex column (300 × 7.8 mm, Bio-Rad, USA) and a Refractive Index Detector (RID; Agilent, USA). The mobile phase consists of 5 mM H_2SO_4 at 0.6 mL/min. The column temperature was controlled at 45°C. The collected samples were initially treated by centrifugation at 10,000 x *g*, at 25°C for 10 min, in a microtube centrifuge

(MiniSpin®, Eppendorf, Germany). The supernatant was diluted 10-fold with Milli-Q water and filtered through a 0.22 μm cellulose acetate membrane (Minisart® NML Syringe Filters, Sartorius, Germany). Then, the supernatant was analyzed by HPLC. The glucose and fermentation products concentration were determined by calibration curves of standard substances (Merck KgaA, Darmstadt, Germany). Fermentation parameters were analyzed by the t-test, with a 95% confidence level (α=0.05).

2.4 Fermentation parameters

Three production factors were used to evaluate fermentation efficiency and define the best nitrogen source for large-scale application: yield, selectivity, and culture medium costs of 2,3-BDO. The yield was calculated based on the ratio between 2,3-BDO production and glucose consumption ($Y_{P/S;}$ g/g) and the ratio between 2,3-BDO production and cell biomass ($Y_{P/X}$; g/g). The 2,3-BDO selectivity was calculated based on the percentage composition between *levo*-2,3-BDO, *meso*-2,3-BDO, and acetoin (C4-compounds).

Furthermore, the DO mass balance was used to determine the oxygen transfer rate (OTR) and oxygen uptake rate (OUR) used in the scale-up process, according to Equation 1. While OTR was defined from kLa (Equation 2), OUR was given as a function of the oxygen uptake specific rate (q_{O2}) (Equation 3). Considering that the oxygen supply was wholly consumed during the fermentation, no DO accumulation was observed in this study, allowing the q_{O2} determination from kLa, according to Equation 4.

$$\frac{dC}{dt} = OTR - OUR \qquad (1)$$

$$OTR = kLa\ (CS-C) \qquad (2)$$

$$OUR = -\ q_{O2}.X \qquad (3)$$

$$q_{O2} = \frac{kLa\ (CS-C)}{X} \qquad (4)$$

Where C_S is the saturated DO concentration at 32 °C (g/L), C is the DO concentration in the culture medium (g/L), and X is the DCW (g/L). The oxygen parameter units are: OTR and OUR = g_{O2}/L/h, kLa= h^{-1}, and q_{O2}= g_{O2}/g_{DCW}/h.

3. Results and discussion

3.1 Effects of C/N ratio on 2,3-BDO metabolism

The C/N ratio was set by keeping the glucose concentration constant and changing the YE concentration to evaluate the actual nitrogen source effect on the 2,3-BDO production by *P. peoriae* NRRL BD-62. Furthermore, it was expected to achieve more significant savings using lower YE concentrations. The time courses of each assay are presented in Figure 5.1.

Except for C/N= 8.5 g/g, the investigated C/N ratios showed statistically similar behaviors for glucose consumption (Fig. 5.1a), cell biomass formation (Fig. 5.1b), and 2,3-BDO production (Fig. 5.1c). Glucose consumption was more pronounced in the assays at C/N= 8.5 g/g, in that the nitrogen appears to have been sufficient for efficient assimilative carbon metabolism by *P. peoriae* NRRL BD-62 (Fig. 5.1a). As a result, about 31% of the substrate was consumed in 2 h and approximately 81% in 24 h. In contrast, under low nitrogen supply, glucose consumption seems to have been compromised by the culture medium nutritional limitation, ended up leading to a carbon consumption of only 50-60% in 24 h, even at a substrate consumption of 0.30 g/L/h similar to that observed at C/N= 8.5 g/g in 2-24 h (Fig. 5.1a).

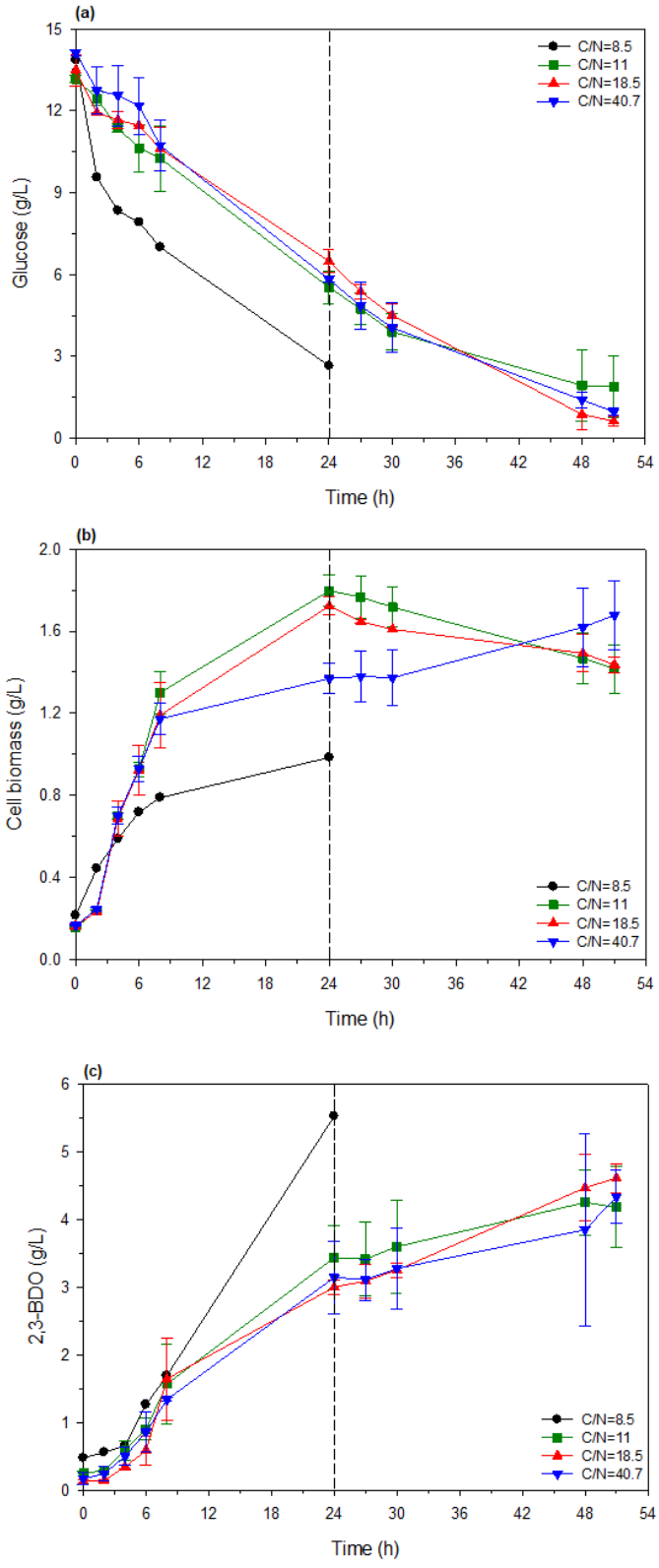

Figure 5.1: Time courses of 2,3-BDO production by *P. peoriae* NRRL BD-62 from different glucose/yeast extract ratios in batch fermentation: (a) glucose; (b) cell biomass; (c) 2,3-BDO. The C/N=8.5 g/g assay was performed for 24 h (limited by the dashed lines), while the other C/N ratios were performed for 51 h.

The fermentation time was extended to 51 h for assays at C/N> 8.5 g/g to enhance glucose uptake. It allowed a final substrate consumption of approximately 85-95%, as presented in Table 5.1. Therefore, high C/N ratios led to a slow reaction rate, whose fermentation time had to be extended to allow maximum 2,3-BDO production by *P. peoriae* NRRL BD-62.

Table 5.1: Effects of carbon:nitrogen ratio on 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and yeast extract.

Time (h)	C/N ratio (g/g)	DCW (g/L)	2,3-BDO (g/L)	EtOH (g/L)	Acetoin (g/L)	SC (%)
24	8.5	0.77 ± 0.03^{a}	5.05 ± 0.32^{a}	0.03 ± 0.00		80.8 ± 0.0^{a}
24	11.0	1.64 ± 0.08^{b}	3.18 ± 0.45^{b}	0.22 ± 0.31		58.0 ± 4.1^{b}
	18.5	1.56 ± 0.06^{b}	2.87 ± 0.13^{b}	0.00 ± 0.00		51.7 ± 5.3^{b}
	40.7	1.20 ± 0.08^{b}	2.97 ± 0.45^{b}	0.11 ± 0.15	ND*	58.7 ± 1.1^{b}
51	11.0	1.26 ± 0.12^{b}	3.92 ± 0.47^{b}	0.36 ± 0.06		85.7 ± 9.8^{a}
	18.5	1.27 ± 0.02^{b}	4.48 ± 0.24^{b}	0.08 ± 0.11		95.3 ± 1.4^{b}
	40.7	1.51 ± 0.17^{b}	4.16 ± 0.31^{b}	0.22 ± 0.02		93.1 ± 0.9^{b}

*ND= not detected.
C/N ratio= carbon/nitrogen ratio; DCW= dry cell weight (cell biomass); 2,3-BDO= 2,3-BDO titer (*meso-* + *levo*-isomer titers); EtOH= ethanol titer; SC= substrate consumption. The means followed by the same letter do not show significant differences concerning the C/N=8.5 by t-test ($\alpha = 0.05$).

Most of the carbon assimilated at C/N= 8.5 g/g was directed to the 2,3-BDO production (Fig. 5.1c). In comparison, the carbon consumed in the other C/N ratios was directed preferentially to cell plasticity (Fig. 5.1b). Due to the higher nitrogen concentration, a higher cell concentration was expected in the assays at C/N= 8.5 g/g, resulting from the enhanced assimilative metabolism observed in this condition (ANZOLA-ROJAS, GONÇALVES DA FONSECA, *et al.*, 2015). However, the low oxygen supply seems to have contributed to the so-called Pasteur effect, in which the glycolytic pathway was favored, and the respiratory process was prioritized over the fermentation process under nutritional limitations and an anaerobic environment. This behavior was observed in the assays at high C/N ratios, showing a higher initial specific cell growth rate (μ) and a higher dry cell weight (DCW). While the assays at C/N= 8.5 presented μ of around 0.16 h^{-1}, the other conditions presented μ around 0.32 h^{-1} (Fig. 5.1c). The DCW at C/N= 11 and 18.5 g/g and C/N= 40.7 g/g were 2-fold and 1.5-fold higher than at C/N= 8.5 g/g in 24 h, respectively (Table 5.1).

The 2,3-BDO production reached a $Y_{P/S}$ of 0.45 g/g, equivalent to 90% of the theoretical value at C/N= 8.5 g/g, with a titer of 5.05 g/L and glucose consumption of

about 81% in 24 h (Table 5.1). Statistically similar yields were obtained at C/N= 11 and 18.5 g/g in 24 h, with an average 2,3-BDO production of 3 g/L and only 52-58% glucose consumption. For the assays at C/N= 40.7 g/g, the $Y_{P/S}$ was reduced by 20% concerning that obtained at C/N= 8.5 g/g, although 2,3-BDO production and glucose consumption were similar to the C/N= 11 and 18.5 g/g ratios. Therefore, nitrogen nutritional limitation was responsible for incomplete glucose conversion preferentially used for cell plasticity. So, a lower 2,3-BDO production by *P. peoriae* NRRL BD-62 was verified in 24 h. ANZOLA-ROJAS, GONÇALVES DA FONSECA, *et al.* (2015) also found an inverse relationship between biohydrogen yield and cell biomass accumulated by natural fermentation from synthetic wastewater in an up-flow fixed-bed anaerobic reactor. Figure 5.2 compares $Y_{P/S}$, $Y_{X/S}$, and $Y_{P/X}$ of investigated C/N ratios.

The $Y_{P/S}$ at high C/N ratios decreased concerning assays at C/N= 8.5 g/g, reaching about 70% of the theoretical value, on average equal to 0.34 g/g, in 51 h (Fig. 5.2a). A lower $Y_{X/S}$ of 0.11 g/g on average was also observed, with reduced cell biomass resulting from limited nitrogen supply (Fig. 5.2b). The average 2,3-BDO production was only 4.2 g/L for assays in 51 h (Table 5.1). Therefore, the additional assimilated carbon appears to have been directed towards cell maintenance processes and other metabolic pathways rather than microbial biomass formation and 2,3-BDO synthesis.

It was recently reported that increased glycolytic flux to compensate for the intracellular ATP lack was responsible for increased glucose consumption (KOU, CUI, *et al.*, 2022). Furthermore, the by-product formation, such as ethanol and organic acids, can indicate the carbon balance shift to metabolic pathways other than respiration and the 2,3-BDO production (Table 5.1). SUNARNO, PRASERTSAN, *et al.* (2019) reported similar behavior for producing by-products with an increasing C/N ratio. In the authors' study, 2,3-BDO was considered a by-product of the *Enterobacter aerogenes* TISTR 1468 metabolism, whose main product was ethanol. With the increased C/N ratio resulting from the decreased tuna condensate, an alternative low-cost carbon source, the 2,3-BDO formation was favored at the expense of cell growth and ethanol production from a complex medium using crude glycerol and tuna condensate.

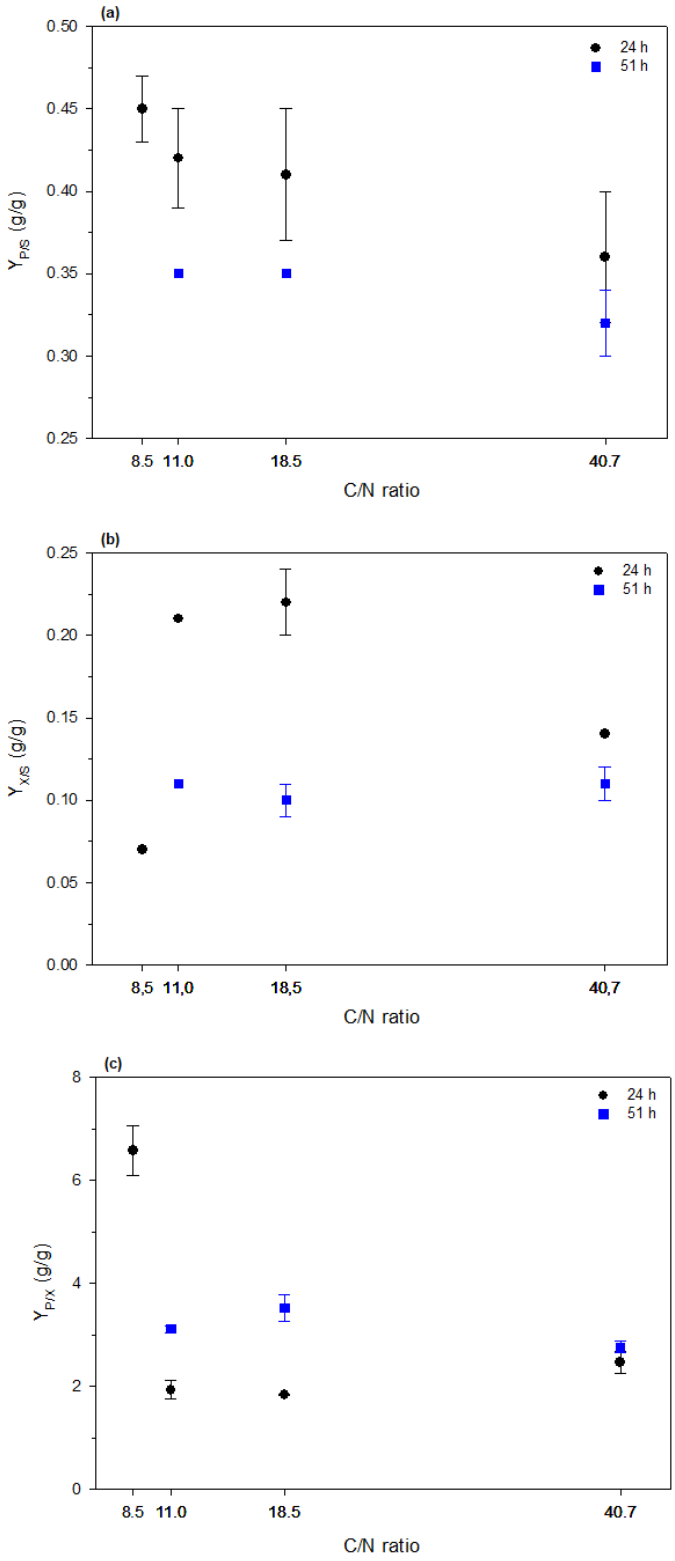

Figure 5.2: Carbon:nitrogen ratio effects on 2,3-BDO yields by *P. peoriae* NRRL BD-62 in batch fermentation: (a) $Y_{P/S}$ (2,3-BDO/glucose yield); b) $Y_{X/S}$ (cell dry weight/glucose yield); and (c) $Y_{P/X}$ (2,3-BDO/cell dry weight yield).

In contrast, DAS, PRAKASH, *et al.* (2021) reported lower lactic acid and formic acid formation under limited nitrogen supply when reducing YE to cheapen the culture medium for the 2,3-BDO production by a newly *Bacillus licheniformis* BL1. The optimal N concentration was defined as 0.37 g/L. Under these conditions, the 2,3-BDO titer and yield were not affected, but the fermentation time for maximum 2,3-BDO production was extended.

Since 2,3-BDO is considered a product partially associated with cell growth (TINÔCO, DE CASTRO, *et al.*, 2021), the balance between respiration and fermentation processes must be established. One parameter used to verify this balance's efficiency is the $Y_{P/X}$. The highest $Y_{P/X}$ was achieved at C/N= 8.5 g/g, equal to 6.57 g/g (Fig. 5.2c). Increasing the C/N ratio decreased the $Y_{P/X}$ in 24 h, mainly due to the increased cell biomass resulting from the previously discussed Pasteur effect. Lower $Y_{P/X}$ on average of 3.13 g/g at high C/N ratios were verified in 51 h, despite the reduced cell biomass (Fig. 5.2b). Therefore, the $Y_{P/X}$ results are in line with the observations made about the additional carbon assimilated at higher C/N ratios, in which the carbon flux was directed to the production of 2,3-BDO metabolism by-products, thus reducing its final yield.

The 2,3-BDO production depended on the C/N ratio, although it did not show statistically significant differences between the assays at C/N> 8.5 g/g. However, compared to C/N= 8.5 g/g, the high C/N ratios were responsible for a slower reaction rate and incomplete carbon consumption, leading to a lower 2.3 -BDO titer and yield in 24 h. The nutritional balance given by C/N= 8.5 g/g was found to be adequate for the metabolic activities of *P. peoriae* NRRL BD-62, leading to yields close to the theoretical values. Therefore, an increased C/N ratio to reduce the culture medium costs was not considered productively efficient, having been replaced by the investigation of alternative nitrogen sources (residual and commercial) at C/N= 8.5 g/g.

3.2 Effects of alternative nitrogen sources on 2,3-BDO metabolism

3.2.1 Organic nitrogen sources

Two commercial organic nitrogen sources (YE brewer and urea) and two residual organic nitrogen sources (YE Senai and CSL) were used to replace YE (control). They

were chosen based on their chemical composition, availability, and market prices (Tables S1 and S2 - Supplementary material). The results obtained for 2,3-BDO production, glucose consumption, and bioprocess yields are presented in Figure 5.3.

None of the four alternative organic nitrogen sources allowed an equal or higher 2,3-BDO titer than YE (~5.05 g/L) in 24 h (Fig. 5.3a). A production of approximately 3 g/L was obtained using YE Senai, YE brewer, and CSL, while just over 0.5 g/L from urea was achieved. With the fermentation extension, the 2,3-BDO production from CSL reached close to the control in 48 h. A 2,3-BDO production of 3.8-4.5 g/L from YE Senai and YE brewer was obtained in 30 h and 48 h, respectively. It was equal to CSL, considering statistical deviations. Again, a lower 2,3-BDO titer of only 3.6 g/L from urea was obtained at 71 h.

Commercial urea is a chemically defined organic compound of high purity (~98%). On the other hand, YE and CSL exhibit complex chemical composition, regardless of their residual or commercial nature, formed by proteins, free peptides, and other growth factors (ÁVILA, ROCHÓN, *et al.*, 2021, THAPA, LEE, *et al.*, 2019). Therefore, the nutritional capacity of the investigated organic nitrogen sources may explain the differential 2,3-BDO production by *P. peoriae* NRRL BD-62. The amino acids and vitamins absence in urea possibly contributed to a 2,3-BDO production about 10-fold and 1.4-fold lower than that obtained using YE at 24 h and 71 h, respectively, since these nutrients are used to enhance cell plasticity and, consequently, the synthesis of metabolites associated with microbial growth.

As in this study, XIAO, WANG, *et al.* (2012) investigated CSL and urea effects on the 2,3-BDO and acetoin production by *Geobacillus* sp. XT15, a novel thermophilic strain, from glucose in batch fermentation. The authors found a production of approximately 7.7 g/L acetoin and 14.5 g/L 2,3-BDO from 60 g/L CSL, equivalent to 69% product yield. A poor acetoin titer of less than 0.5 g/L was obtained using urea as a nitrogen source. AMRAOUI, NARISETTY, *et al.* (2021) investigated the CSL and urea supplementation of 2 g/L in flask cultures with a synthetic medium containing xylose for 2,3-BDO production by a mutant *E. ludwigii* strain. While a marginal impact on cell growth and no change in 2,3-BDO production were observed using urea, $OD_{600\ nm}$ and 2,3-BDO titer increased by about 12-16% with CSL supplementation.

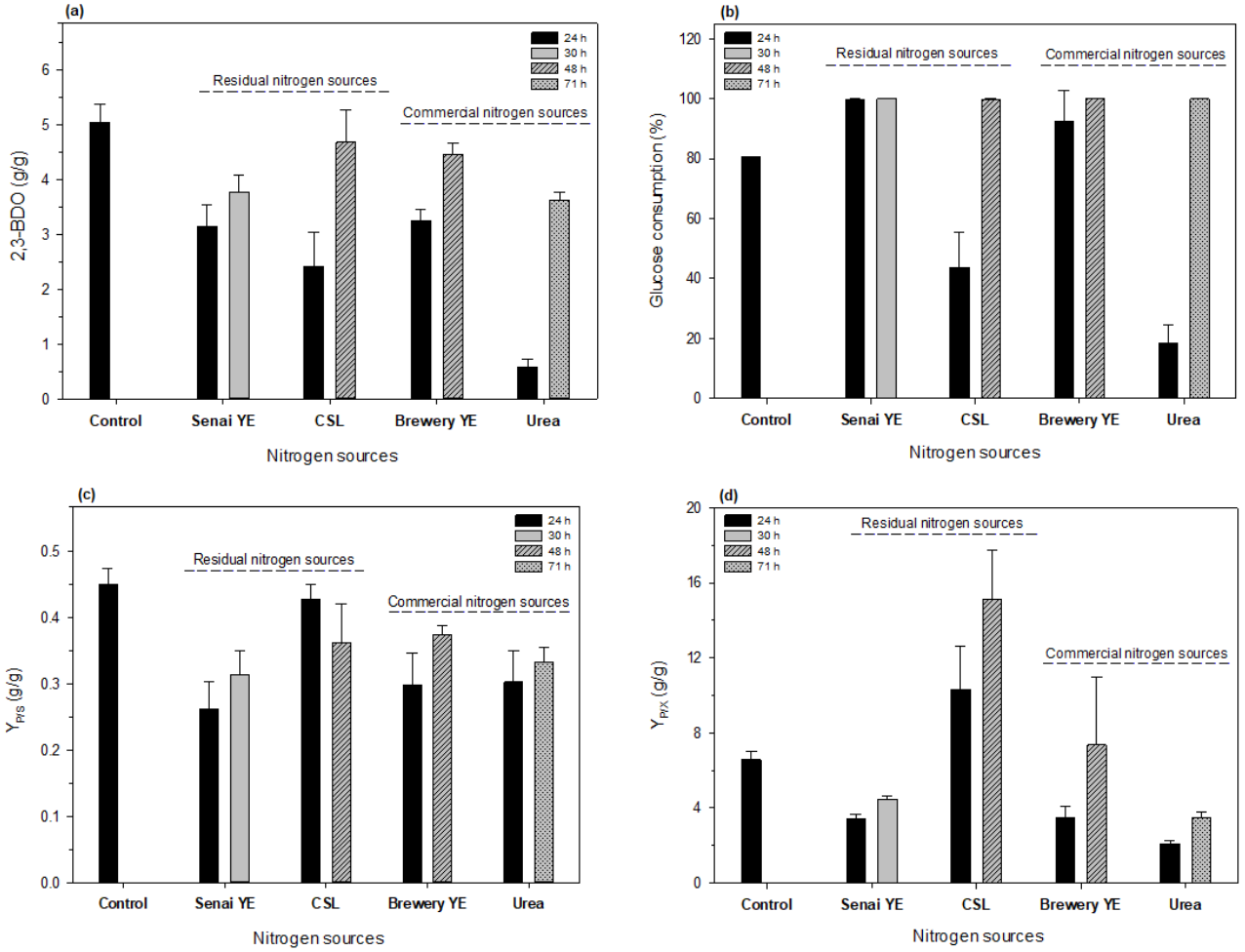

Figure 5.3: Alternative organic nitrogen sources effects on (a) 2,3-BDO production; (b) glucose consumption; (c) $Y_{P/S}$ (2,3-BDO/glucose yield); (d) $Y_{P/X}$ (2,3-BDO/cell dry weight yield) by *P. peoriae* NRRL BD-62 in batch fermentation.

Differently, TSIGORIYNA, GANCHEV, *et al.* (2021) found a negative CSL effect in the range of 0-20 g/L on 2,3-BDO production by *B. licheniformis* 24 from glucose when performing a Plackett–Burman media components design. The authors excluded CSL from the final culture medium composition. DAI, Jian Ying, ZHAO, *et al.* (2015) also observed a negative CSL effect on 2,3-BDO production by *E. cloacae* (CGMCC 6053) from molasses. No apparent difference in 2,3-BDO titer was observed in fermentation assays without CSL. Moreover, reduced foaming was observed. WONG, HUANG, *et al.* (2012) reported positive urea effects on 2,3-BDO production by an indigenous *Klebsiella* sp. Zmd30 from agricultural waste in batch fermentation. Urea showed the best productive and economic performance among nine nitrogen sources investigated for t2,3-BDO production, including NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, KNO_3, $NaNO_3$, NH_4HCO_3, peptone, and tryptone. Maximum 2,3-BDO production of 56 g/L and a yield of 68% was achieved from 160 g/L glucose and 2 g/L urea. This urea supplementation was relatively low compared to previous studies, as JI, Xiao-Jun, HUANG, *et al.* (2009) reported.

Glucose was completely metabolized by *P. peoriae* NRRL BD-62 in the medium containing YE Senai and YE brewer in 24 h, considering the statistical deviations (Fig. 5.3b). These results were better than the control, which achieved about 80% glucose consumption in the same period. In the medium containing CSL and urea, glucose uptake was compromised, so its exhaustion occurred in 48 h and 71 h, respectively.

The absence of residual CSL pretreatment may have contributed to delayed total glucose consumption due to cell growth inhibitory compounds. The cell oxygen supply was also compromised due to the suspended solids observed mainly after autoclaving the culture medium (REBECCHI, PINELLI, *et al.*, 2018). The 50% (v/v) water composition must also have contributed to increased cell growth limiting compounds since twice as much CSL was required to maintain C/N= 8.5 g/g. Therefore, cell growth was correlated to glucose consumption by *P. peoriae* NRRL BD-62 to understand better the organic nitrogen source effects (Fig. S1 – Supplementary material).

A net cell formation of only 0.32 g/L was achieved in 48 h using CSL, while approximately 1.2 g/L was obtained using commercial YE, YE Senai, and YE brewer in 24 h. Despite having different origins, the three YE types were pretreated, freeze-dried,

and maintained with a nutritional composition consisting of proteins, amino acids, vitamins, sodium chloride, and ash. Unlike this study, SONG, RATHNASINGH, *et al.* (2018) observed no differences in the maximum $OD_{600\ nm}$ when using commercial CSL and YE in glucose fed-batch fermentation for 2,3-BDO production by *B. licheniformis* GSC3102; however, the average time to reach an $OD_{600\ nm}$ of 17-17.2 was about 2.3-fold shorter using CSL.

The low free amino acids concentration in residual CSL compared to the three YE types may also explain the low cell growth and low glucose consumption in 24 h. According to LIU, DANTOFT, *et al.* (2016), CSL acid hydrolysis resulted in the formation of the free peptide that was taken up and degraded by *Lactococcus lactis* CS4435 via an intracellular peptidases system. Pretreatment increased the availability of amino acids by solubilizing the proteins present in CSL and improved the final ethanol production. In this study, CSL hydrolysis was not performed since total glucose consumption was achieved in 48 h, thus indicating that the fermentation extent was sufficient to promote 2,3-BDO production by *P. peoriae* NRRL BD-62 in the CSL-based medium.

The $Y_{P/S}$ was calculated for all alternative organic nitrogen sources (Fig. 5.3c). The control showed a 2,3-BDO yield of approximately 0.45 g/g, considered high for a GRAS (generally recognized as safe) microorganism. Traditional microbial producers such as *Klebsiella*, *Enterobacter*, and *Serratia*, whose biosecurity level requires greater control, especially on a large scale (HAKIZIMANA, MATABARO, *et al.*, 2020), have reported yields above 0.40 g/g.

A 2,3-BDO yield of 0.42 g/g was achieved using CSL in 24 h. This result was similar to the control, considering the statistical deviations. Furthermore, it was maintained high even after glucose depletion in 48 h. YE brewer also showed a high $Y_{P/S}$, around 0.37 g/g, which was statistically equivalent to CSL at 48 h. YE Senai and urea showed 0.25-0.35 g/g, commonly reported by *Paenibacillus* strains (TINÔCO, PATERAKI, *et al.*, 2021). To the best of our knowledge, this was the first time that a 2,3-BDO yield greater than 0.40 g/g has been reported by a safe wild-type *P. peoriae* strain using CSL as a nitrogen source.

The best results obtained using CSL were confirmed by the $Y_{P/X}$ (Fig. 5.3d). With values of 10.3 and 15.1 g/g, about 1.5 and 2.3-fold higher than the control in 24 and 48 h, respectively, CSL was able to ensure an adequate balance between cell respiration process and 2,3-BDO production, outperforming $Y_{P/X}$ of YE. Except for the YE brewer that showed $Y_{P/X}$ equal to 7.3 g/g in 48 h, no other nitrogen source besides CSL was able to overcome the control yield of 6.6 g/g. Despite the good result for the YE brewer, the yield obtained was approximately 2-fold lower than the CSL yield in 48 h. The medium with urea had the lowest $Y_{P/X}$, resulting from the relative cell biomass accumulation instead of 2,3-BDO in 71 h.

CSL is a cheap by-product from corn wet-milling composed of nitrogen, water-soluble vitamins, amino acids, minerals, reducing sugars, organic acids, enzymes, and other growth stimulants, able to favor cell growth and, consequently, enhance the 2,3-BDO production (YANG, Tao-Wei, RAO, *et al.*, 2013, ZHOU, YU, *et al.*, 2022). Due to its productive and economic advantages, CSL has been widely investigated.

ADLAKHA, YAZDANI (2015) obtained a 2,3-BDO yield of 0.33 g/g from 65 g/L cellulosic hydrolysates in a medium containing 15 g/L CSL, similar to that obtained using commercial YE. The authors concluded that CSL could not affect the 2,3-BDO metabolism by *P. polymyxa* ICGEB2008, thus considered an attractive alternative nitrogen source. OH, LEE, *et al.* (2018) observed similar 1,3-propanediol (1,3-PDO) and 2,3-BDO production by a mutant *K. pneumoniae* when replacing 1 g/L YE with 10 g /L CSL in a medium containing 20 g/L crude glycerol. A 1,3-PDO titer and yield of 0.41 g/g and 2.5 g/L were achieved under these conditions, respectively. HAZEENA, BINOD, *et al.* (2019) investigated eight organic nitrogen sources (meat extract, beef extract, sesame oil cake, coconut oil cake, groundnut oil cake, peptone, YE, and CSL) and obtained an increased 2,3-BDO production by *E. cloacae* SG1 using CSL as nitrogen source. The authors reported a 2,3-BDO yield of 0.36 g/g from an optimized medium containing 70 g/L glucose, 8 g/L CSL, and 1 g/L sodium citrate. In contrast, PSAKI, MAINA, *et al.* (2019) reported a lower 2,3-BDO yield of only 0.35 g/g using synthetic media containing 80 g/L molasses supplemented with 30 g/L untreated CSL, while a yield of 0.40 g/g was obtained using 15.3 g /L YE by a wild-type *E. ludwigii* strain in shake flask cultures.

3.2.2 Inorganic nitrogen sources

The inorganic nitrogen sources ($NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$) were defined based on the nutritional requirements of *P. peoriae* NRRL BD-62 through the chemical composition of the optimized culture medium, which already contained chloride, sulfate and phosphate salts. This selection prevented unwanted or unknown effects from affecting the 2,3-BDO metabolism. All four nitrogen sources were commercial compounds. The results obtained for the 2,3-BDO titer and yield and glucose consumption are presented in Figure 5.4.

The 2,3-BDO production from $NaNO_3$ as the sole nitrogen source was relatively low, reaching only 1.35 g/L in 24 h (Fig. 5.4a). A glucose conversion of only 25% (Fig. 5.4b) and a reduced cell growth in 8 h were also observed (Figure S1 - Supplementary material). These results indicated an impaired assimilative glucose metabolism by *P. peoriae* NRRL BD-62, leading to inconsistent and unreliable $Y_{P/S}$ and $Y_{P/X}$ values (Fig. 5.4c-d). Therefore, $NaNO_3$ was found inefficient in this study to replace YE.

FERNÁNDEZ-GUTIERREZ, VEILLETTE, *et al.* (2020) investigated nitrogen supplementation using $NaNO_3$ or urea in a medium containing 1 g/L NH_4Cl. Low glucose consumption and limited growth of genetically modified *Escherichia coli* MG1655 in the $NaNO_3$ presence were observed, which resulted in an inefficient 2,3-BDO and acetoin production in batch fermentation. According to the authors, the 2,3-BDO metabolism was compromised by nitrite accumulation, considered a toxic compound to cells resulting from the catalytic reaction of nitrate reduction by *E. coli* strains. Although the ability to reduce nitrate to nitrite by *P. peoriae* NRRL BD-62 is unknown, the results suggest a possible 2,3-BDO metabolism inhibition by nitrite accumulation.

The 2,3-BDO production from the other three alternative inorganic nitrogen sources did not exceed the production from YE, even with the fermentation extension to 48 h (Fig. 5.4a). While NH_4Cl and $(NH_4)_2HPO_4$ showed similar 2,3-BDO production in the range of 3.5-4.1 g/L in 24 h, considering the statistical deviations, production of only 2.6 g/L was obtained using $(NH_4)_2SO_4$ in the same period. The maximum 2,3-BDO production was approximately 4.0 g/L (27 h), 3.7 g/L (48 h), and 4.6 g/L (48 h) using NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$, respectively.

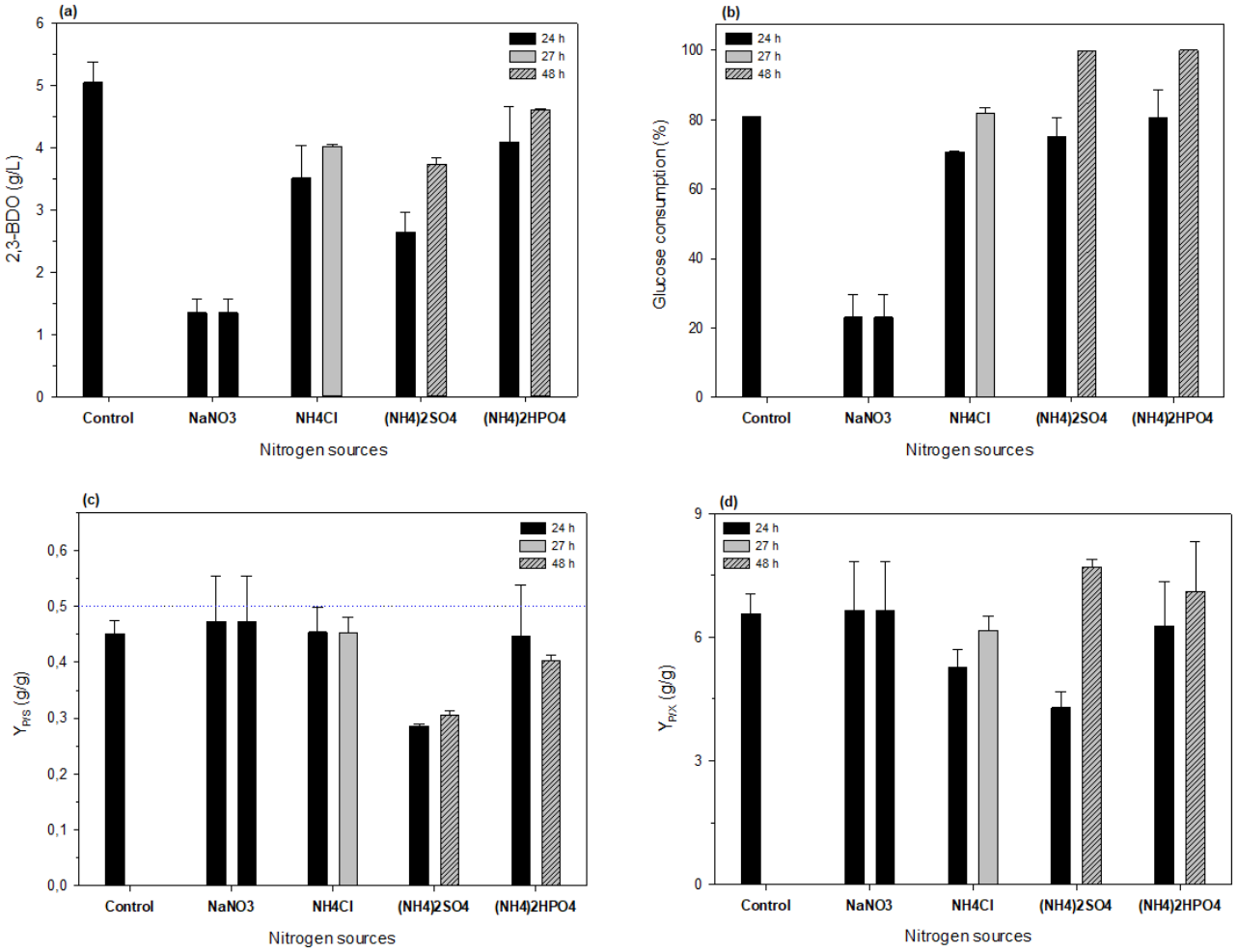

Figure 5.4: Alternative inorganic nitrogen sources effects on (a) 2,3-BDO production; (b) glucose consumption; (c) $Y_{P/S}$ (2,3-BDO/glucose yield); (d) $Y_{P/X}$ (2,3-BDO/cell dry weight yield) by *P. peoriae* NRRL BD-62 in batch fermentation.

YUAN, HE, *et al.* (2017) evaluated the effects of adding five nitrogen sources, including YE and $(NH_4)_2SO_4$, on 2,3-BDO production by *S. marcescens* H30 from sweet sorghum juice. Compared to the control, adding exogenous nitrogen enhanced 2,3-BDO production and cell biomass formation, which reached the highest values with the YE addition. While about 18 g/L of 2,3-BDO were produced with the $(NH_4)_2SO_4$ addition, about 22 g/L of 2,3-BDO was obtained using YE. KONGJAN, JARIYABOON, *et al.* (2021) evaluated the YE and $(NH_4)_2SO_4$ effects on 1,3-PDO production by *Enterobacter* sp. MU-01 from glycerol. The highest 1,3-PDO titers were achieved in assays containing YE as a nitrogen source. A 2,3-BDO production of 0.88 g/L from 10 g/L glycerol supplemented with 1 g/L YE was also verified. THAPA, LEE, *et al.* (2019) found an increased 2,3-BDO production by *E. aerogenes* SUMI02 (ΔptaΔldhA) by increasing the $(NH_4)_2SO_4$ added to the culture medium. The highest 2,3-BDO titer and cell biomass were obtained with 7 g/L $(NH_4)_2SO_4$ in shake flask cultures.

Glucose consumption using NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$ was close to control in 24 h (Fig. 5.4b). On average, about 75% glucose was consumed in this period. With the fermentation extension, glucose assimilation exceeded 80%, exhausted in the medium containing $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ in 48 h. Therefore, *P. peoriae* NRRL BD-62 could use the three nitrogen sources without significant difficulties.

BERBERT-MOLINA, SATO, *et al.* (2001) observed total sucrose consumption by *K. pneumoniae* NRRL B199 upon supplementing sugarcane juice-based medium with at least 5 g/L $(NH_4)_2HPO_4$ in 48 h. A proportional and increasing relationship was observed between 1-5 g/L $(NH_4)_2HPO_4$ and sucrose consumption. GUO, WANG, *et al.* (2017) found an increased lactose consumption rate by two *K. pneumoniae* strains with the external nitrogen addition in cheese whey powder culture. Supplementation was done with 3 g/L YE and 2 g/L $(NH_4)_2SO_4$. On the other hand, TIAN, FAN, *et al.* (2016) reported residual glucose not metabolized by *B. subtilis* SF4-3 when using 3.3 g/L $(NH_4)_2HPO_4$ as a nitrogen source. Glucose consumption was stopped in 48 h, leading to the lowest 2,3-BDO production among the inorganic nitrogen sources investigated.

The $Y_{P/S}$ using NH_4Cl and $(NH_4)_2HPO_4$ were similar to the control and equal to approximately 0.45 g/g in 24 h, accounting for statistical deviations (Fig. 5.4c). As the fermentation progressed, the 2,3-BDO yield did not change significantly in medium

containing NH_4Cl in 27 h. However, a slight reduction was observed in a medium containing $(NH_4)_2HPO_4$, which became 0.40 g/ g in 48 h. The yield using $(NH_4)_2SO_4$ showed a slight increase, reaching about 0.30 g/g in 48 h. Again, to the best of our knowledge, this was the first time that yields greater than 0.40 g/g were reported by a safe and wild-type *P. peoriae* strain using inorganic compounds as the sole nitrogen source.

YANG, Taowei, ZHANG, *et al.* (2012) obtained a yield of 0.38 g/g when using ammonium citrate ($C_6H_5O_7(NH_4)_3$) in shake flasks for 2,3-BDO production by *B. amyloliquefaciens* B10-127 from glucose. Low acetoin production was also verified. The authors then optimized the culture medium, including organic nitrogen sources, and increased the production scale. Again, a yield of 0.38 g/g was obtained in batch fermentation, where the medium consisted of 5.58 g/L ammonium citrate, 31.9 g/L CSL, and 22 g/L soybean meal. MA, WANG, *et al.* (2009) reported a 2,3-BDO yield of about 0.43 g/g by *K. pneumoniae* SDM from glucose in batch fermentation. The culture medium was optimized, consisting of 8.27 g/L CSL and 4.91 g/L $(NH_4)_2HPO_4$, which was considered the most critical factor for the 2,3-BDO accumulation under the conditions investigated. CHAN, KANCHANATAWEE, *et al.* (2018) achieved the highest 2,3-BDO yield of 0.50 g/g by a mutant *K. oxytoca* KMS005 from fed-batch glucose fermentation using an inorganic nitrogen-based medium. The authors optimized the oxygen consumption and kept the kLa constant at around 25.2 h^{-1}. The culture medium contained only mineral salts, including $(NH_4)_2HPO_4$ and $NH_4H_2PO_4$ as nitrogen sources.

With a similar net cell biomass of around 0.60 g/L (Figure S2 – Supplementary material), the $Y_{P/X}$ using NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$ were directly proportional to the 2,3-BDO production (Fig. 5.4d). Thus, the 41% increase in 2,3-BDO titer using $(NH_4)_2SO_4$ resulted in an approximately 1.8-fold higher $Y_{P/X}$, from 4.3 g/g in 24 h to 7.7 g/g in 48 h. The $Y_{P/X}$ using $(NH_4)_2HPO_4$ and NH_4Cl increased by only 13.5% and 17% in 27 h and 48 h, respectively, since the final 2,3-BDO production varied little with the fermentation length, considering the statistical deviations.

WANG, KIM, *et al.* (2020) investigated different nitrogen sources for the poly-γ-glutamic acid and 2,3-BDO coproduction by a newly isolated *B. subtilis* CS13. Higher 2,3-BDO production was achieved from inorganic nitrogen sources (NH_4Cl, $(NH_4)_2SO_4$, $C_6H_5O_7(NH_4)_3$, KNO_3, and $NaNO_3$), while enhanced cell growth was observed using

organic nitrogen sources (YE, peptone, beef extract, and CSL). According to the authors, ammonium citrate was responsible for the highest 2,3-BDO titer due to the high viscosity, reducing the culture medium's oxygen supply. CHO, KIM, *et al.* (2015) found increased cell biomass of *K. oxytoca* M1 by adding YE and casamino acid in a medium containing $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$. A higher 2,3-BDO yield was also observed compared to the medium without adding nutrients.

3.2.3 Selectivity of 2,3-BDO

Figure 5.5 presents the 2,3-BDO selectivity for the alternative nitrogen sources investigated. No acetoin production was detected.

Unlike the control, *meso*-2,3-BDO production was detected in all assays. Although the oxygen supply was low to cause acetoin accumulation, it was probably sufficient to simultaneously activate the BDH and DAR enzymes, responsible for directing the carbon flux to the *levo*- and *meso*-2,3-BDO synthesis, respectively (DIAS, LIMA, *et al.*, 2018, TINÔCO, PATERAKI, *et al.*, 2021). Furthermore, the fermentation broth viscosity may have affected oxygen mass transfer according to the nitrogen source. As a result, the $NADH/NAD^+$ balance varied in each culture medium, leading to the different 2,3-BDO chiral ratios.

HÄßLER, SCHIEDER, *et al.* (2012) found an increased *levo*-2,3-BDO selectivity with an increased YE in the culture medium. While 93% *levo*-2,3-BDO was obtained using 5 g/L YE, the high selectivity of 98% was achieved by *P. polymyxa* DMS 365 using 60 g/L YE. The broth viscosity effects on the *levo*-2,3-BDO selectivity were also observed with the Tween80® addition. It caused an approximately 12% reduction in the culture medium viscosity, leading to a final *levo*-2,3-BDO optical purity of only 83 %. TIAN, FAN, *et al.* (2016) observed an initial 2,3-BDO accumulation rather than acetoin with increasing fermentation broth viscosity. The authors investigated increasing YE and CSL and suggested the microaerobic environment formation from 20 g/L of these compounds, responsible for the preferential 2,3-BDO production. A different 2,3-BDO accumulation profile was also observed for each nitrogen source due to the different broth viscosities.

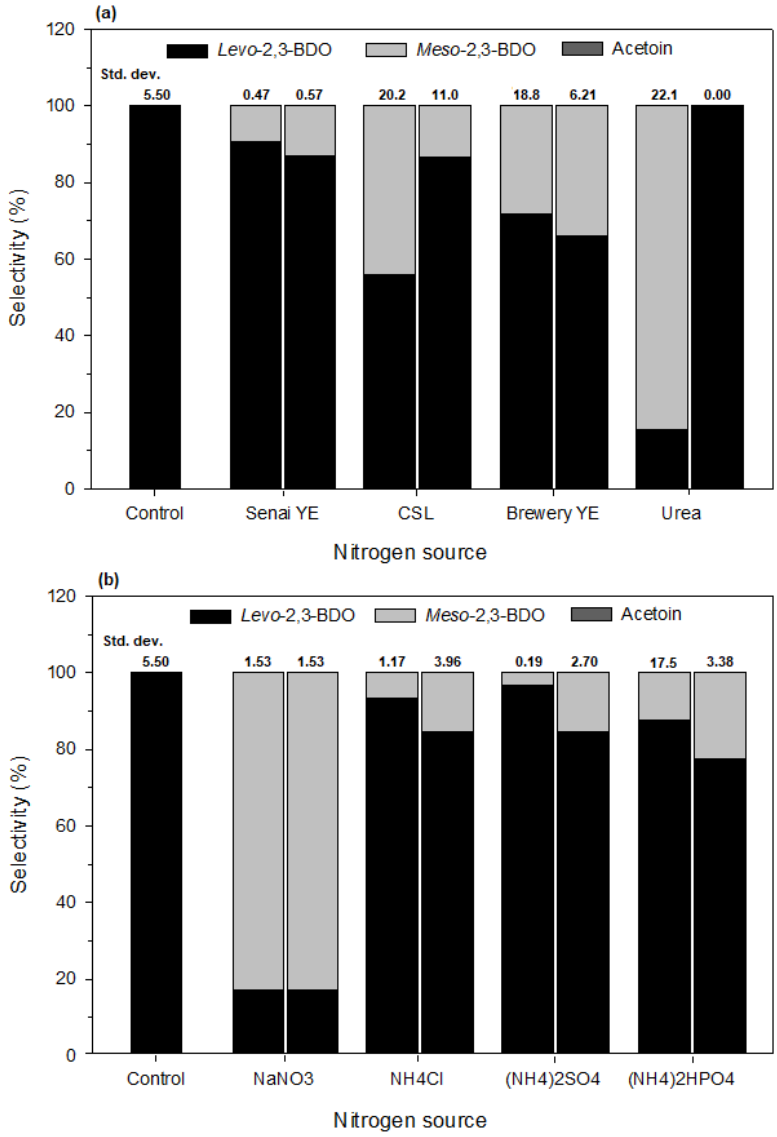

Figure 5.5: Selectivity of 2,3-BDO (*levo*- and *meso*-isomers) and acetoin by *P. peoriae* NRRL BD-62 using: (a) organic nitrogen sources; (b) inorganic nitrogen sources. The control corresponds to commercial yeast extract (YE). The first bar of each investigated nitrogen source corresponds to the selectivity at 24 h, while the second corresponds to the selectivity at the time of maximum 2,3-BDO production: 24 h ($NaNO_3$), 27 h (NH_4Cl); 48 h (Senai YE, corn steep liquor - CSL, Brewery YE, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$; and 71 h (urea).

Meso-2,3-BDO production was mainly observed in the first 24 h using organic nitrogen sources such as CSL and urea, reaching about 44% and 84%, respectively (Fig. 5.5a). Comparably, the *meso*-2,3-BDO selectivity using YE brewer was lower, around 28%. However, this result was similar to the selectivity obtained using CSL, considering

the statistical deviations. The highest *levo*-2,3-BDO selectivity, around 91%, was obtained using YE Senai. With the fermentation extension, *levo*-2,3-BDO increased using CSL and urea, reaching 87-100% in 48-71 h. No statistically significant change was observed for the *levo*-2,3-BDO selectivities using YE Senai and YE brewer, equal to 87% and 66% in 30 h and 48 h, respectively.

LI, Lixiang, ZHANG, *et al.* (2013) verified a *levo*-/*meso*-2,3-BDO ration of 1:1 by *B. licheniformis* 10-1-A from glucose in a two-step agitation fed-batch fermentation. The culture medium was optimized, consisting of 5.8 g/L YE, 14.7 g/L CSL, and 6.5 g/L sodium acetate. A low acetoin production of 2.2 g/L was found. SONG, RATHNASINGH, *et al.* (2018) reported a *meso*-2,3-BDO selectivity of approximately 62.5% by *B. licheniformis* GSC3102 from glucose added to 10 g/L YE or 40 g/L CSL in a two-step oxygen supply fed-batch fermentation. The results indicated that the YE replacement with CSL could not affect the 2,3-BDO selectivity. Recently, SONG, KWON, *et al.* (2022) observed a *meso*-2,3-BDO selectivity of approximately 70.4% by *B. licheniformis* 4071 in a CSL-based medium containing glucose and L-glutamate. Assays were conducted in shake flasks at 45°C. Without L-glutamate addition, the *meso*-2,3-BDO selectivity was similar to the previous study, equaling approximately 62.5%.

Except for $NaNO_3$, inorganic nitrogen sources showed higher *levo*-2,3-BDO selectivities than organic sources, around 88-97% in 24 h (Fig. 5.5b). Assays using NH_4Cl and $(NH_4)_2SO_4$ showed low statistical deviations, not exceeding 1.2%. With the fermentation extension, a slight increase in *meso*-2,3-BDO selectivity was observed using NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$, reaching about 15-23% in 27-48 h. Despite the reduction, the *levo*-2,3-BDO selectivities remained high, averaging 82%. The experimental deviations decreased as fermentation progressed using all nitrogen sources. The lowest deviations were found in the 2.7-4% range using inorganic sources such as NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$. These results highlight the high experimental inorganic nitrogen reproducibility and the more uniform 2,3-BDO isomers production.

GAO, Jian, XU, *et al.* (2010) reported a *levo*-2,3-BDO production by *P. polymyxa* ZJ-9 with optical purity greater than 98% after optimizing the culture medium for one-step inulin extract fermentation from Jerusalem artichoke tubers. The authors identified NH_4Cl as a critical factor for the 2,3-BDO production and determined its optimal

concentration to be equal to 0.93 g/L. The medium also contained other nutrients, including YE and peptone. ZHANG, LI, *et al.* (2016) observed selectivity of 87% 2,3-BDO and 13% acetoin by *E. cloacae* CICC 10011 from corncob-derived xylose in a medium containing 20 g/L CSL and 3.05 g/L $(NH_4)_2HPO_4$ in fed-batch fermentation.

3.3 Effects of amino acid supplementation on 2,3-BDO metabolism

Supplementation by Arg and Asn was performed in the media containing CSL or NH_4Cl, considered potential candidates to replace YE in large-scale fermentations. These amino acids were chosen based on studies by GAO, J., JIANG, *et al.* (2019). The authors observed a higher 2,3-BDO production from adding 1.5 g/L Arg or Asn at the beginning of inulin fermentation in shake flasks. While Arg was found to be the primary cell growth limiting factor, Asn was responsible for the highest substrate consumption and 2,3-BDO production rates by *P. polymyxa* ZJ-9.

3.3.1 Production of 2.3-BDO

3.3.1.1 Corn steep liquor

Figure 5.6 presents the time courses of 2,3-BDO metabolism using CSL as the primary nitrogen source. The Arg addition to the culture medium (CSL+Arg medium) enhanced glucose uptake (Fig. 5.6a) and 2,3-BDO production (Fig 5.6c) in 24 h. A 2,3-BDO production of approximately 4.5 g/L was obtained with glucose depletion, representing an increase of more than 85% over the production using a pure CSL medium. Despite the enhanced production, the 2,3-BDO yield decreased from 0.43 g/g to 0.35 g/g (Table 5.2), accompanied by a more pronounced *P. peoriae* NRRL BD-62 cell growth. , whose DCW was 2.7-fold higher than that obtained without adding amino acids (Fig. 5.6b).

The decreased $Y_{P/X}$ confirmed the carbon balance shift for cell plasticity from 10.3 g/g to 6.9 g/g. However, this result was statistically similar to the medium without adding Arg, indicating that its supplementation could not favor the 2,3-BDO production at the cell growth expense. In contrast, both 2,3-BDO production and cell biomass formation benefited (Table 5.2).

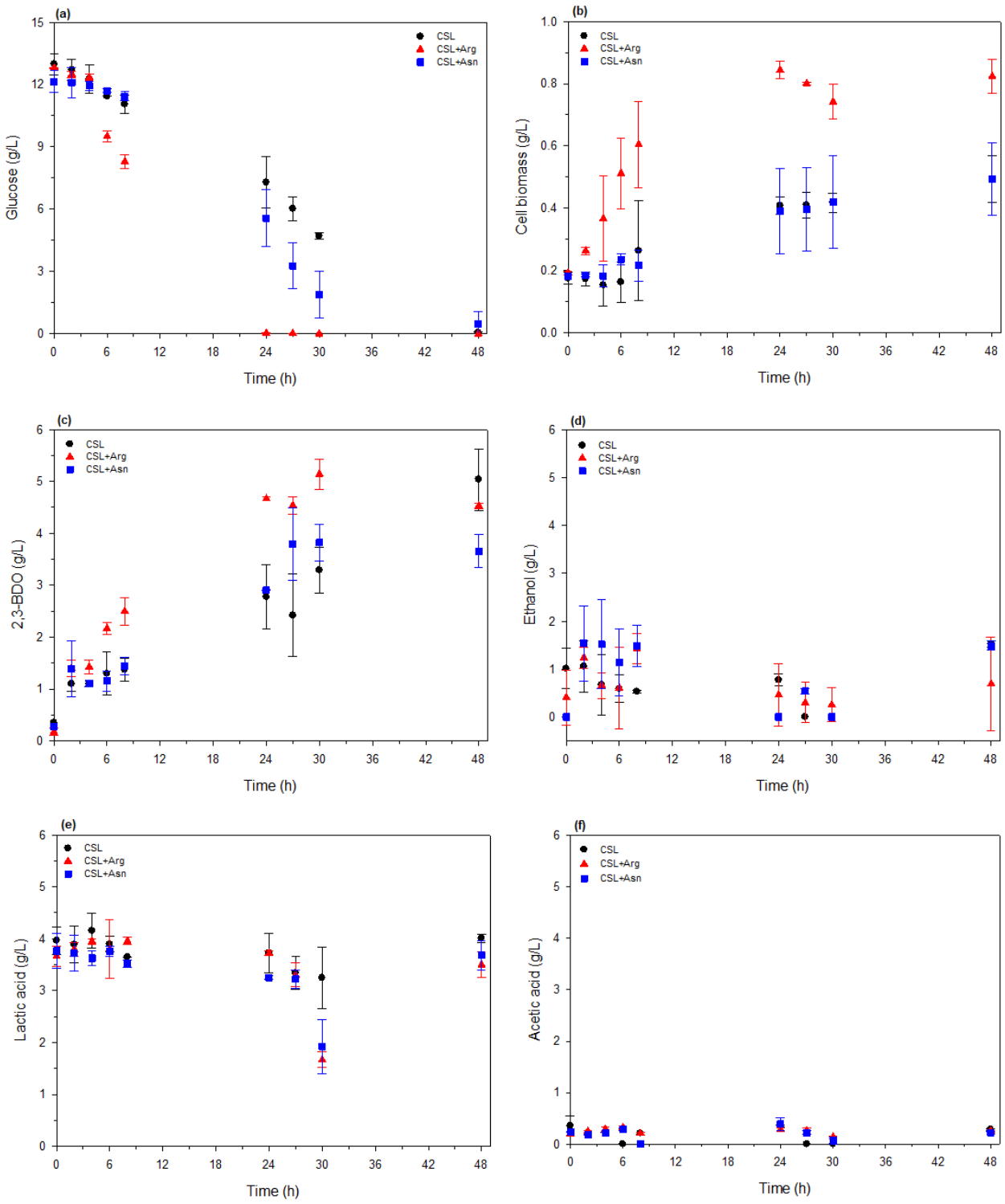

Figure 5.6: Time courses of 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 using corn steep liquor (CSL)-based medium supplemented with Arg and Asn: (a) glucose consumption; (b) dry cell weight; (c) 2,3-BDO; (d) ethanol; (e) lactic acid; (f) acetic acid.

In turn, adding Asn to the CSL medium (CSL+Asn medium) had little influence on glucose assimilation and 2,3-BDO production. The $Y_{P/S}$ and $Y_{P/X}$ and substrate consumption showed no statistically significant differences from the results found using a medium without amino acid supplementation in 24 h (Table 5.2).

With the fermentation extension, glucose assimilation increased for CSL and CSL+Asn media, reaching consumptions higher than 80% in 48 h and 30 h and statistically equal to that seen for CSL+Arg medium in 30 h, respectively (Table 5.2). Similarities were also observed in the 2,3-BDO titer and yield, which showed mean values of 4.4 g/L and 0.37 g/g, respectively. Considering the statistical deviations, the 2,3-BDO yields were similar to those found using YE, although the fermentation time was longer than 24 h.

P. peoriae NRRL BD-62 showed a different cell growth pattern between media. While there was a similarity between CSL and CSL+Asn media, the DCW was about 2-fold higher for the CSL+Arg medium (Fig. 5.6b). The Arg addition led to a statistically significant decreased $Y_{P/X}$ from 15.1 g/g to 9.1 g/g in 30 h, suggesting that its supplementation in CSL medium favored cell formation at the 2,3-BDO production expense (Table 5.2).

Table 5.2: Effects of amino acid supplementation on 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and corn steep liquor at $C/N_{initial}$=8.5 g/g.

Nitrogen source	Time (h)	2,3-BDO (g/L)	DCW (g/L)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)	SC (%)
CSL	24	2.42 ± 0.62^{a}	0.23 ± 0.01^{a}	0.43 ± 0.02^{a}	10.3 ± 2.3^{a}	43.6 ± 11.7^{a}
CSL + Arg		4.49 ± 0.03^{b}	0.65 ± 0.02^{b}	0.35 ± 0.00^{b}	6.9 ± 0.3^{a}	99.8 ± 0.1^{b}
CSL + Asn		2.63 ± 0.01^{a}	0.21 ± 0.14^{a}	0.42 ± 0.12^{a}	15.9 ± 10.3^{a}	53.9 ± 13.2^{a}
CSL	48^{mp}	4.69 ± 0.60^{c}	0.32 ± 0.09^{c}	0.36 ± 0.06^{c}	15.1 ± 2.6^{c}	99.6 ± 0.4^{c}
CSL + Arg	30^{mp}	4.97 ± 0.22^{c}	0.55 ± 0.05^{d}	0.39 ± 0.02^{c}	9.1 ± 0.4^{d}	100.0 ± 0.0^{c}
CSL + Asn		3.54 ± 0.35^{c}	0.24 ± 0.15^{c}	0.35 ± 0.09^{c}	19.0 ± 13.4^{c}	84.2 ± 9.9^{c}

C/N ratio= carbon/nitrogen ratio; CSL= corn steep liquor; Arg= arginine; Asn= asparagine; mp= maximum production; 2,3-BDO= 2,3-BDO titer (*meso*- + *levo*-isomer titers); DCW= dry cell weight (cell biomass); $Y_{P/S}$= 2,3-BDO/glucose yield; $Y_{P/X}$= 2,3-BDO/dry cell weight yield; SC= substrate consumption. The means followed by the same letter do not show significant differences concerning the CSL at 24 h and 48 h by t-test ($\alpha = 0.05$).

Arginine is a metabolically versatile amino acid used as carbon, nitrogen, and energy sources for the growth of a bacteria variety (LU, Chung-Dar, 2006). NOVAK, KUTSCHA, *et al.* (2020) verified a more significant cell biomass formation in the 2,3-BDO production by an engineered *E. coli* strain from acetate supplemented with Arg. The

increased cell growth possibly resulted from activating an Arg-mediated acid resistance system (ARS), responsible for protecting the cells from the low pH throughout fermentation. The medium's acidification can alter the homeostatic cell balance and compromise cell viability through changes in lipid content (saturated and unsaturated fatty acids) and membrane fluidity. In particular, Arg-based ARS acts mainly in the microbial growth exponential phase by consuming intracellular protons in the Arg decarboxylation reaction, which makes the internal pH less acidic and more suitable for cell development (XU, BEHR, *et al.*, 2019). At this step, μ_{max} was observed, favoring cell plasticity processes at the 2,3-BDO synthesis expense.

The Arg catabolism involved in intracellular pH control and microbial growth is related to the arginine deiminase pathway (ADP), activated under limited oxygen conditions (XIONG, TENG, *et al.*, 2016). Enzymes of this pathway were located in *P. polymyxa* HK4 (SONI, NANJANI, *et al.*, 2021), a strain phylogenetically related to *P. peoriae* (TINÔCO, PATERAKI, *et al.*, 2021). Arg-mediated pH regulation was also observed in other *Bacillus* strains (SENOUCI-REZKALLAH, SCHMITT, *et al.*, 2011, YANG, MENG, *et al.*, 2015). Therefore, this Arg-based metabolic mechanism was expected in *P. peoriae* NRRL BD-62.

Other studies have reported the effects of different amino acids on microbial growth. LI, Jinshan, WANG, *et al.* (2013) identified methionine (Met) as the main growth-limiting factor of *P. polymyxa* ATCC 12321 in a vigorous oxygen-limiting glucose fermentation for the *levo*-2,3-BDO production. From the investigation of 18 amino acids randomly combined into 4 groups and supplemented in an optimized culture medium, Met showed a substantial effect on microbial growth, increasing $OD_{600\ nm}$ by approximately 8-fold. Met is an amino acid of the aspartate (Asp) family, which can be obtained by converting Asn. Therefore, in contrast to this study, Asn supplementation was expected to promote the *P. polymyxa* ATCC 12321 growth. MASER, PEEBO, *et al.* (2020) reported a 2-fold higher μ for *E. coli* BW25113 cultured in a glucose-containing medium supplemented with 20 amino acids than medium without added nutrients. Approximately 72% of carbon and more than 140 mg/g_{DCW} of nitrogen used for biosynthesis resulted from amino acids, among which serine (Ser) accounted for about 41% and 58% of the total carbon and nitrogen consumed for cell plasticity, respectively.

The third largest nitrogen contribution was made by Arg, which allocated more than 7.6% nitrogen to cell biomass formation.

Ethanol, lactic acid, and acetic acid showed no changes in their production by adding Arg and Asn in the CSL medium (Fig. 5.6d-f). Metabolic flux appeared to be little affected with amino acid addition, as the parallel pathways to 2,3-BDO remained equally active with or without these compounds. No net organic acid production was detected. According to XU, BEHR, *et al.* (2019), the 2,3-BDO production at the acetate and lactate expense resulted from a metabolic NADH-driven and ADP up-regulation to relieve intracellular acid stress. Therefore, the results align with those previously discussed, mainly related to Arg supplementation.

Although Arg and Asn supplementation enhanced 2,3-BDO production and reduced fermentation time to total glucose consumption, 2,3-BDO yields did not show statistically significant improvements to justify these compounds' addition to the CSL-based medium. Arg and Asn were possibly already part of the CSL composition since approximately half of its organic nitrogen is found in the free amino acid form (LOY, LUNDY, 2019). Therefore, adding these amino acids ended up causing little or no net effect on the bioprocess parameters.

Furthermore, Arg seems to have been the primary growth factor in residual CSL since the *P. peoriae* NRRL BD-62 growth was favored at the 2,3-BDO production expense. The Arg excess resulting from nutritional supplementation in the CSL-based medium containing Arg intensified possibly the cell plasticity processes. Similar observations were made by LU, Mingshou, PARK, *et al.* (2014) using a culture medium with excess branched-chain amino acids (BCAA). According to the authors, cell growth of an engineered *K. pneumoniae* strain was favored by the α-acetolactate decarboxylase (ALDC) and excess BCAA. Under these conditions, the ALDC enzyme promoted the mutant *K. pneumoniae* growth by overexpressing the *budA* gene instead of converting α-acetolactate to R-acetoin in a YE-based medium.

Therefore, since Arg and Asn supplementation did not contribute to improved production parameters, this strategy was disregarded for the CSL medium.

3.3.1.2 Ammonium chloride

The Arg and Asn supplementation was then carried out in the NH_4Cl-based medium. Figure 5.7 presents the time courses of 2,3-BDO metabolism for NH_4Cl media with or without amino acids. The adding Arg to the culture medium (NH_4Cl+Arg medium) had little influence on glucose uptake in 24 h. However, it caused a reduction in its consumption rate in 24-30 h, equal to 0.14 g/L/ h versus 0.30 g/L/h for pure NH_4Cl medium (Fig. 5.7a). The slower consumption was accompanied by lower cell growth (Fig. 5.7b), and lower 2,3-BDO production (Fig. 5.7c), suggesting that the Arg reduced the *P. peoriae* NRRL BD-62 metabolic activity. As a result, the $Y_{P/S}$ decreased from 0.45 g/g to 0.30 g/g for a substrate consumption of approximately 70% in 24 h (Table 5.3). In contrast, the $Y_{P/X}$ remained statistically equal to the pure NH_4Cl medium, confirming that adding Arg could not favor the 2,3-BDO production by *P. peoriae* NRRL BD-62.

In turn, adding Asn enhanced glucose assimilation, whose uptake exceeded 80% in 24 h (Fig. 5.7a). A 2,3-BDO production of about 4.6 g/L was achieved (Fig. 5.7c), along with cell biomass of 0.75 g/L (Fig. 5.7b). Despite these improved results, no statistical difference was observed compared to pure NH_4Cl medium. The $Y_{P/S}$ and $Y_{P/X}$ also remained statistically equal to the medium without Asn, reaching 0.41 g/g and 6.2 g/g in 24 h, respectively (Table 5.3).

With the fermentation extension, glucose was exhausted in all investigated media in 48 h (Fig. 5.7a). The maximum 2,3-BDO production varied for each condition, reached in 27, 48, and 30 h for NH_4Cl, NH_4Cl+Arg, and NH_4Cl+Asn media, respectively (Fig. 5.7c). The 2,3-BDO titer was statistically similar between media with and without Arg, on average equal to 4.2 g/L. In contrast, adding Asn increased by approximately 17%, reaching about 4.9 g/L (Table 5.3). However, the $Y_{P/S}$ showed no significant improvement with adding Asn, even with glucose depletion (Table 5.3). The adding Arg was also unable to improve the 2,3-BDO yield, which reduced about 25% from 0.45 g/g to 0.36 g/g (Table 5.3). Again, this result suggested a 2,3-BDO metabolism impairment by *P. peoriae* NRRL BD-62 in NH_4Cl medium supplemented with Arg.

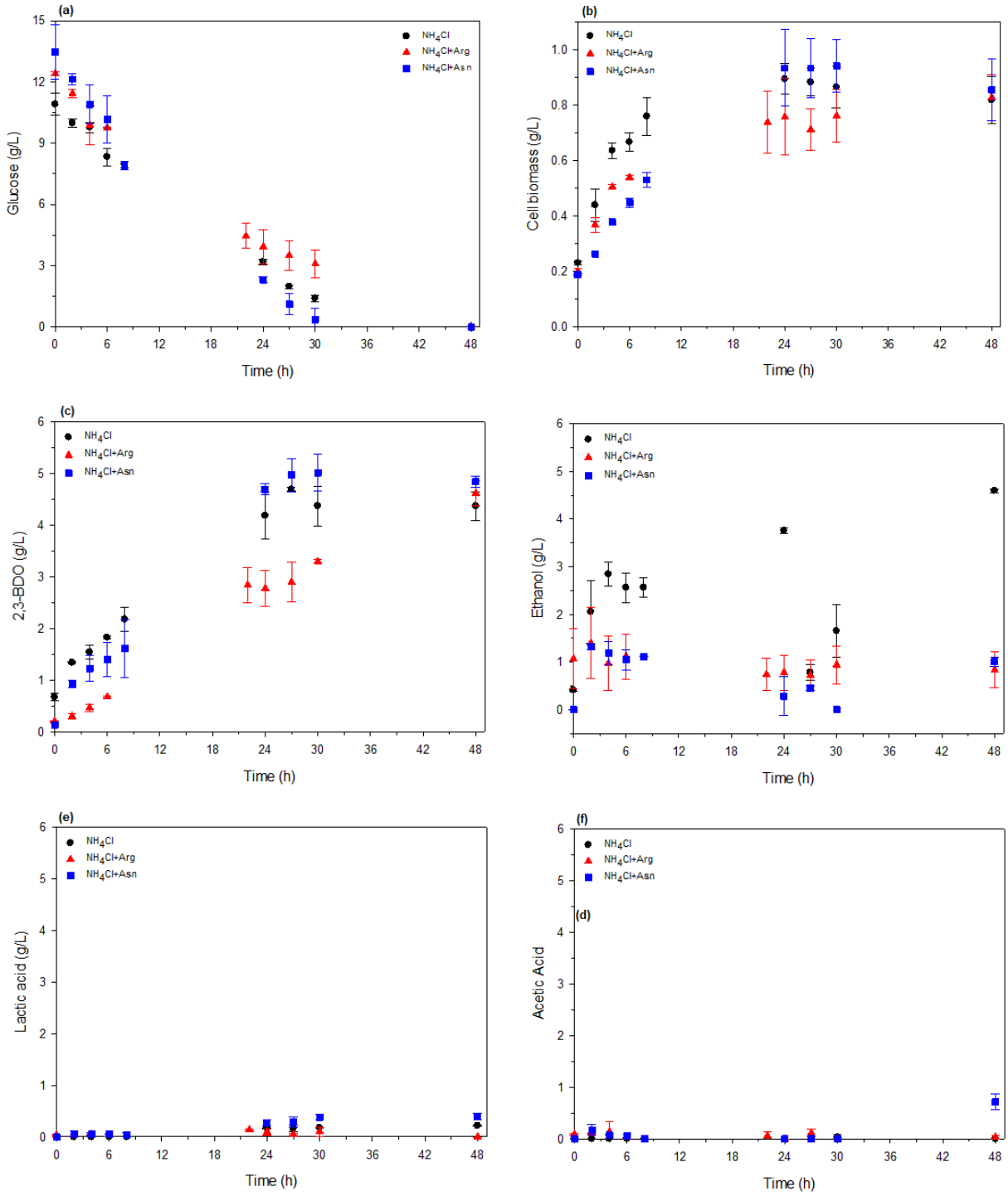

Figure 5.7: Selectivity of 2,3-BDO (*levo*- and *meso*-isomers) and acetoin by *P. peoriae* NRRL BD-62 using: (a) corn steep liquor (CSL)-based medium supplemented with Arg and Asn; (b) NH_4Cl-based medium supplemented with Arg and Asn. The first bar of each investigated medium corresponds to the selectivity at 24 h, while the second corresponds to the selectivity at the time of maximum 2,3-BDO production: 27 h (NH_4Cl); 30 h (CSL+Arg, CSL+Asn, NH_4Cl+Asn); and 48 h (NH_4Cl+Arg).

As previously discussed, two pathways in bacteria can accomplish the Arg catabolism: the arginase pathway (AP) and ADP. The first pathway involves the urea cycle, and the second involves ornithine production, releasing NH_3, CO_2, and ATP as metabolism regulators (LU, Chung-Dar, 2006, XIONG, TENG, *et al.*, 2016). In this study, the NH_4Cl medium possibly inhibited the ADP enzymes by feedback since the NH_4^+ is found in equilibrium with NH_3. Therefore, the Arg catabolism must have been forced to follow mainly the AP, leading to the urea accumulation. Under these conditions, 2,3-BDO metabolism was compromised since urea was considered an inefficient nitrogen source for the 2,3-BDO production by *P. peoriae* NRRL BD-62, able to reduce glucose consumption rate, $Y_{P/S,}$ and $Y_{P/X}$, as observed in this study.

Table 5.3: Effects of amino acid supplementation on 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and NH_4Cl at $C/N_{initial}$=8.5 g/g.

Nitrogen source	Time (h)	2,3-BDO (g/L)	DCW (g/L)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)	SC (%)
NH_4Cl	24	3.51 ± 0.53^a	0.66 ± 0.05^a	0.45 ± 0.04^a	5.3 ± 0.4^a	70.8 ± 0.1^a
NH_4Cl + Arg		2.57 ± 0.34^a	0.56 ± 0.13^a	0.30 ± 0.01^b	4.7 ± 0.5^a	68.3 ± 6.9^a
NH_4Cl + Asn		4.56 ± 0.11^a	0.75 ± 0.13^a	0.41 ± 0.04^a	6.2 ± 1.0^a	82.6 ± 2.8^b
NH_4Cl	27^{mp}	4.02 ± 0.03^c	0.65 ± 0.04^c	0.45 ± 0.03^c	6.2 ± 0.3^c	81.8 ± 1.7^c
NH_4Cl + Arg	48^{mp}	4.41 ± 0.24^c	0.63 ± 0.07^c	0.36 ± 0.02^d	7.1 ± 1.2^c	99.8 ± 0.2^d
NH_4Cl + Asn	30^{mp}	4.88 ± 0.35^d	0.75 ± 0.09^c	0.37 ± 0.03^c	6.5 ± 0.3^c	96.9 ± 4.3^d

C/N ratio= carbon/nitrogen ratio; Arg= arginine; Asn= asparagine; mp= maximum production; 2,3-BDO= 2,3-BDO titer (*meso*- + *levo*-isomer titers); DCW= dry cell weight (cell biomass); $Y_{P/S}$= 2,3-BDO/glucose yield; $Y_{P/X}$= 2,3-BDO/dry cell weight yield; SC= substrate consumption. The means followed by the same letter do not show significant differences concerning the NH_4Cl at 24 h and 27 h by t-test ($\alpha = 0.05$).

Cell growth was statistically similar in 24-48 h for media with and without added amino acids (Fig. 5.7b), while 2,3-BDO production increased with Asn supplementation (Fig. 5.7c). However, this increase was not enough to change the carbon balance. Therefore, the $Y_{P/X}$ did not show statistically significant differences between the three media, equal to 6.6 g/g (Table 5.3).

Some bacteria can synthesize the coenzyme NAD from Asp, obtained by the Asn catabolism. This coenzyme participates in the cell redox balance by converting NADH into a reduced form in catabolic reactions (KATOH, UENOHARA, *et al.*, 2006). The 2,3-BDO production is closely associated with adjusting the $NADH/NAD^+$ ratio, whose redox balance affects the metabolic flux distribution between acetoin and 2,3-BDO (DAI, Jun-Jun, CHENG, *et al.*, 2014). Therefore, the higher 2,3-BDO production from the

NH_4Cl+Asn medium can be attributed to *P. peoriae* NRRL BD-62 catabolizing this amino acid via the NAD biosynthesis pathway.

Previous studies have correlated Asn/Asp catabolism and NADH production. NOVAK, KUTSCHA, *et al.* (2020) obtained higher 2,3-BDO production from acetate supplemented with Asn/Asp. According to the authors, problems involving the critical cell redox status and the NADH supply were partially overcome by adding these amino acids to the culture medium since they are considered essential precursors of "de novo biosynthesis" NADH, used in the acetoin conversion to 2,3-BDO. The adding Asn/Asp was only necessary to start the fermentation process, and no significant improvements in the biomass yields or specific uptake and production rates were identified in fed-batch fermentation. LIAO, YANG, *et al.* (2019) also used Asp as a NADH biosynthesis precursor for acetone–butanol–ethanol (ABE) production by *Clostridium acetobutylicum* ATCC 824.

Again, ethanol, lactic acid, and acetic acid were detected as 2,3-BDO metabolism by-products in NH_4Cl medium supplemented with Arg and Asn (Fig. 5.7d-f). In 0-30 h, ethanol production showed variations for all three media, possibly resulting from the carbon balance dynamics, in which the ethanol formed was converted into other compounds during the 2,3-BDO metabolism. Therefore, no significant titers were detected in this period. On the other hand, in 30-48 h, net ethanol production of 3 g/L was verified in the pure NH_4Cl medium. With adding amino acids, the ethanol metabolic pathway expression seems to have been reduced, leading to a production of only 1 g/L in the Asn medium. In the Arg-supplemented medium, no net ethanol production was detected (Fig. 5.7d). In turn, the lactic acid production profile was very similar between media, suggesting no significant Arg and Asn effects on its metabolic pathway (Fig. 5.7e). Finally, acetic acid production seems to have been favored with adding Asn in 48 h. However, its titer was low, around only 0.5 g/L.

Ethanol, lactic acid, acetic acid, and 2,3-BDO exhibit NAD(H)-dependent pathways directly affected by cell redox balance (BAO, ZHANG, *et al.*, 2015). While ethanol, lactic acid, and 2,3-BDO compete for NADH, acetic acid requires NAD^+ to convert pyruvate from the glycolytic pathway (TINÔCO, PATERAKI, *et al.*, 2021). The

adding Arg and Asn can provide additional NADH that can redirect carbon flux preferentially to one of the four pathways under analysis.

Ethanol production requires NAD^+ for pyruvate conversion to acetyl-CoA by pyruvate dehydrogenase. Two subsequent NADH reoxidation reactions lead to ethanol synthesis (TINÔCO, PATERAKI, *et al.*, 2021). In contrast, the 2,3-BDO production does not depend on NAD^+ at the beginning of the pyruvate conversion, only on the ALS (α-acetolactate synthase) and ALDC action, forming α-acetolactate and acetoin, respectively. The 2,3-BDO pathway enzymes are activated to maintain pH homeostasis and control the NADH/NAD^+ balance (MONNET, NARDI, *et al.*, 2003). Therefore, the ALS and ALDC enzymes of *P. peoriae* NRRL BD-62 were possibly preferentially activated to pyruvate dehydrogenase in the Arg/Asn-based NADH presence, directing the metabolic flux to the 2,3-BDO synthesis.

ISHII, MORITA, *et al.* (2018) used a high-activity ALS-expressing *Saccharomyces cerevisiae* strain to demonstrate the effects of the pyruvate carbon flux tugging strategy on the 2,3-BDO and ethanol production. The authors introduced additional ALDC and BDH genes and modified the aeration conditions to promote the pyruvate conversion to α-acetolactate rather than acetyl-CoA. A high 2,3-BDO titer and no ethanol sub-generation were achieved. KIM, Jin-Woo, KIM, *et al.* (2016) obtained a high 2,3-BDO titer yield by NADH oxidase expression in pyruvate decarboxylase-deficient *S. cerevisiae*. The redox imbalance was overcome through this metabolic strategy, and the pyruvate and NADH accumulation favored the 2,3-BDO production instead of ethanol.

In turn, acetic acid production can be related to amino acid catabolism. SANGAVAI, CHELLAPANDI (2017) proposed the Asn catabolic pathway from the MetaCyc database for *C. sticklandii* DSM 519, a GRAS bacterium capable of producing biofuels and organic acids. According to the authors, L-Asn is converted into oxaloacetate and L-glutamate, which in turn is transformed into acetate via acetyl-CoA after a metabolic reaction sequence with n-butanoate as an intermediate product. These observations suggested that the Asn catabolism by *P. peoriae* NRRL BD-62 must have occurred via a similar acetate production pathway, resulting in acetic acid formation in 48 h. The net acetic acid production absence in the Arg-supplemented medium may be

attributed to the AP expression, previously discussed, since no acetate release is observed in the urea cycle.

Furthermore, a synergism between the acetic acid and 2,3-BDO pathways can be verified. The NAD^+ released in the acetoin conversion to 2,3-BDO can be used in the first step of pyruvate-to-acetate conversion by pyruvate dehydrogenase so that the increased NADH supply results in 2,3-BDO and acetic acid coproduction of (TINÔCO, PATERAKI, *et al.*, 2021). The pyruvate dehydrogenase complex activation in the presence of the amino acid was verified by MASER, PEEBO, *et al.* (2020), who observed higher acetate excretion when *E. coli* BW25113 was cultured in a medium supplemented with amino acids, including Arg, Asn, and Ser. About 16.3 mmol/g_{DCW} of acetate was released using a nutrient-rich medium versus 2.6 mmol/g_{DCW} using a medium without added amino acids. YANG, Yiling, M. POLLARD, *et al.* (2015) found that the Asn degradation into acetate by *E. coli* NCM3722 occurred exclusively under anaerobic conditions and was one of the first amino acids to be consumed by this bacterium.

3.3.2 Selectivity of 2.3-BDO

Figure 5.8 presents the 2,3-BDO selectivity verified using CSL (Fig. 5.8a) and NH_4Cl (Fig. 5.8b) media with or without added Arg and Asn. Acetoin was not detected.

3.3.2.1 Corn steep liquor

Although adding Arg contributed to a *levo*-2,3-BDO selectivity of about 91% versus 56% and 70% for pure CSL and CSL+Asn media in 24 h, respectively, the results did not show statistical differences (Fig. 5.8a). This high selectivity was attributed to the NADH released from the respiratory process by adding Arg, which possibly increased the BDH activity concerning DAR in 24 h (EHSANI, FERNÁNDEZ, *et al.*, 2009, LIANG, SHEN, 2017). These results were in line with the observations made by FU, WANG, *et al.* (2014), who attributed a fundamental role to NADH in the 2,3-BDO chiral production. The authors found more significant than 99% *levo*-2,3-BDO optical purity by *B. subtilis* 168, increased NADH pool through low DO control, reduced substrates, and rational metabolic engineering.

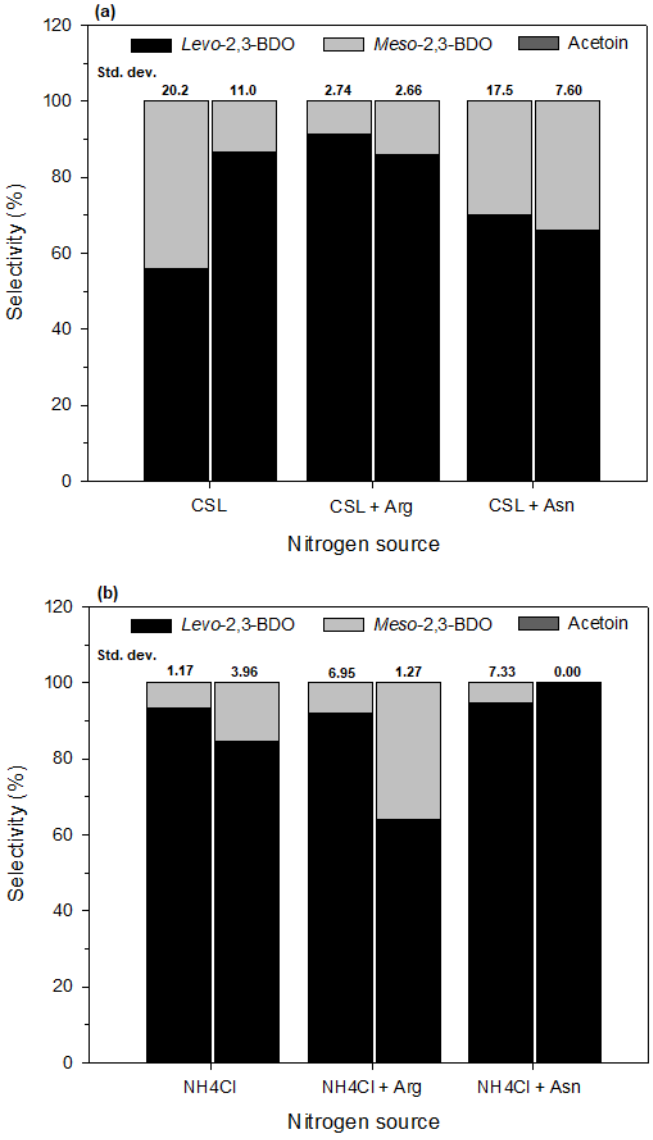

Figure 5.8: Time courses of 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 using NH_4Cl-based medium supplemented with Arg and Asn: (a) glucose consumption; (b) dry cell weight; (c) 2,3-BDO; (d) ethanol; (e) lactic acid; (f) acetic acid.

With the fermentation extension to maximum 2,3-BDO production, the *levo*-2,3-BDO selectivity again showed no statistical differences between the conditions investigated. An approximately 1.5-fold increase was seen in pure CSL medium, from 56 to 87% *levo*-2,3-BDO. The *meso*-2,3-BDO variation was slight in the media supplemented with amino acids, suggesting that the redox potential was efficiently maintained by the NADH/NAD^+ balance in the Arg and Asn presence. Overall, the experimental errors were generally reduced in 30 h, below 2.7% for the CSL+Arg medium and 8% for the CSL+Asn medium.

3.3.2.2 Ammonium chloride

The NH_4Cl-based media showed a *levo*-2,3-BDO selectivity higher than 90% in v24 h, with no significant differences among them (Fig. 5.8b). However, with the fermentation extension, the NH_4Cl+Asn medium showed an exclusive production *levo*-2,3-BDO in 30 h, similar to that obtained using commercial YE-based medium in 24 h. As previously discussed, Asn's ability to form NADH via the NAD biosynthesis pathway makes it an essential *levo*-2,3-BDO inducer.

On the other hand, a higher *meso*-2,3-BDO production was seen with adding Arg, whose average selectivity was 36% in 48 h. Again, this result can be attributed to the previously discussed Arg metabolism, in which ADP must have been deactivated by excess NH_4^+. In the urea presence from AP, cell growth was affected, reducing the NADH released in the respiratory process. Therefore, the 2,3-BDO selectivity was in line with discussions about the 2,3-BDO production in the NH_4Cl-based medium supplemented with amino acids.

The experimental deviations for conditions evaluated were minor, remaining below 4% at the fermentation end. These results suggested that high fermentation reproducibility can be achieved on a large scale. Furthermore, the high optical purity and non-acetoin accumulation make the downstream step (2,3-BDO separation, recovery, and purification) less costly. Finally, NH_4Cl requires less rigorous upstream pretreatment steps for the 2,3-BDO production, further favoring the bioprocess economy, especially compared to complex nitrogen sources such as CSL (MASER, PEEBO, *et al.*, 2020).

3.4 Effects of pH autoregulation on 2,3-BDO metabolism

3.4.1 Production of 2.3-BDO

The pH effects on 2,3-BDO production by *P. peoriae* NRRL BD-62 were evaluated by removing its external control in the NH_4Cl-based medium, previously selected to replace the YE-based medium. The NH_4Cl medium at pH=5 without amino acid supplementation was used as a control. Figure 5.9 presents the time courses of fermentations without external pH control (NH_4Cl, NH_4Cl+Arg, and NH_4Cl+Asn).

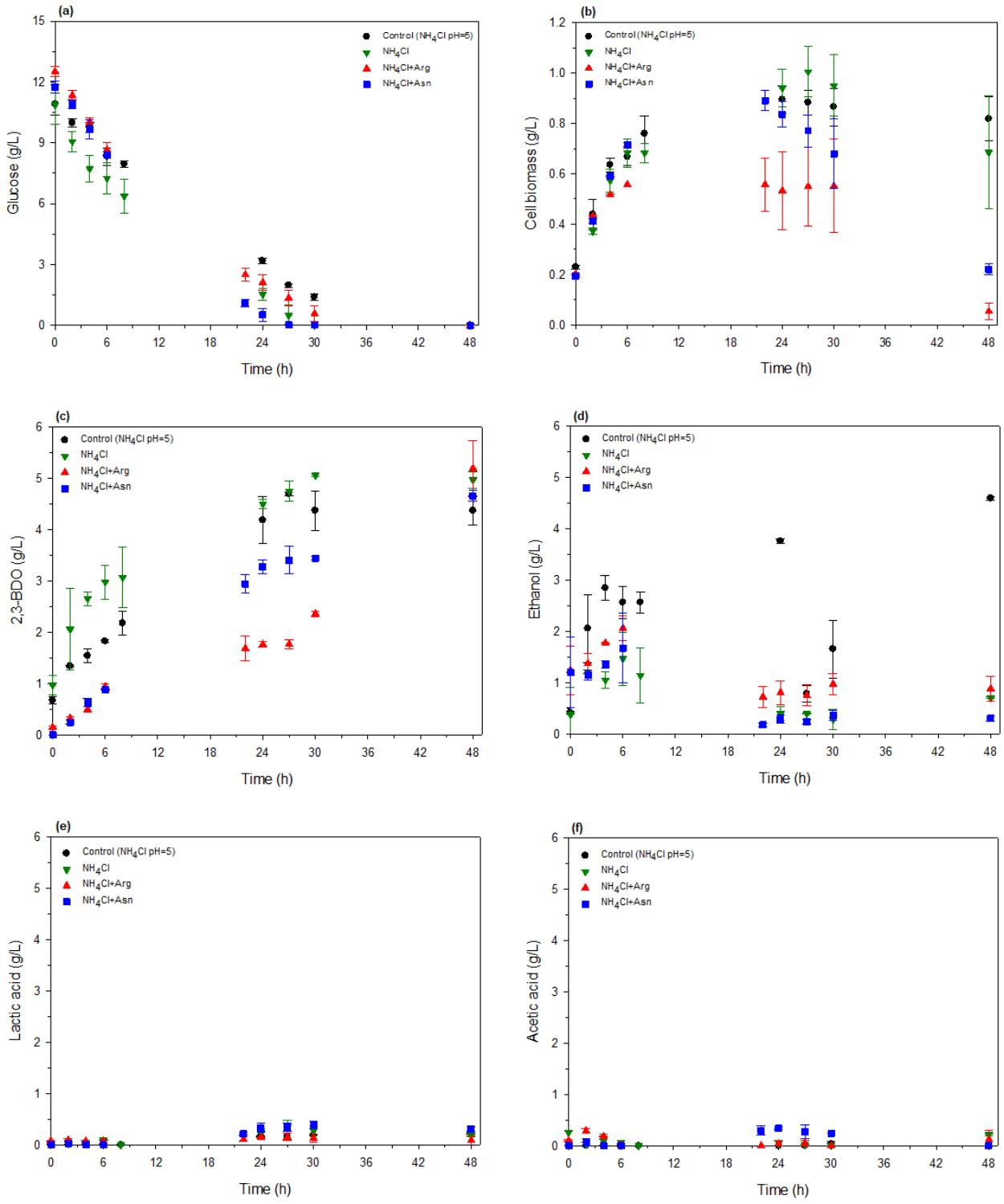

Figure 5.9: Time courses of 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 using NH_4Cl-based medium supplemented with Arg and Asn without pH control: (a) glucose consumption; (b) dry cell weight; (c) 2,3-BDO; (d) ethanol; (e) lactic acid; (f) acetic acid.

Initially, the external pH control led to an increased glucose consumption rate in 24 h, from 0.32 g/L/h (pH=5) to 0.36 g/L/h (without pH control). With adding Arg and Asn, this rate increased further, reaching about 0.42 and 0.47 g/L/h, respectively (Fig. 5.9a). Glucose consumption increased by an average of 24% concerning the medium at pH=5 in this period, exceeding 80% for the conditions without pH control. In particular, adding Asn favored higher substrate assimilation, equal to approximately 95% (Table 5.4).

The 2,3-BDO production (Fig. 5.9c) and the cell biomass formation (Fig. 5.9b) showed no statistically significant differences between control and NH_4Cl media, equal on average to 3.52 g/L and 0.70 g/L in 24 h, respectively. The medium supplemented with Asn also showed similar results to those reported. In contrast, the adding Arg seems to have affected the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 since the fermentative parameters were halved.

The productive similarity was also observed in $Y_{P/S}$ for control and NH_4Cl media, equaling 0.41 g/g (Table 5.4). The adding Asn reduced to 0.29 g/g, although it was found to be statistically similar to the amino acid-free medium. The adverse effects of adding Arg fell on the $Y_{P/S}$, equal to only 0.16 g/g in 24 h. The $Y_{P/X}$ showed no statistical differences between the media with and without amino acid supplementation, equal to, on average, 5.05 g/g.

OURIQUE, ROCHA, *et al.* (2020) observed an increased substrate consumption rate and diol production by *Pantoea agglomerans* BL1 from nitrogen supplementation in pH uncontrolled culture media containing soybean hull acid hydrolysate. Although adding YE favored cell biomass, no improvement was seen in 2,3-BDO volumetric productivity and yield. On the other hand, YEN, Hong Wei, LI, *et al.* (2014) observed a slowness in glycerol consumption by *Klebsiella* sp. Ana-WS5 in pH uncontrolled batch diol fermentation. After 24 h, the pH was dropped to about 5, contributing to a slow 1,3-PDO and 2,3-BDO accumulation, although the diol yields were higher than that observed under pH control, reaching about 86%. MARWOTO, NAKASHIMADA, *et al.* (2002) observed lower xylose consumption by *P. polymyxa* ATCC 12321 in uncontrolled pH 2,3-BDO batch fermentation. The authors reported lower 2,3-BDO production as the pH decreased to 5.3-5.4 after 24 h, resulting from the decreased BDH activity. Despite the

increased initial xylose concentration, the 2,3-BDO production showed no significant improvement. OLIVEIRA, RÖHRENBACH, *et al.* (2022) also observed a reduced 2,3-BDO titer in pH uncontrolled continuously gassed batch processes. *C. ragsdalei* DSM 15248 was used in an autotrophic syngas fermentation to convert CO, CO_2, and H_2 into organic acids and alcohols, including 2,3-BDO. The final pH dropped from 6 to 4, decreasing the total CO uptake.

Table 5.4: Effects of uncontrolled pH on 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and NH_4Cl with and without amino acid supplementation at $C/N_{initial}$=8.5 g/g.

Nitrogen source	Time (h)	2,3-BDO (g/L)	DCW (g/L)	$Y_{P/S}$ (g/g)	$Y_{P/X}$ (g/g)	SC (%)
Control*	24	3.51 ± 0.53^{a}	0.66 ± 0.05^{a}	0.45 ± 0.04^{a}	5.3 ± 0.4^{a}	70.8 ± 0.1^{a}
NH_4Cl		3.53 ± 0.01^{ae}	0.74 ± 0.08^{ae}	0.38 ± 0.04^{ae}	4.8 ± 0.3^{ae}	86.0 ± 1.3^{be}
NH_4Cl + Arg		1.61 ± 0.02^{f}	0.33 ± 0.15^{f}	0.16 ± 0.00^{f}	5.5 ± 0.5^{e}	83.0 ± 2.8^{e}
NH_4Cl + Asn		3.28 ± 0.13^{e}	0.64 ± 0.05^{e}	0.29 ± 0.03^{e}	5.1 ± 0.6^{e}	95.4 ± 2.8^{f}
Control*	27^{mp}	4.02 ± 0.03^{c}	0.65 ± 0.04^{c}	0.45 ± 0.03^{c}	6.2 ± 0.3^{c}	81.8 ± 1.7^{c}
NH_4Cl	30^{mp}	4.09 ± 0.21^{cg}	0.75 ± 0.12^{cg}	0.38 ± 0.05^{cg}	5.5 ± 0.6^{cg}	100.0 ± 0.0^{dg}
NH_4Cl + Arg	48^{mp}	5.04 ± 0.56^{g}	0.00 ± 0.00^{h}	0.40 ± 0.05^{g}	UD**	99.9 ± 0.05^{g}
NH_4Cl + Asn	48^{mp}	4.66 ± 0.10^{h}	0.03 ± 0.02^{h}	0.40 ± 0.02^{g}	UD**	100.0 ± 0.0^{g}

* Control= NH_4Cl at pH=5

**ND= undetermined due DCW~ 0.0 g/L

C/N ratio= carbon/nitrogen ratio; Arg= arginine; Asn= asparagine; mp= maximum production; 2,3-BDO= 2,3-BDO titer (*meso-* + *levo*-isomer titers); DCW= dry cell weight (cell biomass); $Y_{P/S}$= 2,3-BDO/glucose yield; $Y_{P/X}$= 2,3-BDO/dry cell weight yield; SC= substrate consumption. The means followed by the same letter do not show significant differences between Control - NH_4Cl, and NH4Cl - NH4Cl+Arg/Asn by t-test ($\alpha = 0.05$).

The initial pH of the uncontrolled NH_4Cl medium was approximately 5, the same maintained by adding NaOH. Under these conditions, low lactic acid (Fig. 5.9e) and acetic acid (Fig. 5.9f) production were observed. In the presence of these by-products, the intracellular pH showed a slight variation, seeming to have been easily overcome by the 2,3-BDO production by *P. peoriae* NRRL BD-62. Different microorganisms produce 2,3-BDO to control intracellular pH due to its neutral and non-inhibitory character (CELIŃSKA, GRAJEK, 2009, JI, Xiao-jun, HUANG, *et al.*, 2011). Since the organic acid production was less than 1 g/L, the 2,3-BDO released was sufficient to maintain the pH around 5 throughout the fermentation, not requiring external control.

PETROV, PETROVA (2009) concluded that the 2,3-BDO production by *K. pneumoniae* G31 from glycerol resulted from an adaptive pH autoregulation mechanism. The alternation mediated the regulation of the glycerol catabolism oxidative pathway between acetic acid and 2,3-BDO. Higher acetic acid production was observed in the

exponential cell growth phase, reducing the medium's pH. Immediately, 2,3-BBDO was produced to control the pH variation. The most significant 2,3-BDO accumulation was observed at an initial pH of 8 due to the greater variation amplitude, whose final pH was equal to 5.1 in 280 h.

In contrast, the 2,3-BDPO yield was affected by the increased lactate and ethanol under basic conditions. AMRAOUI, PRABHU, *et al.* (2022) found a systematic carbon flux shift for 2,3-BDO synthesis by *E. ludwigii* from brewers' spent grain hydrolysate as a cell pH maintenance strategy. A 2,3-BDO yield of 0.41 g/g was obtained by regularly dropping the pH from 7 to slightly acidic conditions around 5.8. This yield was 28% higher than that obtained in pH-controlled assays.

The external pH control removal intensified the adverse effects of adding Arg in the NH_4Cl medium, which was responsible for neutralizing ammonia and preventing feedback inhibition of ADP enzymes. As a result, as previously discussed, a more significant urea amount must have been released, affecting the 2,3-BDO titer and yield. Furthermore, the acid resistance promoted by Arg was lost with ADP blocking, madding the bioprocess more vulnerable to pH lowering.

With the fermentation extension, glucose was depleted in the pH uncontrolled media (Fig. 5.9a). Cell growth (Fig. 5.9b), maximum 2,3-BDO production (Fig. 5.9c), and $Y_{P/S}$ and $Y_{P/X}$ (Table 5.4) were statistically similar for the control and the NH_4Cl media, although the fermentation time was equal to 27 h and 30 h, respectively. MITREA, VODNAR (2019) reported equal glycerol consumption in assays with and without pH control for the 1,3-PDO and 2,3-BDO production by *K. pneumoniae* DSMZ 2026 in 24 h. However, total substrate consumption and metabolite synthesis were delayed with uncontrolled pH.

The average increased 2,3-BDO production from 3.52 g/L to 4.05 g/L was accompanied by the same average cell biomass of 0.70 g/L, which led to an increase of approximately 16% on average $Y_{P/X}$ for the control and NH_4Cl media (Table 5.4). In contrast, the $Y_{P/S}$ remained on average equal to 0.41 g/g, and the pH was maintained at around 5 throughout the fermentation. Again, producing high-yield 2,3-BDO by *P. peoriae* NRRL BD-62 was biologically sufficient to regulate and maintain pH under

optimal cultivation conditions. VIVIJS, MOONS, *et al.* (2014) evaluated 2,3-BDO fermentation as a cell strategy to protect against low pH. The authors attributed proton consumption throughout 2,3-BDO metabolism as the mechanism responsible for pH control since this behavior is similar to that of amino acid decarboxylase reactions, such as arginine decarboxylase, conferring acid resistance to several bacteria. Besides external pH control, 2,3-BDO fermentation was related to intracellular control by improving pH homeostasis from the abovementioned proton consumption.

The *P. peoriae* NRRL BD-62 growth was strongly compromised in the pH uncontrolled NH_4Cl medium supplemented with amino acids, showing a net cell biomass of almost zero in 48 h (Fig. 5.9b). Despite the cell viability loss, a maximum 2,3-BDO production was seen in this period, statistically similar to NH_4Cl media with and without Arg, and about 14% higher for Asn-based medium (Fig 5.9c). The $Y_{P/S}$ showed no statistical differences with adding amino acids, remaining around 0.40 g/g, a value similar to the yield found for the control and NH_4Cl media. The $Y_{P/X}$ could not be calculated due to the cell death observed in the amino acid-supplemented fermentations (Table 5.4).

Cell death by Arg was attributed to the acid resistance mechanism impairment caused by ADP blockade, while cell death by Asn was related to its catabolism in the NH_4Cl medium. Naturally, NH_4Cl has acidic properties, with a pH of around 5-5.5. In Asn catabolism, NH_4^+ is released during conversion to Asp (www.metacyc.org), causing a decreased pH below 5. Under these conditions, the *P. peoriae* NRRL BD-62 growth was compromised, madding challenging to maintain the cell growth stationary phase. Furthermore, the maximum 2,3-BDO production was delayed, reaching a longer fermentation time and at a lower titer than that found for NH_4Cl+Asn at pH=5 (Table 5.3). Therefore, the amino acid supplementation strategy to pH uncontrolled NH_4Cl medium was considered inefficient, despite the 2,3-BDO production in 48 h, since the cell viability of *P. peoriae* NRRL BD-62 was not maintained throughout the entire fermentation.

In general, the 2,3-BDO production is strongly compromised with cell death, especially in late fermentation steps, because it depends on both μ and DCW. Furthermore, it is impossible to apply strategies to increase the 2,3-BDO production on a large scale, such as fed-batch fermentation or continuous cultivation, since high cell

viability is required. Conducting the fermentation until cell reduction is observed can also not overcome the problem since the substrate not consumed represents an additional cost with feedstock in the upstream production step. Therefore, cell death makes the bioprocess economically unfeasible and industrially inoperable and should be avoided (TINÔCO, BORSCHIVER, *et al.*, 2020).

DURGAPAL, KUMAR, *et al.* (2014) verified the metabolic activity loss of an engineered *K. pneumoniae* capable of producing 1,3-PDO and 2,3-BDO as a by-product from glycerol when the pH dropped below 5. Microbial glycerol assimilation was stopped in 24 h in an uncontrolled pH medium, leading to an accumulation of approximately 18 g/L. MITREA, VODNAR (2019) also observed a cell viability loss and reduced microbial biomass with decreasing pH to values around 4.5. CHAN, JANTAMA, *et al.* (2016) reported reduced substrate uptake and cell biomass formation of an engineered *K. oxytoca* KMS005 in 2,3-BDO micro aerated batch fermentation at pH below 6. LEE, Sang Jun, CHOI, *et al.* (2017) verified only 0.89 g/L 2,3-BDO by *E. aerogenes* SUMI014 in batch fermentation at pH= 5.5. The authors reported reduced glucose consumption and cell growth under low pH conditions.

Finally, ethanol formation at the fermentation end was minimized in an uncontrolled pH medium with and without amino acid addition, and no significant net production was detected in 48 h (Fig. 5.9d). Lactic acid and acetic acid showed similar production profiles to those observed for pH-controlled media supplemented or not with amino acids, except for the Asn-based medium, which showed no acetate accumulation at the fermentation end (Fig. 5.9e -f).

The pH is closely linked to the metabolic pathway activation, changing the ALS activity, and diverting the carbon flux to other metabolites instead of 2,3-BDO (CHAN, JANTAMA, *et al.*, 2016). DURGAPAL, KUMAR, *et al.* (2014) found higher ethanol, succinate, acetate, and formate production with increasing pH, while an inverse relationship was observed for 2,3-BDO production. The highest 2,3-BDO titer of 18.5 g/L was achieved in an uncontrolled pH medium in 24 h. BIEBL, ZENG, *et al.* (1998) obtained similar results for the 2,3-BDO and by-product production by *K. pneumoniae* GT1 from glycerol by gradually reducing the pH from 7.3 to 5.4 in continuous cultures. A maximum 2,3-BDO yield was reached at pH 5.5, accompanied by decreased acetate

and ethanol. YEN, Hong Wei, LI, *et al.* (2014) reported lower succinic, lactic, and acetic acid production in uncontrolled pH batch fermentation when investigating the pH effects on the 1,3-PDO and 2,3-BDO production by *Klebsiella* sp. Ana-WS5 from glycerol. Despite low by-product production, the volumetric diol productivity was impaired by uncontrolled pH.

3.4.2 Selectivity of 2.3-BDO

Figure 5.10 presents the 2,3-BDO selectivity for the pH uncontrolled media. No acetoin accumulation was detected. A *levo*-2,3-BDO selectivity higher than 90% was verified for all conditions in 24 h. Again, the adding Arg and Asn contributed to a highly uniform *levo*-2,3-BDO production, with experimental deviations of less than 1%.

With the fermentation extension to the maximum 2,3-BDO production, the *levo*-2,3-BDO selectivity decreased, reaching about 40% and 60% for the media containing Arg and Asn, respectively. Experimental deviations also increased, although they were below 8.5% in 48 h. These results were expected due to the effects on the $NADH/NAD^+$ balance caused by adding amino acids to the pH uncontrolled NH_4Cl-based medium, as previously discussed.

The highest *levo*-2,3-BDO selectivity was achieved in the pH uncontrolled NH*4*Cl medium without adding amino acids, reaching 87% in 30 h. Under these conditions, a low experimental deviation of only 5.6% was verified. These results were similar to those obtained in the control medium, whose selectivity was equal to approximately 85%, with an experimental deviation of less than 4%.

Unlike this study, GAO, J., JIANG, *et al.* (2019) concluded that Arg and Asn supplementation was an efficient strategy to enhance the *levo*-2,3-BDO production by *P. polymyxa* ZJ-9 from inulin or glucose. The adding Arg increased the *levo*-2,3-BDO production by 16.5% in pure inulin fermentation. This amino acid was a limiting factor for cell growth due to its presence in large amounts in the inulin extract from Jerusalem artichoke tubers. Adding Asn seems to have increased the gene expression related to the *levo*-2,3-BDO synthesis.

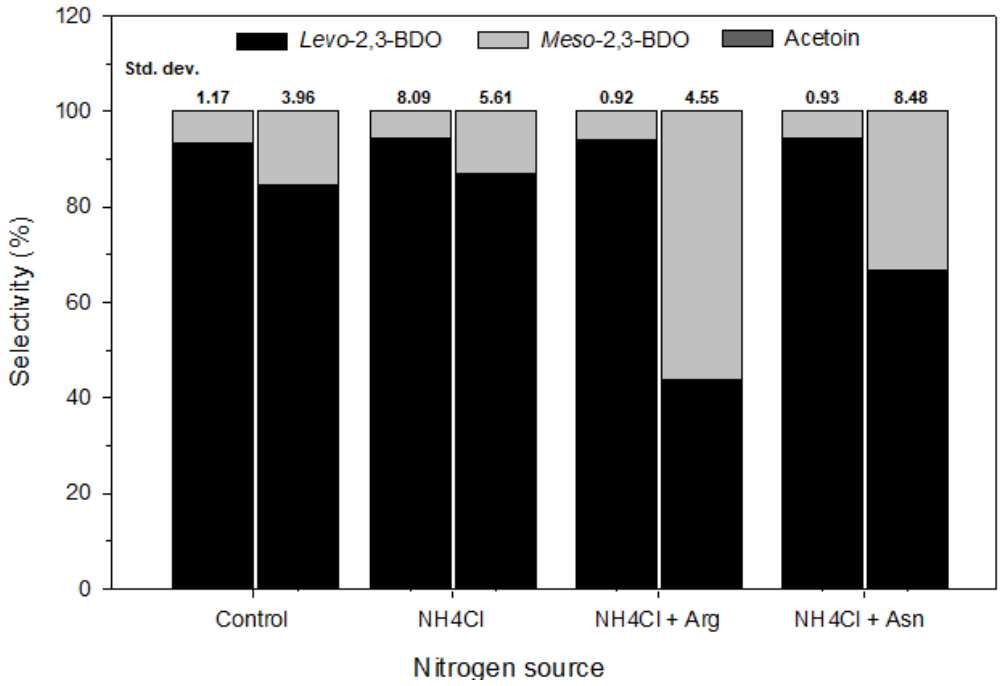

Figure 5.10: Selectivity of 2,3-BDO (*levo*- and *meso*-isomers) and acetoin by *P. peoriae* NRRL BD-62 using NH_4Cl-based medium supplemented with Arg and Asn without pH control. The control medium corresponds to pure NH_4Cl at pH=5. The first bar of each investigated medium corresponds to the selectivity at 24 h, while the second corresponds to the selectivity at the time of maximum 2,3-BDO production: 27 h (control); 30 h (NH_4Cl); and); and 48 h (NH_4Cl+Arg, NH_4Cl+Asn).

3.5 Validation of 2,3-BDO production in NH_4Cl-based medium

The microbial pH autoregulation by *P. peoriae* NRRL BD-62 was equivalent to its external control mediated by NaOH in the NH_4Cl medium without Arg or Asn. This strategy was metabolically efficient and economically advantageous since fermentative parameters, and cell viability were kept high in a lower-cost culture medium. A similar result for 2,3-BDO production at uncontrolled pH was reported by DAS, PRAKASH, *et al.* (2021). Therefore, the uncontrolled pH pure NH_4Cl-based medium was chosen to replace the YE-based medium at pH=5 to produce high-yield 2,3-BDO by *P. peoriae* NRRL BD-62.

3.5.1 Batch fermentation

The 2,3-BDO production validation by *P. peoriae* NRRL BD-62 from uncontrolled pH pure NH_4Cl-based medium was initially investigated in batch fermentation. The assays were carried out in the same 200 mL reaction system to investigate the different nitrogen sources, with an initial glucose concentration higher than 10 g/L, maintaining C/N= 8.5 g/g. Figure 5.11 presents the batch fermentation time courses.

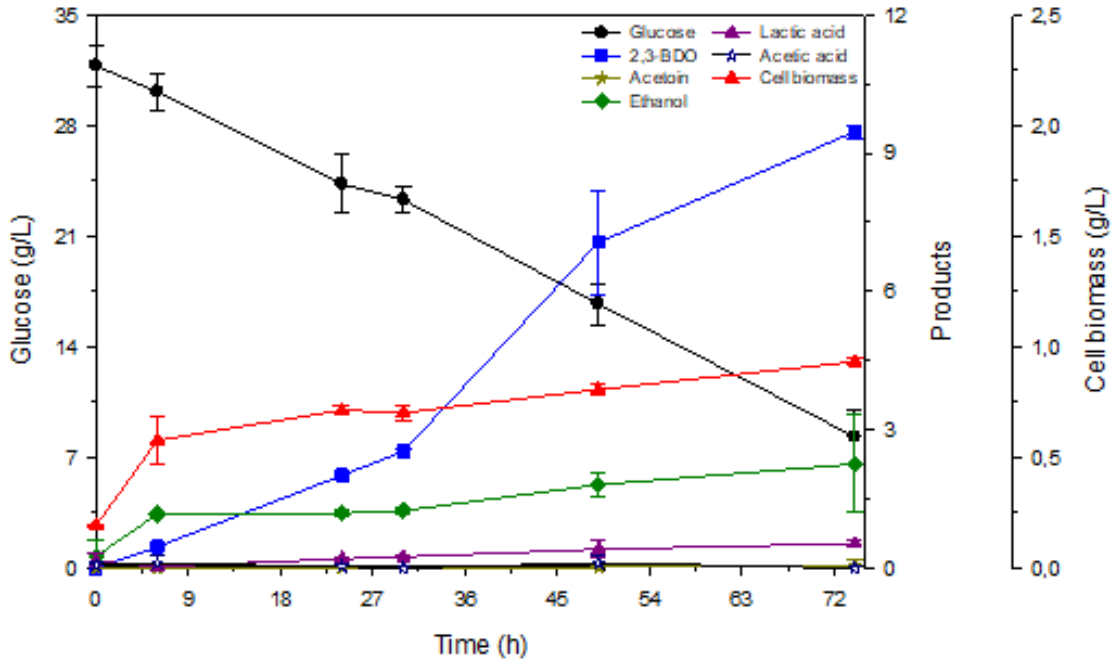

Figure 5.11: Validation of 2,3-BDO production by *P. peoriae* NRRL BD-62 using pure NH_4Cl-based medium without pH control in batch fermentation (Dasbox 200 mL).

The initial glucose of 30 g/L was set based on preliminary cell inhibition assays caused by excess substrate under low oxygen supply (data not shown). Higher NH_4Cl concentrations were required to maintain the C/N ratio at 8.5 g/g. In cultures with more than 40 g/L glucose, the NH_3 accumulation, resulting from the equilibrium with $NH4^+$, may have inhibited the *P. peoriae* NRRL BD-62 growth, as did excess chloride. Ammonia is toxic to many microorganisms because it is uncharged and can easily cross the cell membrane (REN, CHEN, *et al.*, 2015). In turn, chloride can reduce cell growth and substrate uptake rates, inhibiting glucose fermentation, as investigated by CASEY, MOSIER, *et al.* (2013).

Excess NH_4Cl can also compromise the specific glucose uptake rate (q_{Glu}), responsible for activating the expression of genes involved in the acetate, acetoin, and 2,3-BDO production. Changes in q_{Glu} affect the yield of these bioproducts, as reported by CRISTIANO-FAJARDO, FLORES, *et al.* (2019). The authors found higher 2,3-BDO + acetoin production by *B. amyloliquefaciens* 83 in continuous cultures at high q_{Glu}, while cell yield was favored at low q_{Glu}. DE MAS, JANSEN, *et al.* (1988) observed lower 2,3-BDO production rates by *P. polymyxa* ATCC 12321 at low q_{Glu} in fed-batch fermentation. In both studies, 2,3-BDO production and glucose uptake were correlated with cell oxygen supply and demand.

The low and uncontrolled pH also appears to have affected the high initial substrate concentrations for the 2,3-BDO production. Similar observations were reported by CHAN, JANTAMA, *et al.* (2016), who found a high residual maltodextrin accumulation in uncontrolled pH fermentation, significantly below 6. The authors suggested that the increased acidity caused a pH gradient collapse across the cell membrane, compromising the ATP production and, consequently, the maltodextrin uptake by the mutant *K. oxytoca* KMS005. Compromised substrate consumption caused by the high pH gradient between the intracellular and extracellular medium was also reported by THAPA, LEE, *et al.* (2019).

Approximately 9.5 g/L of 2,3-BDO was produced from 74% consumption glucose in 74 h (Fig. 5.11). The 2,3-BDO production was mainly observed in the microbial growth stationary phase, whose cell biomass reached about 0.74 g/L at the fermentation end. The $Y_{P/S}$ was equal to 0.40 g/g, the same obtained in the previous NH_4Cl selection assays, indicating that the high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62 was validated. The $Y_{P/X}$ confirmed the uncontrolled pH pure NH_4Cl-based medium validation since its high value of 12.8 g/g reflected a carbon flux directed preferentially to 2,3-BDO synthesis rather than cell growth (Table 5.5).

AMRAOUI, NARISETTY, *et al.* (2021) reported a high-yield 2,3-BDO production from xylose in an uncontrolled pH batch fermentation. Approximately 40 g/L xylose was rapidly assimilated and converted to 2,3-BDO, achieving a yield of 0.44 g/g in 26 h. The pH dropped below 5.5. ALMUHAREF, RAHMAN, *et al.* (2019) reported a

2,3-BDO yield of 0.40 g/g by *S. proteamaculans* SRWQ1 from glycerol in uncontrolled pH flask cultures.

The minimization of parallel 2,3-BDO metabolism pathways was verified by the low ethanol and lactic acid production, whose titers were approximately 2 g/L and 0.4 g/L, respectively. No net acetic acid production was detected. However, a slight acetoin accumulation of around 0.07 g/L was observed in 74 h (Fig. 5.11). This accumulation possibly resulted from decreased NADH supply and increased DO at the late fermentation steps. In the microbial growth stationary phase, a cell activity loss can be observed, leading to inefficient oxygen uptake. Excess oxygen affects the $NADH/NAD^+$ ratio, so the 2,3-BDO metabolism redox balance promotes the reverse conversion reaction to acetoin, justifying its presence even under low aeration conditions (GURAGAIN, VADLANI, 2017).

Table 5.5: Validation of 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and NH_4Cl at $C/N_{initial}$=8.5 g/g and uncontrolled pH in batch and fed-batch fermentation.

Cultivation conditions	Fermentation	
	Batch (Dasbox 200 mL)	**Fed-batch (Bioreactor 1L)**
2,3-BDO (g/L)	9.47 ± 0.14	7.50 ± 0.10
DCW (g/L)	0.74 ± 0.02	0.60 ± 0.00
$Y_{P/S}$ (g/g)	0.40 ± 0.01	0.34 ± 0.00
$Y_{P/X}$ (g/g)	12.8 ± 0.2[a]	12.4 ± 0.2[a]
SC (%)	74.0 ± 4.1	99.7 ± 0.0
Time (h)	74	96

C/N ratio= carbon/nitrogen ratio; 2,3-BDO= 2,3-BDO titer (*meso-* + *levo*-isomer titers); DCW= dry cell weight (cell biomass); $Y_{P/S}$= 2,3-BDO/glucose yield; $Y_{P/X}$= 2,3-BDO/dry cell weight yield; SC= substrate consumption. The means followed by the same letter do not show significant differences by t-test (α = 0.05).

3.5.2 Scale-up process

An initial attempt to bioprocess scale-up was carried out in a 2 L bioreactor. Maintaining similar hydrodynamic and mass transfer conditions at different scales is impossible, and the most critical control variable must be chosen (GARCIA-OCHOA, GOMEZ, *et al.*, 2010). Typically, maintaining the oxygen transfer rate (OTR) by the kLa control is used as a scale-up strategy for aerobic fermentations (GARCIA-OCHOA, GOMEZ, 2009), while constant oxygen absorption rate (OUR) is used for microaerobic fermentation (CONVERTI, PEREGO, *et al.*, 2003). The OTR is considered a key

parameter for the 2,3-BDO yield and volumetric productivity. For this reason, it has been investigated as a control variable for scale-up, although there is no consensus on its efficiency for microaerobic fermentation (REBECCHI, PINELLI, *et al.*, 2018).

In this study, the constant OTR strategy was adopted by adjusting kLa. A controlled kLa around 5 h^{-1} and an uncontrolled DO, decreased toward zero (DO<1% saturation) throughout the batch fermentation in 200 mL bioreactor, contributed to a low and approximately constant OTR of 1.125 $mmol_{O2}$/L/h, used to validate the results found in 2 L bioreactor. FLORES, MEDINA-VALDEZ, *et al.* (2020) obtained the highest 2,3-BDO + acetoin titer by *B. velezensis* 83 in glucose shake flasks at a maximum OTR of 2.5 $mmol_{O2}$/L/h, using $(NH_4)_2SO_4$ as a nitrogen source. HEYMAN, LAMM, *et al.* (2019) proposed a shake flask methodology for assessing the OTR effects on 2,3-BDO production by *B. licheniformis* DSM 8785. The authors obtained the highest 2,3-BDO titer at 4 $mmol_{O2}$/L/h. This methodology was easily adaptable to other bioreactor configurations and scales. Therefore, the maintaining OTR criterion at a low and constant value was considered adequate because it is based on the aeration extrapolation, considered a critical parameter for 2,3-BDO production, whose efficiency depends on a low oxygen supply (JI, Xiao-jun, HUANG, *et al.*, 2011).

Figure 5.12 presents the fed-batch fermentation time courses in the bioreactor with an initial volume of 1 L. The initial glucose concentration was approximately 10 g/L, the same used in the previous NH_4Cl selection assays. This concentration was chosen to allow a comparative analysis of the substrate consumption profiles in the two production scales during the first fermentation hours. A pulse feed was performed in 28 h when about 70% glucose had been consumed, and the cells were in the exponential growth phase.

Approximately 7.5 g/L 2,3-BDO was produced from total glucose uptake in 96 h. On average, the total glucose assimilated by *P. peoriae* NRRL BD-62 in batch and fed-batch fermentation was equal to 22 g/L. However, the $Y_{P/S}$ was lower in fed-batch fermentation, reaching only 0.34 g/g (Table 5.5). In contrast, the $Y_{P/X}$ of 12.4 g/g was statistically similar to that observed in batch fermentation (Table 5.5) since 2,3-BDO production increased in 72-96 h, while cell biomass reduced to 0.60 g/L at the interval end. No net acetoin, ethanol, lactic acid, and acetic acid production was detected (Fig. 5.12).

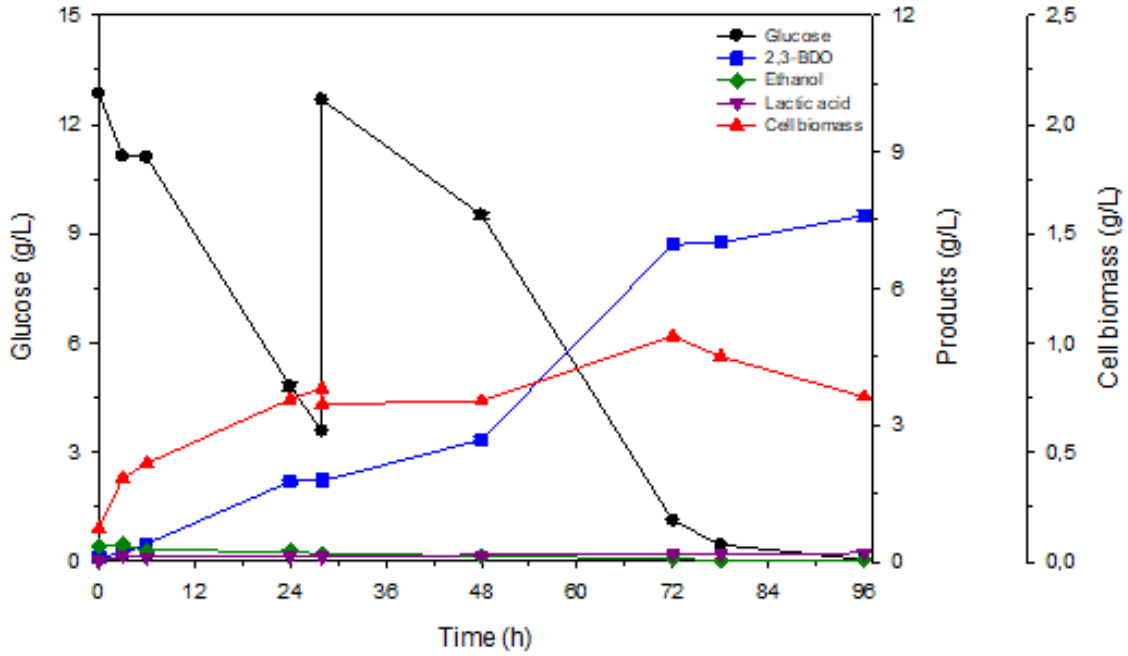

Figure 5.12: Validation of 2,3-BDO production by *P. peoriae* NRRL BD-62 using pure NH_4Cl-based medium without pH control in fed-batch fermentation (Bioreactor 1 L).

GURAGAIN, VADLANI (2017) investigated the optimization of 2,3-BDO fed-batch fermentation by *K. oxytoca* ATCC 8724 from glucose. One set of experiments was performed without pH control and with a two-step aeration scheme to favor cell biomass (high aeration) and 2,3-BDO production (low aeration). The ethanol, lactic acid, and acetic acid production were minimized in the low aeration step, while sugar was channeled almost exclusively to the 2,3-BDO production by reducing the pH from 7 to 5.3. However, due to slow glucose uptake, 2,3-BDO volumetric productivity significantly decreased after the first feed pulse. DAMASCENO, ROSSI, *et al.* (2022) investigated the 1,3-PDO and 2,3-BDO production from glycerol in a fluidized bed reactor with cell-immobilized *K. pneumoniae* BLh-1. The authors found an inhibition of ethanol and lactic acid production and a 2,3-BDO accumulation when the pH dropped to 5.5 in uncontrolled pH continuous culture. Low acetic acid concentrations were also obtained, while no succinic acid production was observed under these conditions.

Despite the apparent minimization of 2,3-BDO parallel metabolism pathways, the low $Y_{P/S}$ and cell activity loss at the late fermentation steps made the initial attempt to 2,3-BDO production scale-up in the 2 L bioreactor unsuccessful. One possible explanation for the low 2,3-BDO yield was the nutritional limitation caused by NH_4Cl in

glucose-fed medium, whose C/N value was increased during fed-batch fermentation. Nitrogen limitation associated with the absence of other growth factors, such as those in complex sources such as YE, seems to have compromised the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62. Similar observations were made by REBECCHI, PINELLI, *et al.* (2018). The authors found an almost 50% reduction in the $Y_{P/S}$ of 0.44 g/g by *B. licheniformis* ATCC9789 when replacing the culture medium containing glucose, beef extract, and soy peptone with a cheap medium based on molasses and chicken meat and bone meal.

Another possible explanation would be the geometric difference between the systems used since OTR depends on the bioreactor dimensions (SEIDEL, MASCHKE, *et al.*, 2021). Although environmental conditions, mainly oxygen supply, were maintained to preserve the *P. peoriae* NRRL BD-62 physiological activity and allow an adequate glucose conversion to 2,3-BDO in uncontrolled pH pure NH_4Cl-based medium, the area/volume ratio of the systems was different. The hydrostatic pressure possibly suffered variations with the change in the dimensions adopted, which may have altered the oxygen solubility (GARCIA-OCHOA, GOMEZ, *et al.*, 2010). Consequently, heat and mass transfers and the surface aeration level must have been modified, compromising the 2,3-BDO synthesis.

The auxiliary oxygen supply equipment may also have contributed to the reduced $Y_{P/S}$, as different aerators and agitators were used in each system. This difference may have modified the contact surface area of air bubbles and the flow type established in the reaction medium. The turbulence caused by the different vessel geometries and the impeller type must have affected the resistance to molecular oxygen diffusion, modifying its cell absorption (CLARKE, CORREIA, 2008). Again, the 2,3-BDO metabolism was altered, even though the oxygen supply was maintained by constant kLa.

3.5.3 Oxygen uptake specific rate (q_{O_2})

The same OTR value can provide different DO levels in microaerobic processes performed at different scales (REBECCHI, PINELLI, *et al.*, 2018). The oxygen uptake specific rate (q_{O_2}) must be kept within a range that allows the balance between the respiration and fermentation processes to control this parameter (DE MAS, JANSEN, *et*

al., 1988). Any change in the q_{O_2} can cause an imbalance in the carbon balance and the OTR and DO values, directly affecting the 2,3-BDO yield and volumetric productivity (REBECCHI, PINELLI, *et al.*, 2018).

REBECCHI, PINELLI, *et al.* (2018) developed and implemented an OTR optimization procedure that allowed obtaining a wide q_{O_2} range to be applied to the 2,3-BDO bioprocess scale-up. For q_{O_2} ranging from 1.4–7.9 $mmol_{O_2}/g_{DCW}/h$, a high 2,3-BDO yield was achieved. CONVERTI, PEREGO, *et al.* (2003) varied q_{O_2} from 0 to 72.7 $mmol_{O_2}/g_{DCW}/h$ to evaluate the available oxygen effects on 2,3-BDO metabolism by *E. aerogenes* NCIMB 10102 in glucose batch fermentation. A 2,3-BDO yield of 69%, a glucose consumption for cell growth of 59.4%, and an enhanced energy yield were verified with increasing q_{O_2}.

Figure 5.13 presents the q_{O_2} found for producing 2,3-BDO by *P. peoriae* NRRL BD-62. While the q_{O_2} remained practically the same in the 200 mL bioreactor with 10 and 30 g/L initial glucose, an increase was observed in the 2 L bioreactor in fed-batch fermentation. Although small, the increased q_{O_2} may have compromised the 2,3-BDO yield by altering the $NADH/NAD^+$ balance due to differences in the aeration systems. Furthermore, naturally, DO can vary throughout cell growth, remaining constant during the exponential phase and variable in the stationary phase (SEIDEL, MASCHKE, *et al.*, 2021), when 2,3-BDO production is highest. Therefore, q_{O_2} control is critical to the 2,3-BDO metabolism control.

The q_{O_2} effects on 2,3-BDO selectivity were also investigated. Figure 5.14 presents the results found in batch and fed-batch fermentation. The increased initial glucose concentration seems to have affected the *levo*-2,3-BDO synthesis, reaching only 55% at the batch fermentation end. A small acetoin accumulation of about 0.7% was detected in the same period. The reduced *levo*-2,3-BDO was even more pronounced in fed-batch fermentation, reaching only 18% in 96 h. However, no acetoin accumulation was detected.

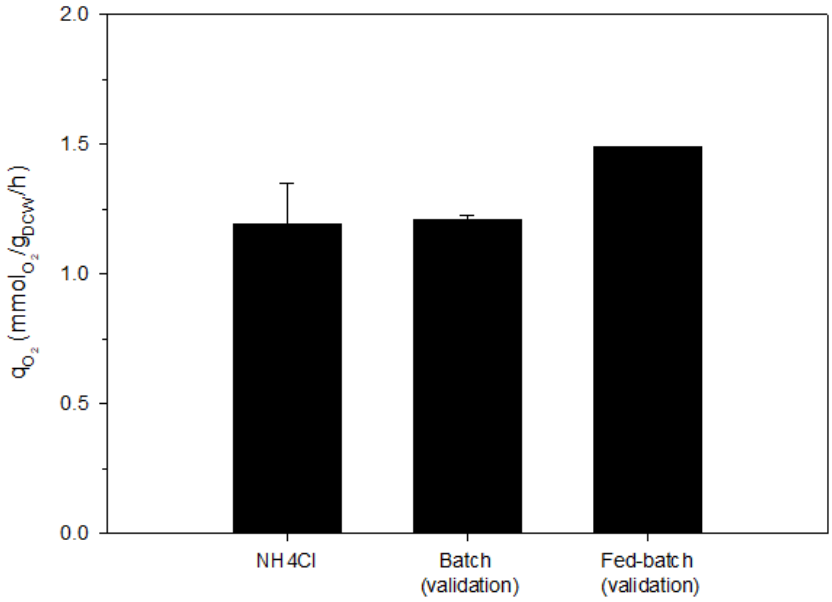

Figure 5.13: Oxygen consumption specific rate of *P. peoriae* NRRL BD-62 in: (a) NH_4Cl batch fermentation without pH control using 10 g/L glucose; (b) validation in batch fermentation; (c) validation in fed-batch fermentation.

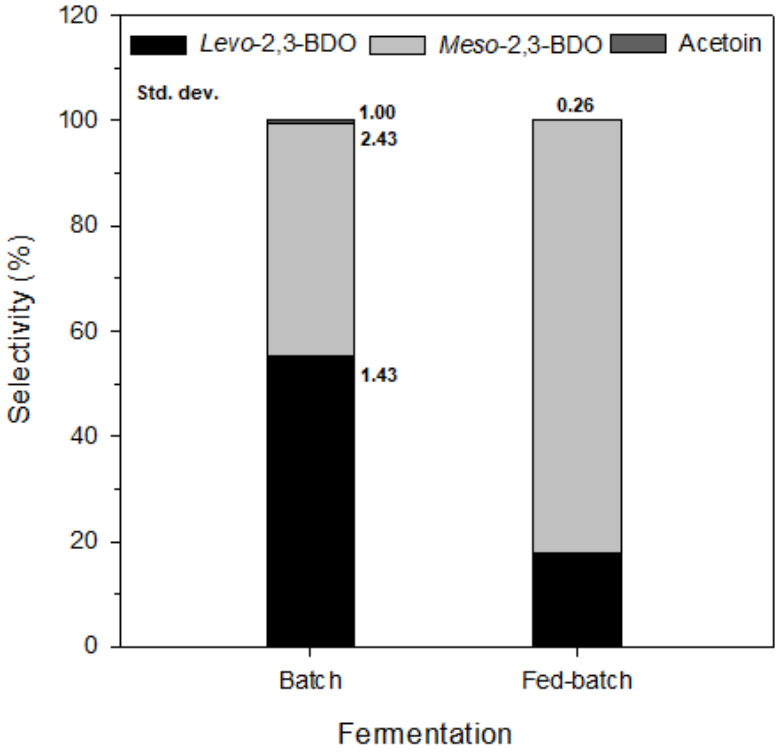

Figure 5.14: Selectivity of 2,3-BDO (*levo*- and *meso*-isomers) and acetoin by *P. peoriae* NRRL BD-62 for batch and fed-batch validations.

The high *meso*-2,3-BDO production in fed-batch fermentation seems to have been caused by the 24% increase in the q_{O_2}, which reached about 1.5 $mmol_{O_2}/g_{DCW}/h$. Under these conditions, the carbon flux was shifted preferentially to *meso*-2,3-BDO synthesis, possibly by activating the DAR enzyme rather than BDH in 2,3-BDO metabolism. However, the q_{O_2} variation was not enough to cause an acetoin accumulation, being in line with the results found.

SU, WILLIS, *et al.* (2015) observed a more expressive *meso*-2,3-BDO production instead of *levo*-2,3-BDO by *Spathaspora passalidarum* NN245 in xylose shake flasks when q_{O_2} increased by 18.2%, ranging from 0.33 to 0.39 $mmol_{O2}/g_{DCW}/h$. DE MAS, JANSEN, *et al.* (1988) found reduced yield and *levo*-2,3-BDO production rate with increased q_{O_2} greater than 4.5 $mmol_{O_2}/g_{DCW}/h$. NAKASHIMADA, KANAI, *et al.* (1998) obtained a *levo*-isomer optical purity of 93% by *P. polymyxa* ATCC 12321 in chemostat culture at q_{O_2} of 2.6 $mmol_{O_2}/g_{DCW}/h$. WANG, OH, *et al.* (2021) observed an inverse relationship between DO and *levo*-2,3-BDO. Increasing the agitation and aeration rate from 400 rpm to 500-600 rpm and from 1 L/min to 2 L/min, respectively, enhanced the *meso*-2,3-BDO purity to 90.5% while reducing the *levo*-2,3-BDO by *B. subtilis* CS13. The authors concluded that small changes in oxygen supply could affect the 2,3-BDO isomer formed and, therefore, the selectivity achieved in each culture condition adopted.

3.6 Comparative analysis of the bio-based 2,3-BDO production

Table 5.6 presents a comparative analysis between the results found in this study and those previously reported in studies investigating using different nitrogen sources to produce bio-based 2,3-BDO. The following fermentation parameters were shown: bioprocess operation mode, pH, 2,3-BDO yield, and 2,3-BDO selectivity. It was possible to note the non-GRAS microorganisms' predominance in the studies whose 2,3-BDO yield and selectivity were higher than 0.40 g/g and 80%, respectively. The use of complex nitrogen sources such as YE was also highlighted.

3.7 Culture medium costs evaluation

Table 5.7 summarizes the costs per culture medium volume (m^3) for each alternative nitrogen source investigated and its relation to commercial YE, considering

an initial glucose concentration of 10 g/L and C/N= 8.5 g/g. Free-on-board (FOB) prices (COMEXSTAT, 2022) and the prices traded by companies in the bioprocess sector (ANGEL YEAST, 2022) were used to more realistically calculate the culture medium costs for large-scale 2,3-BDO production by *P. peoriae* NRRL BD-62 (Table S2 – Supplementary material).

As expected, YE (commercial and brewer) presented the highest cost, with a FOB price of 6.00 US$/kg. All other nitrogen sources presented a FOB price below 1.00 US$/kg, except $NaNO_3$, which presented 2.82 US$/kg. The lowest culture medium cost was achieved using urea, equal to 0.59 US$/m³. However, urea was disregarded to replace the commercial YE due to the previously observed low 2,3-BDO production and the limitations caused by the *P. peoriae* NRRL BD-62 growth. CSL was the most efficient organic compound for the 2,3-BDO production, despite its high cost, reaching a total of 20.40 US$/m³, only 37.9% lower than the commercial YE. The medium using CSL was then considered economically unfeasible and discarded.

Overall, inorganic nitrogen sources contributed to more significant savings in culture medium. On average, cost savings were 1.9-fold more significant using these sources than YE brewer, CSL, and urea. Except for $NaNO_3$, 67-68% savings were achieved using NH_4Cl, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$. Besides the cost of 8.02 US$/m³, nitrate contributed to the growth inhibition of *P. peoriae* NRRL BD-62 and was also disregarded in this study. The economy generated by the ammonium salts was enhanced by increasing the initial glucose concentration since higher nitrogen was needed to maintain C/N= 8.5 g/g. Notably, the validation assays using 30 g/L initial glucose and NH_4Cl as a nitrogen source without pH control contributed to the reduced culture medium costs by 75%, reaching a final value of 41.61 US$/m³ versus 171.87 US$/m³ using commercial YE.

Table 5.6: Comparative analysis of the bio-based 2,3-BDO metabolism parameters from different nitrogen sources.

Strain	Risk fact	Fermentation mode	Nitrogen source	pH	$Y_{2,3\text{-BDO/S}}$ (g/g)	Selectivity (%)	Reference
Enterobacter ludwigii	2	Fed-batch	Yeast extract	5-7 (cyclic control)	0.33-0.40	-	AMRAOUI, NARISETTY, *et al.* (2021)
E. cloacae CICC 10011	2	Fed-batch	Corn steep liquor and $(NH_4)_2HPO_4$	7 (initial) - 6 (controlled)	0.39	88% (2,3-BDO) 12% (acetoin)	ZHANG, LI, *et al.* (2016)
E. cloacae CGMCC 6053	2	Batch	Corn steep liquor powder and urea	7 (initial) - 5.8 (controlled)	0.39	93% (2,3-BDO) 7% (acetoin)	DAI, Jian Ying, ZHAO, *et al.* (2015)
Serratia sp.	2	Shake flask	NH4Cl and peptone	Uncontrolled	0.40	93% (2,3-BDO) 7% (acetoin)	HUANG, JIANG, *et al.* (2013)
S. marcescens 30	2	Fed-batch	Yeast extract	6	0.43	~98% (2,3-BDO) ~2% (acetoin)	YUAN, HE, *et al.* (2017)
Klebsiella pneumoniae H3	2	Batch	$(NH_4)_2SO_4$	6	0.38	~88% (2,3-BDO) ~12% (acetoin)	DAI, Jian Ying, GUAN, *et al.* (2020)
K. pneumoniae OU7	2	Fed-batch	Urea	6.5	0.38	~98% (2,3-BDO) ~2% (acetoin)	GUO, OU, et al. (2020)
K. oxytoca KMS005	2	Fed-batch	$(NH_4)_2HPO_4$ and $NH_4H_2PO_4$	6	0.41	-	CHAN, JANTAMA, *et al.* (2016)
K. pneumoniae HR521 LDH	2	Batch	Yeast extract and $(NH_4)_2SO_4$	Uncontrolled	0.47	-	WANG, Xiao Xiong, HU, *et al.* (2016)
Bacillus sp. H-18W	1	Shake flask	Amino acid from soybean meal hydrolysates	Uncontrolled	0.20	48% (2,3-BDO) 52% (acetoin)	ZHONG, WANG, *et al.* (2020)

Bacillus spp. FJ-4	1	Fed-batch	Yeast extract and peptone	6.5	0.44	88% (*levo*) 12% (acetoin)	YAN, FENG, *et al.* (2017)
B. vallismortis B-14891	1	Microtiter plate shaker	Yeast extract, $(NH_4)_2SO_4$ and $(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$	Uncontrolled	0.37	~77% (*levo*) ~23% (acetoin)	KALLBACH, HORN, *et al.* (2017)
Paenibacillus polymyxa DMS 365	1	Batch	Yeast extract and tryptone	6-6.5	0.42	93% (*levo*) 7% (acetoin)	OKONKWO, UJOR, *et al.* (2017)
P. peoriae NRRL BD-62	1	Batch (200 mL)	NH_4Cl + 10 g/L glucose	5	0.45	85% (*levo*) 15% (*meso*) 0% (acetoin)	This study
P. peoriae NRRL BD-62	1	Batch (200 mL)	NH_4Cl + 10 g/L glucose	Uncontrolled	0.38	87% (*levo*) 13% (*meso*) 0% (acetoin)	This study
P. peoriae NRRL BD-62	1	Batch (200 mL)	NH_4Cl + 30 g/L glucose	Uncontrolled	0.40	55% (*levo*) 44% (*meso*) 1% (*acetoin*)	This study

2,3-BDO: 2,3-butanediol; Risk fact: 1=GRAS, 2=non-GRAS; GRAS= Generally Recognized As Safe; $Y_{2,3\text{-BDO/S}}$= 2,3-BDO/substrate yield, Selectivity: relative amount of *levo*-/*meso*-2,3-BDO, and acetoin from the total 4-carbon compounds.

Table 5.7: Costs of alternative nitrogen sources per culture volume and culture medium economy by replacing commercial yeast extract for the 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose at $C/N_{initial}$=8.5 g/g.

Nitrogen source		FOB price per net weight (US$/kg)	Concentration (kg/m³)[a]	Cost/m³ (US$/m³)[b]	Total culture medium cost (US$/m³)[b]	Cost reduction (%)
Type	**Compound**					
Control	Yeast Extract (commercial)	6.00	7.50	45.00	64.96	control
Organic	Yeast Extract (Senai)	NA	NA	NA	NA	NA
	Yeast Extract (brewer)	6.00	7.50	45.00	64.96	0
	Corn steep liquor	0.97	21.00	20.40	40.36	38
	Urea	0.54	1.10	0.59	20.55	68
Inorganic	$NaNO_3$	2.82	2.85	8.02	27.98	57
	NH_4Cl	0.88	1.79	1.58	21.54	67
	$(NH_4)_2SO_4$	0.35	2.20	0.77	20.72	68
	$(NH_4)_2HPO_4$	0.65	2.20	1.43	21.39	67
Amino acid	Arginine	5.65	1.50	8.48	*	*
	Asparagine	12.90	1.50	19.34	*	*

[a]Based on initial 10 g/L glucose to maintain C/N=8.5; [b]culture medium nutrient costs are in Supplementary Material (Tables S1 and S2);
*used as a supplement and not as an alternative nitrogen source to yeast extract;
NA= not available;
C/N ratio= carbon/nitrogen ratio; FOB price= free on board price.

The external pH control removal also contributed to cost reduction of the 2,3-BDO bioprocess since it was not necessary to add 1M NaOH during the batch fermentation. The FOB price for NaOH was 0.48 US$/kg (Table S2 – Supplementary material). Although not shown, the final costs with NaOH had already been reduced by replacing commercial YE with NH_4Cl. As previously discussed, the initial pH in the pure NH_4Cl-based medium was approximately 5, an optimal condition for producing 2,3-BDO by *P. peoriae* NRRL BD-62. Therefore, a smaller NaOH volume was used for pH adjustment, reducing inputs and water costs. According to JI, Xiao-Jun, HUANG, et al. (2009), ammonium salts can cause acidification, reducing the alkali required for pH control.

The total cost of salts in the culture medium was 11.56 US$/m³, while glucose at 10 g/L accounted for 8.40 US$/m³ (Table S3 – Supplementary material). Only the scale variation can change the costs of the salts used here (K_2HPO_4, KH_2PO_4, KCl, $MnSO_4$) since ADLAKHA, YAZDANI (2015) previously optimized their concentrations. Furthermore, increased initial glucose concentration tends to decrease the relative share of salts in the total culture medium costs, conferring an additional economic advantage. Finally, replacing commercial glucose with residual carbon sources has a higher potential to generate even more savings in the 2,3-BDO bioprocess, an exciting subject in future studies.

REBECCHI, PINELLI, *et al.* (2018) reported a 68.3% savings in the total culture medium cost for producing bio-based 2,3-BDO by replacing glucose, beef extract, and soy peptone with molasses and chicken meat and bone meal. A final value of 13 US$/m³ was achieved. PSAKI, MAINA, *et al.* (2019) estimated a feedstock cost for the bio-based 2,3-BDO production from sugarcane molasses at US$17,761 million/year when optimizing the culture medium containing YE, CSL, ammonium salts, and metal traces. This cost corresponded to an approximate value of 80.97 US$/m³, about 1.95-fold higher than that verified in this study. DAS, PRAKASH, *et al.* (2021) found an average cost of 14.26 US$/m³ when using a modified soy hydrolysate medium to produce 2,3-BDO by *B. licheniformis* BL1. A 2.7-fold reduction was achieved by replacing YE and peptone, whose medium cost was 38.49 US$/m³.

4. Conclusion

After a thorough investigation into the synergistic effects of nitrogen source, oxygen supply, and pH on the metabolism and economy of 2,3-BDO by a newly *P. peoriae* NRRL BD-62, NH4Cl was selected to replace YE in uncontrolled pH culture without nutritional supplementation. The good results obtained using the pure NH_4Cl-based medium were attributed to its inorganic nature, which favored the 2,3-BDO production at the cell growth expense at a cost considerably lower than that of complex nitrogen sources. Furthermore, the NADH/NAD^+ balance from the limited oxygen transfer in the fermentation broth favored *levo*-isomer formation without acetoin accumulation. The external pH control removal stimulated the 2,3-BDO synthesis, used as a metabolic strategy to maintain cell homeostasis. The H^+ uptake by *P. peoriae* NRRL BD-62 helped regulate the distribution of metabolites, minimizing the by-product formation and promoting glucose consumption. As a result, the 2,3-BDO yield was equivalent to that reported for non-GRAS microbial producers, which reduced the bioprocess upstream step costs. Maintaining the initial C/N ratio at 8.5 g/g was nutritionally sufficient for producing 2,3-BDO by *P. peoriae* NRRL BD-62 without adding amino acids. Since Arg and Asn had a few statistically significant effects on the 2,3-BDO yield and selectivity, their high costs became relevant to bioprocess economics. Furthermore, the cell activity loss in the uncontrolled pH medium made it impossible to apply Arg and Asn in large-scale operations. Finally, the uncontrolled pH pure NH_4Cl-based medium was validated and showed the q_{O_2} importance for controlling 2,3-BDO metabolism, especially in scale-up processes. This parameter can affect the intracellular redox balance, altering the 2,3-BDO yield and selectivity, production factors related to the bioprocess economics. Therefore, the pure NH_4Cl-based medium was considered a promising substitute for the YE-based medium, capable of regulating the 2,3-BDO metabolism and reducing the culture medium costs, economically and productively enabling large-scale fermentation.

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CHAPTER 6

Contextualization

In this chapter, fed-batch fermentation was investigated as a strategy to improve the 2,3-BDO production by *P. peoriae* NRRL BD-62, keeping its yield equal to that obtained in batch fermentation, around 0.40 g/g. The cultivation conditions optimized in Chapter 4 were used in the pulse, constant, and exponential feeding assays, as well as ammonium chloride, selected in Chapter 5 as an inexpensive nitrogen source alternative to yeast extract. The bioprocess operation modes and the nitrogen source nature were correlated from different carbon/nitrogen ratio (C/N) conditions and specific cell growth rates (μ) to evaluate their effects on high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62.

Therefore, the specific objectives of this chapter were:

- Investigate the feeding strategies: pulse, constant and exponential;
- Establish the relationship between the feed profiles and the nitrogen source nature used in the 2,3-BDO production;
- Validate the results obtained in Chapters 1 and 2 in fed-batch fermentation at continuous feed rates.

Relevance

Through the investigation carried out in this chapter, it was possible to understand:

- The C/N ratio effects on the intermittent feeding and constant feeding for 2,3-BDO production;
- The μ effects on intermittent feed and exponential feeding for 2,3-BDO production;
- The relationship between the nutritional limitation of the culture medium and the bioprocess type in the 2,3-BDO production.

RELATIONSHIP BETWEEN FEEDING STRATEGIES AND DIFFERENT NITROGEN SOURCES IN THE HIGH-YIELD 2,3-BUTANEDIOL PRODUCTION BY *Paenibacillus peoriae* NRRL BD-62 IN MICROAEROBIC FED-BATCH FERMENTATION

Abstract

The production of bio-based 2,3-butanediol (2,3-BDO), a highly versatile platform chemical, can be improved by applying strategies such as fed-batch fermentation and culture medium nutritional control. In this study, the relationship between different feeding profiles and the nitrogen source nature used was investigated for the high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62, a newly GRAS (generally recognized as safe) strain. The highest 2,3-BDO titer of 28.6 g/L was achieved in fed-batch fermentation at constant feeding using yeast extract (YE) at C/N= 8.5 g/g. Under nitrogen limitation at C/N= 18.5 g/g, the 2,3-BDO production was reduced by 44%. No statistical difference was observed for the results obtained in one-pulse feeding assays at C/N= 8.5 and 18.5 g/g. A 2,3-BDO yield of around 0.40 g/g was achieved in both feeding strategies. YE was replaced by ammonium chloride (NH_4Cl) to reduce culture medium costs. Although the results in exponential feeding were better than in one-pulse feeding, the 2,3-BDO titers were below the expected for the three conditions investigated, based on the cell growth specific rate (μ). However, an average yield of 0.38 g/g was achieved at μ= 0.11, 0.14, and 0.17 h^{-1}. Furthermore, a residual glucose accumulation was verified in exponential feeding and was intensified by the nutritional limitation imposed by NH_4Cl. Therefore, nitrogen supply was the main limiting factor in the high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62 and should be controlled mainly in intermittently and continuously fed-batch cultures.

Keywords: Pulse feeding, constant feeding, exponential feeding, nitrogen supply, GRAS strain.

1. Introduction

One of the most popular strategies to improve bioproduct production is conducting fed-batch cultures (KIM, LEE, *et al.*, 2004). Bio-based 2,3-butanediol (2,3-BDO) is a versatile platform chemical used in different industrial applications such as pharmaceuticals and healthcare, cosmetics, polymers, food, agriculture, fuel, and energy (TINÔCO, BORSCHIVER, *et al.*, 2020). Its production has been reported mainly using pulse-fed-batch fermentation (GURAGAIN, VADLANI, 2017, LEE, Sang Jun, CHOI, *et al.*, 2017, PRIYA, LAL, 2019, REHMAN, KHAIRUL ISLAM, *et al.*, 2021, SONG, KWON, *et al.*, 2022, ZHANG, Li, CAO, *et al.*, 2018). The intermittent substrate addition can improve the 2,3-BDO titer without causing cell inhibition due to its excess in the fermentation broth (MA, Kedong, HE, *et al.*, 2018). Despite the benefits, the bio-based 2,3-BDO production in fed-batch fermentation is not limited to pulse-fed, and there are several other profiles with great potential for application, although little investigated so far.

Nutrient and substrate feeding strategies include methods with and without feedback control (LEE, Sang Yup, 1996). In all cases, a pseudo-steady state is established, allowing information about the cultivation, such as byproduct accumulation and cell biomass formation during fermentation (DE MACEDO ROBERT, GARCIA-ORTEGA, *et al.*, 2019). The methods with feedback control are classified as direct as substrate concentration control (JURCHESCU, HAMANN, *et al.*, 2013, NOVAK, KUTSCHA, *et al.*, 2020, OH, LEE, *et al.*, 2018, RIPOLL, RODRÍGUEZ, *et al.*, 2020), and indirect methods such as DO-stat, pH-stat, carbon dioxide evolution rate, and cell concentration control (AMRAOUI, NARISETTY, *et al.*, 2021, AMRAOUI, PRABHU, *et al.*, 2022, LEE, Ye Gi, SEO, 2019, LIU, ZHANG, *et al.*, 2014, PRIYA, DUREJA, *et al.*, 2016). Methods without feedback control include constant, increasing, and exponential feeding profiles (CHEN, JOHN, *et al.*, 2011, CHENG, LIU, *et al.*, 2010, DE OLIVEIRA, SANTOS, *et al.*, 2021, LI, YANG, *et al.*, 2021, POUDEL, TASHIRO, *et al.*, 2016).

Constant and exponential feeding strategies are based on maintaining the cell growth specific rate (μ) at constant and predetermined values throughout the fermentation ranging from zero to approximately the maximum μ achieved in batch cultures (DING, TAN, 2006). The concentration of limiting nutrients can be kept under appropriate levels

by adjusting μ, which contributes to a higher byproduct yield at the cultivation end (POONTAWEE, LIMTONG, 2020). While feeding can be performed at rates equivalent to the substrate-specific consumption (q_s) in fed-batch fermentation at constant feeding (KOPP, KITTLER, *et al.*, 2020), nutrient addition is performed proportionally to cell growth in exponential feeding (MORCELLI, RECH, *et al.*, 2018), considered a simple open-loop control method (CHAN, KANCHANATAWEE, *et al.*, 2018). Through this strategy, microbial physiology and specific production rate (q_p) can be controlled since they are correlated according to the Luedeking-Piret model (DE MACEDO ROBERT, GARCIA-ORTEGA, *et al.*, 2019). As a result, higher interest bioproduct yields can be verified.

Nutritional control is another strategy to improve bio-based 2,3-BDO production (WONG, Chiao-Ling, HUANG, *et al.*, 2012). Since 2,3-BDO is a metabolite semi-associated with cell growth, the carbon and nitrogen supplied to the cells at adequate levels are crucial for the bioprocess success (TINÔCO, DE CASTRO, *et al.*, 2021). Both nutrients are used in microbial endogenous plasticity and maintenance, resulting in cell biomass formation, which is used in the 2,3-BDO synthesis (THAPA, LEE, *et al.*, 2019). In this sense, applying feeding strategies that extend the fermentation time and, consequently, the cell growth exponential phase can significantly improve the 2,3-BDO titer and yield (WONG, Chiao Ling, YEN, *et al.*, 2014).

Therefore, this study aimed to investigate the relationship between pulse, constant and exponential feeding strategies, and the nitrogen source nature in the high-yield 2,3-BDO production by a newly wild-type *P. peoriae* strain. Nitrogen supply was the main limiting factor for the 2,3-BDO production in fed-batch fermentation. The highest 2,3-BDO titer and a yield of about 0.40 g/g were obtained in the YE-based medium at initial C/N= 8.5 g/g, with glucose feeding at a constant rate. On the other hand, the 2,3-BDO titer was reduced, while its yield was equal to 0.38 g/g on average from the replacement of YE by NH4Cl in fed-batch cultures at exponential glucose feeding. To the best of our knowledge, this is the first time that the 2,3-BDO production by a wild-type *P. peoriae* has been carried out in fed-batch fermentation at constant and exponential feeding.

2. Materials and methods

2.1 Microorganism maintenance and inoculum preparation

Paenibacillus peoriae NRRL BD-62 was maintained at -80 °C in Tryptic Soy Broth – TSB medium (17 g/L tryptone, 3 g/L soytone, 5 g/L NaCl, 2.5 g/L K_2HPO_4, and 2.5 g/L glucose) supplemented with 25% (v/v) glycerol. It was obtained from the culture collection of the Microbial Genetics Laboratory of the Microbiology Institute of the Federal University of Rio de Janeiro (Brazil). The cells were reactivated in 250 mL shake flasks containing 50 mL of TSB medium at 32 °C, 200 rpm, for 24 h by transferring 0.2 mL glycerol stock solution. After reactivation, approximately 1% (v/v) cell biomass was transferred to 1 L shaken flasks containing 350 mL seed medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) and cultured at 32 °C, 200 rpm, for 16 h. The inoculum was used in the fermentation assays at 10% (v/v). All media were sterilized by autoclaving at 121°C for 15 min.

2.2 Fed-batch fermentation

The fermentation medium was composed of (g/L): 0.5 KH_2PO_4, 2.0 K_2HPO_4, 0.0225 $MnSO_4$, and 0.3 KCl (ADLAKHA, YAZDANI, 2015). Glucose was used as a substrate, while yeast extract (YE) and ammonium chloride (NH_4Cl) were used as nitrogen sources at different initial concentrations according to the culture condition investigated using intermittent, constant, and exponential feeding strategies. The assays were carried out in DASbox® Mini Bioreactor – 300 mL (Eppendorf, Germany), designed as a 4-fold system with 16 parallel bioreactors equipped with temperature, pH, and dissolved oxygen (DO) sensors. The temperature was controlled by a liquid-free heating and cooling system, while peristaltic pumps were used for pH control by transferring 2 M NaOH or 1 M H_2SO_4 to the culture medium. The agitation and aeration were controlled by two Rushton-type impellers 3 cm apart and submerged gas supply via an L-sparger (4 mm compression fitting), through which air was injected into a sterile filter (0.22 μm), respectively. Glucose and the other nutrients were separately sterilized by autoclaving at 121 °C for 15 min, and antifoam 204 (Sigma-Aldrich, USA) was used when needed. Fed-batch cultures were performed in biological duplicates.

2.2.1 Intermittent feeding

Pulse-fed-batch fermentations were investigated using a medium containing YE and NH_4Cl as nitrogen sources. Two C/N ratio conditions were evaluated considering an initial glucose concentration of 50 g/L in a YE-based medium: 8.5 g/g (37.5 g/L YE) and 18.5 g/g (12.5 g/L YE). Glucose solution of about 500 g/L was pulse-fed after 96 h to maintain its concentration of around 50 g/L. The NH4Cl-based medium was maintained at the C/N ratio of 8.5 g/g achieved from 10 g/L glucose and 1.79 g/L NH_4Cl. Glucose solution of about 60 g/L was pulse fed after 6 h to maintain its concentration of around 10 g/L. The assays were conducted at 32°C and pH=5. The oxygen supply was based on the volumetric oxygen transfer coefficient (kLa), maintained around 7.5 and 5 h^{-1} for the media containing YE and NH_4Cl, respectively. The agitation and aeration rates were adjusted to 400 rpm and 0.1 vvm with an initial volume of 200 mL for the YE-based medium and 200 rpm and 0.1 vvm with an initial volume of 100 mL for the NH_4Cl-based medium.

2.2.2 Constant feeding

Similar C/N ratio conditions investigated using the YE-based medium were evaluated in fed-batch fermentation at constant feeding. Glucose solution of about 40 g/L was fed at 1.3 mL/h in 96-170 h (C/N= 8.5 g/g) and 120-170 h (C/N= 18.5 g/g). The assays were carried out at 32 °C, pH=5, 400 rpm, and 0.1 vvm (kLa~7.5 h^{-1}), with an initial volume of 200 mL.

2.2.3 Exponential feeding

Three cell growth conditions were evaluated in fed-batch fermentation at exponential feeding based on the cell growth specific rate (μ) of 0.24 h^{-1} of batch cultures. The investigated conditions were 0.11, 0.14, and 0.17 h^{-1}. The fermentation medium contained NH_4Cl as a nitrogen source at an initial C/N ratio of 8.5 g/g achieved from 10 g/L glucose and 1.79 g/L NH_4Cl. The assays were carried out at 32°C, without pH control, 200 rpm, and 0.1 vvm (kLa~5 h^{-1}), with an initial volume of 100 mL. Glucose solution of about 60 g/L was fed according to Equation 1 obtained from the substrate mass balance (NOR, TAMER, *et al.*, 2001), where the cell yield ($Y_{X/S}$) and μ were assumed constant

throughout the fermentation (ZHANG, Liaoyuan, YANG, *et al.*, 2010). The carbon-source limitation application to achieve a constant μ creates the pseudo-steady-state conditions that characterize the well-known substrate-limited fed-batch cultivation. Thus, the instantaneous feed rate (F) of a growth-limiting substrate can also be given by Equation 2.

$$F = \frac{\mu}{Y_{x/s}(Sf-S)} V_o X_o \exp(\mu t) \qquad (1)$$

$$F = F_o \exp(\mu t) \qquad (2)$$

Where S_f is the substrate concentration in the feeding solution (g/L), S is the residual substrate concentration in the culture medium (g/L), V_0 is the initial culture volume (L), X_0 is the initial cell concentration (g/L), t is the feeding time (h), and F_0 is the initial feed rate (L/h). This study assumed S was equal to zero (MORCELLI, RECH, *et al.*, 2018).

2.3 Analytical methods

Culture samples were taken throughout the fed-batch fermentations and analyzed by high-performance liquid chromatography (HPLC; Agilent, USA) to quantify glucose and fermentation products (*levo*- and *meso*-2,3-BDO, acetoin, ethanol, and lactic and acetic acids). HPLC system was equipped with an HPX-87H Aminex column (300 × 7.8 mm, Bio-Rad, USA) and a Refractive Index Detector (RID; Agilent, USA). Sulfuric acid of 5 mM was used as an eluent at 0.6 mL/min, and the column temperature was controlled at 45 °C. Culture samples were centrifuged at 10,000 x *g* at 25 °C for 10 min in a microtube centrifuge (MiniSpin®, Eppendorf, Germany), and the supernatant was diluted 10-fold with Milli-Q water and filtered through cellulose acetate membranes (0.22 μm pore size; Minisart® NML Syringe Filters, Sartorius, Germany). For all HPLC analyses, HPLC grade standards of glucose, 2,3-BDO, acetoin, ethanol, and lactic and acetic acids were used (>99.5% purity). The biomass concentration was determined by dry cell weight (DCW) using a gravimetric method. The centrifuged cells were filtered through a 0.22 μm cellulose acetate membrane (Sartorius, Germany) and dried in an oven at 60 °C until constant weight. Optical density measurements at 600 nm ($OD_{600\ nm}$) were performed in a UV–visible spectrophotometer (Biospectro SP-22, Brazil) and correlated to DCW in the fermentation media as 1 $OD_{600\ nm}$ = 0.392 ± 0.016 g/L dry cell weight. Fermentation

parameters were calculated and analyzed by the t-test, with a 95% confidence level (α=0.05).

2.4 Fermentation parameters

The 2,3-BDO production by *P. peoriae* NRRL BD-62 was evaluated by the $Y_{P/S}$ (g/g) and $Y_{P/X}$ (g/g) yields, defined as the ratio between 2,3-BDO production and glucose consumption, and 2,3-BDO production and cell biomass, respectively. The 2,3-BDO selectivity was calculated based on the molar ratio between *levo*-2,3-BDO, *meso*-2,3-BDO, and acetoin (C4-compounds). The 2,3-BDO specific production rate (q_p) was defined by Equation 3 and rewritten according to Equation 4 from the Luedeking-Piret model simplification to product maintenance negligible (DE MACEDO ROBERT, GARCIA-ORTEGA, *et al.*, 2019). Furthermore, the *P. peoriae* NRRL BD-62 growth was represented by μ (h^{-1}), calculated as the tangent line slope to the cell curve in the microbial growth exponential phase.

$$qp=\frac{1}{X}\frac{dP}{dt} \quad (3)$$

$$q_P=\mu Y_{P/X} \quad (4)$$

Where X is the cell dry weight (g/L), dP is the 2,3-BDO titer variation, and dt is the fermentation time variation. The unit of q_p is $g_{2,3\text{-}BDO}/g_{DCW}/h$.

3. Results and discussion

3.1 Fed-batch fermentation using YE as a nitrogen source

In glucose and YE-based medium, one-pulse and constant feeding profiles were investigated for the high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62. Each fed-batch fermentation was evaluated at a C/N ratio of 8.5 and 18.5 g/g for 219 h. The time courses of 2,3-BDO production, glucose consumption, and cell biomass formation for each condition evaluated are presented in Figures 6.1 and 6.2.

3.1.1 Effects of C/N ratio on one-pulse feeding

Intermittent feeding was performed by a single pulse in 96 h, instant when the residual glucose concentration was in the range of 11-16 g/L and the cells were at the end of the exponential growth phase (Fig. 6.1). Substrate feeding at the exponential phase peak may compromise cell metabolism, diverting carbon flux to lactic acid synthesis due to lactate dehydrogenase activation under high $NADH/NAD^+$ ratio (JANTAMA, POLYIAM, *et al.*, 2015). Therefore, the glucose addition at the cell growth deceleration and stationary phases was adopted in all assays to ensure preferential 2,3-BDO synthesis.

Both C/N ratio conditions showed similar metabolic profiles (Fig. 6.1). Maximum 2,3-BDO production was obtained in 170 h, reaching approximately 21.2 and 18.4 g/L for a C/N ratio of 8.5 and 18.5 g/g, respectively. Compared to the initial batch fermentations, the 2,3-BDO titer was improved by about 74% (C/N=8.5 g/g) and 53.5% (C/N=18.5 g/g). Glucose consumption was 50.1 g/L on average, contributing to the cell biomass formation of about 3.42 g/L. After 170 h, the 2,3-BDO production decreased, possibly due to the culture medium nutritional limitation, while the cell biomass was maintained in the stationary phase until the fermentation end. ZHANG et al., 2016 observed a gradual slowed down 2,3-BDO + acetoin yield in the final fermentation steps at the expense of cell formation in xylose fed-batch cultures.

More than 20 g/L residual glucose was detected in 219 h, suggesting that the one-pulse fed-batch fermentation was inadequate for a complete and rapid sugar metabolism by *P. peoriae* NRRL BD-62. It was attributed to low cellular activity, mainly in late fermentation, regardless of the C/N ratio. The glucose concentration of 50 g/L may also have contributed to its incomplete consumption by *P. peoriae* NRRL BD-62 due to the difficulty in metabolizing the substrate under nutritional limitations (NARISETTY, NARISETTY, *et al.*, 2022).

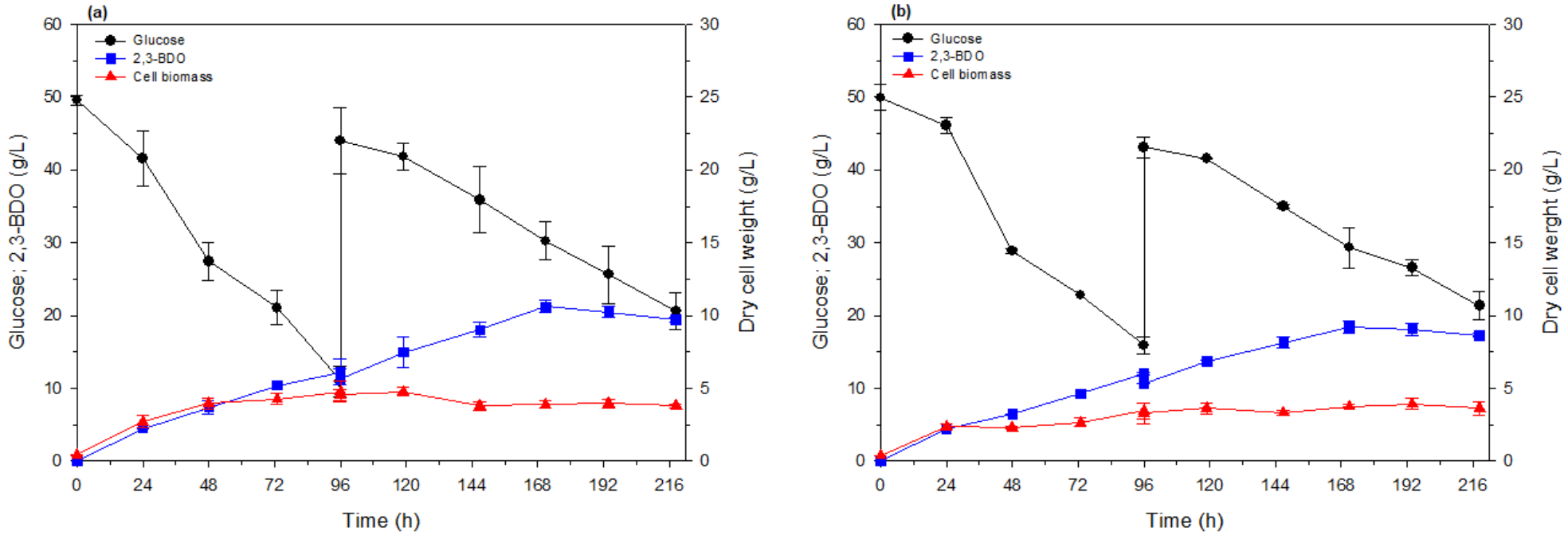

Figure 6.1: Time course of one-pulse fed-batch fermentation for 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and yeast extract at C/N equal to (a) 8.5 g/g; (b) 18.5 g/g.

3.1.2 Effects of C/N ratio on constant feeding

Constant feeding of 0.08 g/h glucose was started at 96 h for C/N=8.5 g/g and 120 h for C/N=18.5 g/g assays (Fig. 6.2). This rate was lower than the sugar consumption rate obtained in batch fermentation to ensure complete sugar metabolization (KHUNNONKWAO, JANTAMA, *et al.*, 2021). A 24 h delay for the feeding start between the investigated conditions was performed to increase glucose consumption at C/N=18.5 g/g, whose residual concentration was approximately 26.5 g/L in 96 h, while no glucose residual was detected at C/N=8.5 g/g in the same period. Constant feeding was performed up to 170 h, resulting in 2,3-BDO production, glucose consumption, and cell biomass formation of approximately 28.6 and 16 g/L, 69.7 and 42.1 g/L, and 4.5 and 3.5 g/L at C/N= 8.5 and 18.5 g/g, respectively.

Constant feeding contributed to a 2,3-BDO increase of more than 41% compared to the respective productions in batch fermentations for both investigated C/N conditions. However, a residual glucose accumulation of approximately 20 g/L was observed at C/N=18.5 g/g, which led to the batch fermentation strategy application to increase the 2,3-BDO production (YANG, RAO, *et al.*, 2011). The assays were then conducted for a further 48 h (Fig. 6.2b). Although no significant residual glucose accumulation was detected, the assays at C/N=8.5 g/g were carried out by batch fermentation from 170 h to 219 h (Fig. 6.2a).

The 2,3-BDO titer decreased after 170 h at C/N=8.5 g/g, possibly due to the glucose absence in the culture medium since *P. peoriae* NRRL BD-62 was able to metabolize this sugar completely. As a result, 2,3-BDO itself was used as a carbon and energy source for endogenous cell maintenance, although a drop in microbial growth was observed in 170-219 h (Fig. 6.2a). In contrast, an improved 2,3-BDO titer was verified at C/N=18.5 g/g, reaching about 17.5 g/L in 219 h, which represented an increase of almost 10% compared to the result obtained at the constant feeding end. The additional 2,3-BDO production was attributed to the residual glucose consumption, which was reduced to 11 g/L at the bioprocess end. The cells were maintained in the microbial growth stationary phase, and no reduction in cell biomass was observed in the period (Fig. 6.2b).

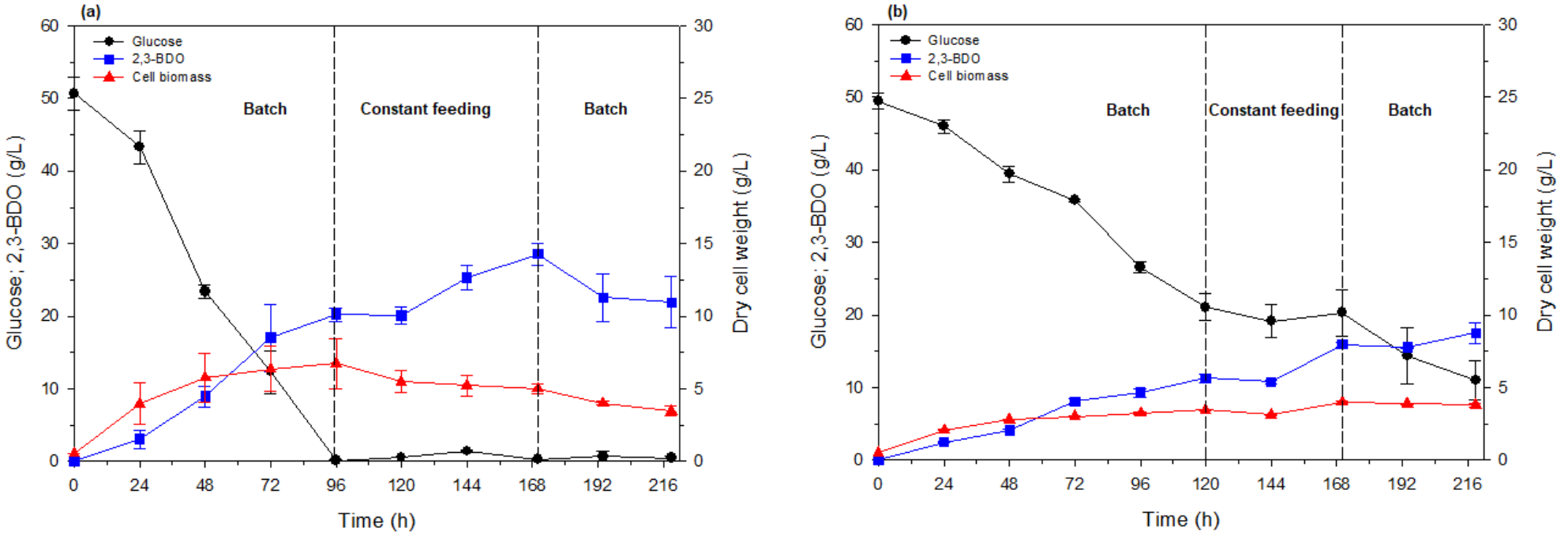

Figure 6.2: Time course of constant fed-batch fermentation for 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and yeast extract at C/N equal to (a) 8.5 g/g; (b) 18.5 g/g.

Therefore, batch fermentation was considered an efficient strategy to overcome the unmetabolized glucose accumulation under nutritional limitations. However, under adequate C/N ratio conditions, fed-batch fermentation at constant feeding was sufficient to ensure total glucose consumption by *P. peoriae* NRRL BD-62, not requiring the application of complementary production strategies.

As well as in this study, CHAN, KANCHANATAWEE, *et al.* (2018) applied batch fermentation after constant feeding to reduce residual sugars in the culture medium. The authors shortened the feeding time, which started at the cell growth phase end, and prolonged the fermentation in batch cultures to ensure total glucose consumption by *K. oxytoca* KMS005. Glucose accumulation decreased from 20 g/L to 4.9 g/L and from 50 g/L to 32.4 g/L in fed-batch fermentation with a feed rate of 2 g/h and initial sugar concentration of 90 and 120 g/L, respectively. Furthermore, the assays were carried out under microaerobic conditions (kLa~25.2 h^{-1}), further improving the final 2,3-BDO titer and yield. ZHANG, Liaoyuan, YANG, *et al.* (2010) terminated a sucrose fed-batch fermentation with respiratory quotient (RQ) control after 26 h of constant feeding and performed the batch fermentation culture for another 7 h to deplete the residual substrate in the culture medium. Sucrose was entirely consumed by *Serratia marcescens* H30, which improved the 2,3-BDO+acetoin titer and yield.

3.1.3 One-pulse and constant feeding comparison

Table 6.1 presents the fermentation parameters $Y_{P/S}$ and $Y_{P/X}$ and the selectivity of 4C-compounds (acetoin and 2,3-BDO isomers) for the investigated conditions. Furthermore, a comparative t-test was performed to determine the most efficient strategy for the 2,3-BDO production by *P. peoriae* NRRL BD-62.

A high YP/S of around 0.40 g/g was achieved in all conditions evaluated. It suggested that glucose feeding by one-pulse and constant profiles could not affect the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 from the YE-based medium since this result was in line with those obtained in previous studies by the group for batch assays (Chapter 4). Furthermore, the $Y_{P/X}$ indicated an adequate biochemical balance between the respiration and fermentation processes, a necessary condition for an efficient 2,3-BDO synthesis (Table 6.1).

Table 6.1: Fed-batch fermentation parameters for 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and yeast extract-based medium in 170 h.

Parameters	One-pulse feeding*		Constant feeding*	
	C/N = 8.5 (g/g)	C/N = 18.5 (g/g)	C/N = 8.5 (g/g)	C/N = 18.5 (g/g)
2,3-BDO (g/L)	21.23 ± 0.88^{aA}	18.43 ± 0.88^{aC}	28.56 ± 1.52^{cB}	15.95 ± 0.34^{dC}
DCW (g/L)	3.45 ± 0.27^{aA}	3.39 ± 0.15^{aC}	4.48 ± 0.32^{cA}	3.51 ± 0.08^{cC}
$Y_{P/S}$ (g/g)	0.41 ± 0.05^{aA}	0.39 ± 0.03^{aC}	0.41 ± 0.01^{cA}	0.38 ± 0.01^{cC}
$Y_{P/X}$ (g/g)	6.16 ± 0.23^{aA}	5.45 ± 0.50^{aC}	6.38 ± 0.12^{cA}	4.54 ± 0.00^{dC}
GC (g/L)	52.39 ± 4.73	47.81 ± 1.32	69.67 ± 2.32	42.14 ± 2.13
Selectivity (*levo:meso*: acetoin)	1:1.10:0	1:1.39:0	1:1.16:0	1:1.35:0

*Glucose feeding started at 96 h inoculation time, except for constant feeding at C/N= 18.5 g/g which was 120 h.

C/N ratio= carbon/nitrogen ratio; 2,3-BDO= 2,3-BDO titer (*meso*- + *levo*-isomer titers); DCW= dry cell weight (cell biomass); $Y_{P/S}$= 2,3-BDO/glucose yield; $Y_{P/X}$= 2,3-BDO/dry cell weight yield; GC= glucose consumption.

The means followed by the same letter do not show significant differences by t-test ($\alpha = 0.05$); The lowercase letters correspond to the analysis of C/N ratios for the same feeding profile, while the capital letters correspond to the analysis of the same C/N ratio for different feeding profiles.

GUO, WANG, *et al.* (2017) also reported a 2,3-BDO yield of around 0.40 g/g when pulse feeding a batch fermentation with 26 g/L whey cheese powder. Unlike this study, feeding was performed at three different fermentation times due to the rapid lactose metabolization after the first two pulses. WANG, OH, *et al.* (2021) obtained a 2,3-BDO yield of 0.42 g/g when using untreated molasses in pulse fed-batch cultures. The final 2,3-BDO production by *Bacillus subtilis* CS13 was formed by 63% and 37% of *meso*- and *levo*-isomer, respectively. AMRAOUI, PRABHU, *et al.* (2022) investigated the 2,3-BDO production by a mutant *Enterobacter ludwigii* from concentrated Brewers' spent grain hydrolysate in intermittent fed-batch cultivation with forced pH fluctuations at 2 vvm and obtained a 2,3-BDO yield of 0.43 g/g. The residual glucose concentration was maintained at around 20 g/L throughout the fermentation. NIE, JI, *et al.* (2011) performed a pH-stat fed-batch culture with glucose feedback to maintain the residual sugar at a low concentration throughout the fermentation. Glucose was fed at a constant rate, which varied according to its consumption by the *K. oxytoca* ME-UD-3 mutant. A 2,3-BDO yield of 0.43 g/g was achieved in 72 h.

Statistically, the two conditions using one-pulse feeding showed similar $Y_{P/S}$ and $Y_{P/X}$ yields, equal to 0.41 and 6.16 g/g at C/N= 8.5 g/g, and 0.39 and 5.45 g/g at C/N =

18.5 g/g in 170 h, respectively. The 2,3-BDO selectivity showed a slight increase in the *meso*-isomer amount, from about 52.4% at C/N= 8.5 g/g to 58.1% at C/N= 18.5 g/g. Acetoin was not detected (Table 6.1). Despite the decrease in *levo*-2,3-BDO optical purity, the acetoin absence suggested that the 2,3-BDO production pathway was completed, and there was no yield loss by acetoin non-conversion. Therefore, the C/N ratio did not significantly influence the 2,3-BDO production by *P. peoriae* NRRL BD-62 in one-pulse fed-batch fermentation.

In contrast, only $Y_{P/S}$ was found to be statistically similar for the conditions using constant feeding, whose values were equal to 0.41 and 0.38 g/g at C/N= 8.5 and 18.5 g/g in 170 h, respectively. The $Y_{P/X}$ was about 1.4-fold higher at C/N= 8.5 g/g, equal to 6.38 g/g against 4.54 g/g at C/N= 18.5 g/g. This result was attributed to the higher 2,3-BDO production observed from a 1.6-fold higher glucose consumption at C/N= 8.5 g/g. As observed in one-pulse fed-batch fermentation, the 2,3-BDO selectivity was given only by the mixture of *levo*- and *meso*-isomers, and no net acetoin production was detected. The *meso*-2,3-BDO amount increased with increasing C/N ratio, from approximately 53.6% at C/N= 8.5 g/g to almost 57.5% at C/N= 18.5 g/g (Table 6.1). Therefore, in fed-batch fermentation at constant feeding, nutritional limitation affected the 2,3-BDO production and isomeric purity by *P. peoriae* NRRL BD-62 since it compromised glucose consumption throughout cultivation.

Fermentation parameters were also statistically compared for the feeding profile, maintaining the C/N ratio equal (Table 6.1). The $Y_{P/S}$ using one-pulse feeding was equal to that achieved using constant feeding at C/N= 8.5 g/g, although glucose consumption was compromised in the intermittent feeding assays, reaching only 52.4 g/L, which led to an approximately 1.3-fold lower 2.3-BDO production. While *P. peoriae* NRRL BD-62 metabolized approximately 99.8% of the constantly added glucose, only 63.4% was consumed in one-pulse fed-batch fermentation. The $Y_{P/X}$ and DCW showed no statistically significant differences between the feeding profiles at C/N= 8.5 g/g and the 2,3-BDO selectivity, which practically did not change, remaining with a *levo: meso* ratio on average equal to 1:1.13.

In contrast, all the fermentation parameters analyzed were statistically similar for the assays at C/N= 18.5 g/g, regardless of the feeding profile used (Table 6.1). These

results suggested a non-influence of the fed-batch fermentation type on 2,3-BDO production by *P. peoriae* NRRL BD-62 under nutritional limitation. This observation was supported by the similarity in glucose consumption and selectivity results, which showed values in the 62-67.5% range and an average ratio of 1:1.37, respectively.

The comparison between the feeding profiles and the C/N ratio effects was extended to the byproducts of 2,3-BDO metabolism. The comparative analysis was based on the statistical deviations of ethanol, lactic acid, and acetic acid production, as presented in Figure 3. In general, the byproduct production was almost the same for the four fed-batch fermentation configurations, except for ethanol, which was not detected in the one-pulse feeding assays. An ethanol concentration lower than 0.5 g/L was verified in fed-batch assays at constant feeding in both C/N ratios. The lowest titers of lactic acid and acetic acid were verified in fed-batch assays at constant feeding at C/N= 18.5 g/g, considering statistical deviations negligible. The most significant experimental variations were observed at C/N= 8.5 g/g.

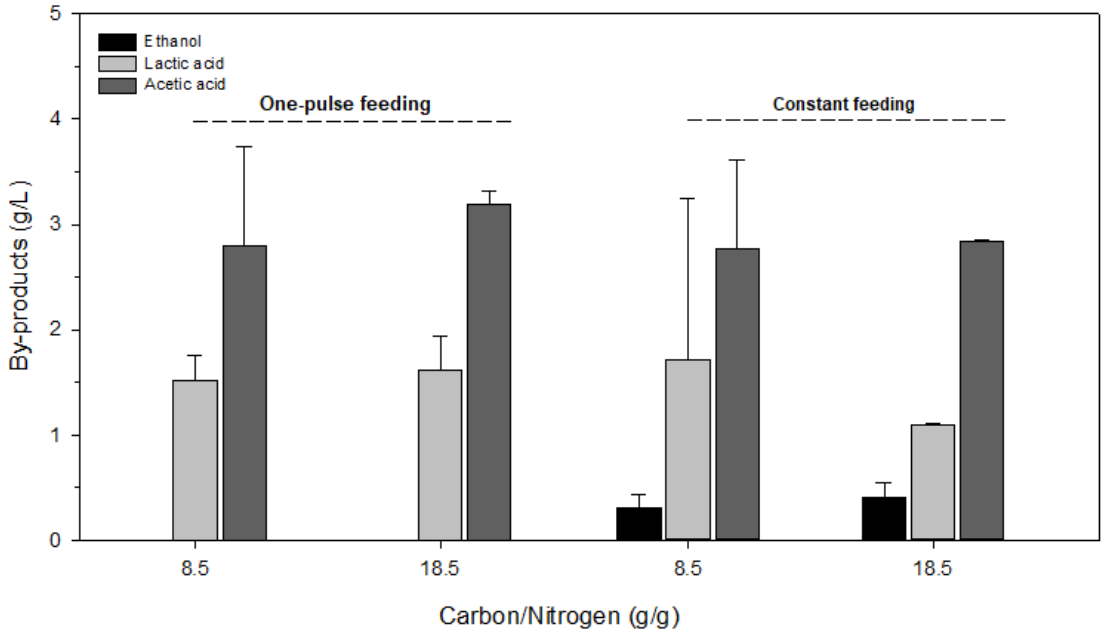

Figure 6.3: By-products formation of 2,3-BDO by *P. peoriae* NRRL BD-62 from glucose and yeast extract in one-pulse and constant fed-batch at $C/N_{initial=}$ 8.5 and 18.5 g/g.

The results found that the 2,3-BDO production and the glucose consumption by *P. peoriae* NRRL BD-62 in the YE-based medium were favored in fed-batch fermentation

at constant feeding at C/N= 8.5 g/g. The nitrogen supplied to the cell culture was considered nutritionally adequate. The continuous addition of small glucose amounts instead of a single-step feeding at higher concentrations contributed to more efficient carbon assimilation, whose flux was preferentially directed to 2,3-BDO synthesis. Consequently, a high 2,3-BDO yield of 0.41 g/g was achieved without residual glucose accumulation, which confirmed the metabolic activity maintenance of *P. peoriae* NRRL BD-62 in fed-batch fermentation at constant feeding. Therefore, the constant feeding profile was considered a promising strategy for bio-based 2,3-BDO production, presenting a potential large-scale application.

KHUNNONKWAO, JANTAMA, *et al.* (2021) found improved 2,3-BDO titer and yield when conducting a fed-batch fermentation under a constant feeding rate of 3.5 g/h starch hydrolysate. Despite the cellular metabolic activity loss at the feeding end, a yield of 0.49 g/g was achieved by engineered *K. oxytoca* KMS006, with a residual sugar accumulation of only 1.1 g/L. Furthermore, a culture medium with fewer trace metals and inorganic ammonium salts was used.

Fed-batch fermentation at constant feeding variations was also investigated. ZHANG, Liaoyuan, YANG, *et al.* (2010) evaluated the effects of three feeding profiles on 2,3-BDO production from sucrose: pulse, exponential, and constant residual substrate concentration. The authors obtained the highest 2,3-BDO + acetoin yield of almost 95% by keeping the residual sucrose concentration constant at 15-25 g/L and the respiratory coefficient (RQ) controlled throughout the fermentation. The 2,3-BDO:acetoin ratio was equal to 21.2:1, considered the highest 2,3-BDO selectivity achieved among the feeding profiles evaluated. MA, Cuiqing, WANG, *et al.* (2009) concluded that the constant glucose concentration fed-batch process could provide a more suitable environment for the 2,3-BDO synthesis than pulse, exponential, or constant rate fed-batch strategies since a high yield of 95% and a 2,3-BDO:acetoin ratio of 15:1 were achieved by maintaining glucose at 20-30 g/L in the culture medium. BAO, ZHANG, *et al.* (2015) extended the acetoin production time by *B. subtilis* 168/pMA5-zwf by constantly feeding glucose to maintain it around 10 g/L. Although the cell biomass amount was reduced in the feeding phase, acetoin production was increased, reaching a yield of 0.32 g/g in 120 h.

3.2 Fed-batch fermentation using NH_4Cl as a nitrogen source

To decrease nitrogen source costs for the high yield 2,3-BDO production by *P. peoriae* NRRL BD-62, YE was replaced by NH_4Cl in a cheap salt medium. CHAN, KANCHANATAWEE, *et al.* (2018) also employed an expensive complex nutrient-free medium composed of simple mineral salts such as ammonium salts and trace metals. Besides lowering culture medium costs, simple nutrients could reduce the obstacles to 2,3-BDO recovery.

Two feeding strategies were investigated: one-pulse and exponential feeding. In fed-batch fermentation at exponential feeding, the μ effects on 2,3-BDO production by *P. peoriae* NRRL BD-62 were investigated. Figures 6.4 and 6.5 present the time courses of 2,3-BDO production, glucose consumption, and cell biomass formation for the evaluated conditions.

3.2.1 One-pulse feeding

One-pulse feeding was performed after 8 h when the residual glucose concentration was equal to half the initial concentration used in batch culture, and the cells were in the final exponential growth phase (Fig. 6.4). With the glucose addition, cell growth was extended, leading to a net microbial biomass amount equal to approximately 1.1 g/L in 48 h. In the same period, a 2,3-BDO production of about 6.1 g/L was observed.

Despite the change in the C/N ratio generated by pulse feeding, which decreased the relative nitrogen amount in the culture medium, about 18.8 g/L of glucose was consumed entirely by *P. peoriae* NRRL BD-62 in 48 h. However, NH_4Cl appears to have compromised the 2,3-BDO formation rate compared to YE-based fermentation at initial C/N= 8.5 g/g after pulse feeding (Fig. 6.1a). A reduction of almost 63% was seen using NH_4Cl, which went from 0.21 $g_{2,3\text{-}BDO}$/L/h in initial batch fermentation to 0.08 $g_{2,3\text{-}BDO}$/L/h in fed-batch fermentation. YE contributed to the maintenance of 0.13 $g_{2,3\text{-}BDO}$/L/h throughout the fermentation.

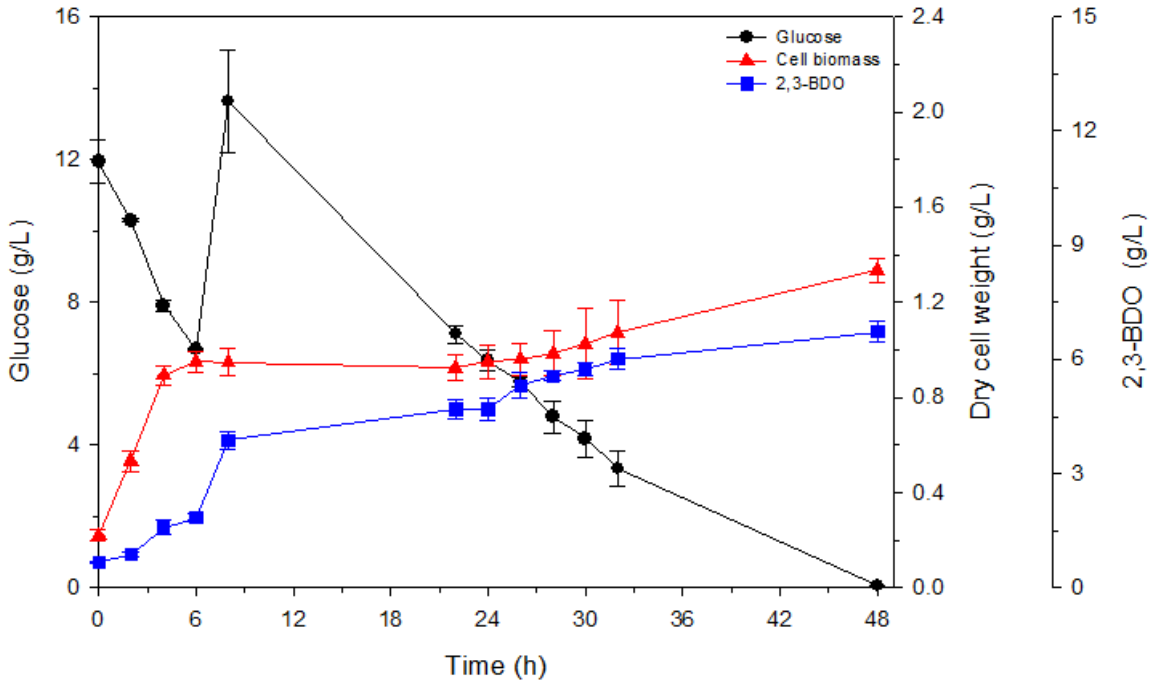

Figure 6.4: Time course of one-pulse fed-batch fermentation for 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and NH_4Cl at $C/N_{initial}$=8.5 g/g.

3.2.2 Effects of μ on exponential feeding

The exponential feeding was applied to overcome the 2,3-BDO production limitation in one-pulse fed-batch fermentation using NH_4Cl. The investigated rates were 0.11, 0.14, and 0.17 h^{-1}, about 2.2-fold, 1.7-fold, and 1.4-fold lower than 0.24 h^{-1} (batch-phase), respectively, to avoid glucose accumulation and cell inhibition by its excess (DE MACEDO ROBERT, GARCIA-ORTEGA, *et al.*, 2019).

Exponential feeding was started after 6 h. At that time, the residual glucose concentration was equal to approximately half the initial concentration used in batch cultures, and the cells were growing at an average μ of 0.24 h^{-1} (Fig. 6.5). The feed rates, together with their characteristic equations for investigated conditions are presented in Table S2 (Supplementary material). A 2,3-BDO production of 10.65 g/L from 21.8 g/L glucose was obtained at μ_{set}= 0.17 h^{-1} in 24 h (Fig. 6.5c). It was approximately 1.9-fold and 1.5-fold higher than the verified from 10.8 g/L glucose at μ_{set}= 0.11 h^{-1} (Fig. 6.5a) and 15.6 g/L glucose at μ_{set}= 0.14 h^{-1} (Fig. 6.5b) in the same period, respectively. In turn, cell biomass formation was reduced in fed-batch fermentation at exponential feeding at

μ_{set} of 0.11 h^{-1} (Fig. 6.5a) and 0.14 h^{-1} (Fig. 6.5b), equal on average to 0.67 g/L. In contrast, the cell biomass increased at μ_{set}= 0.17 h^{-1}, reaching almost 1.4 g/L in 24 h (Fig. 6.5c).

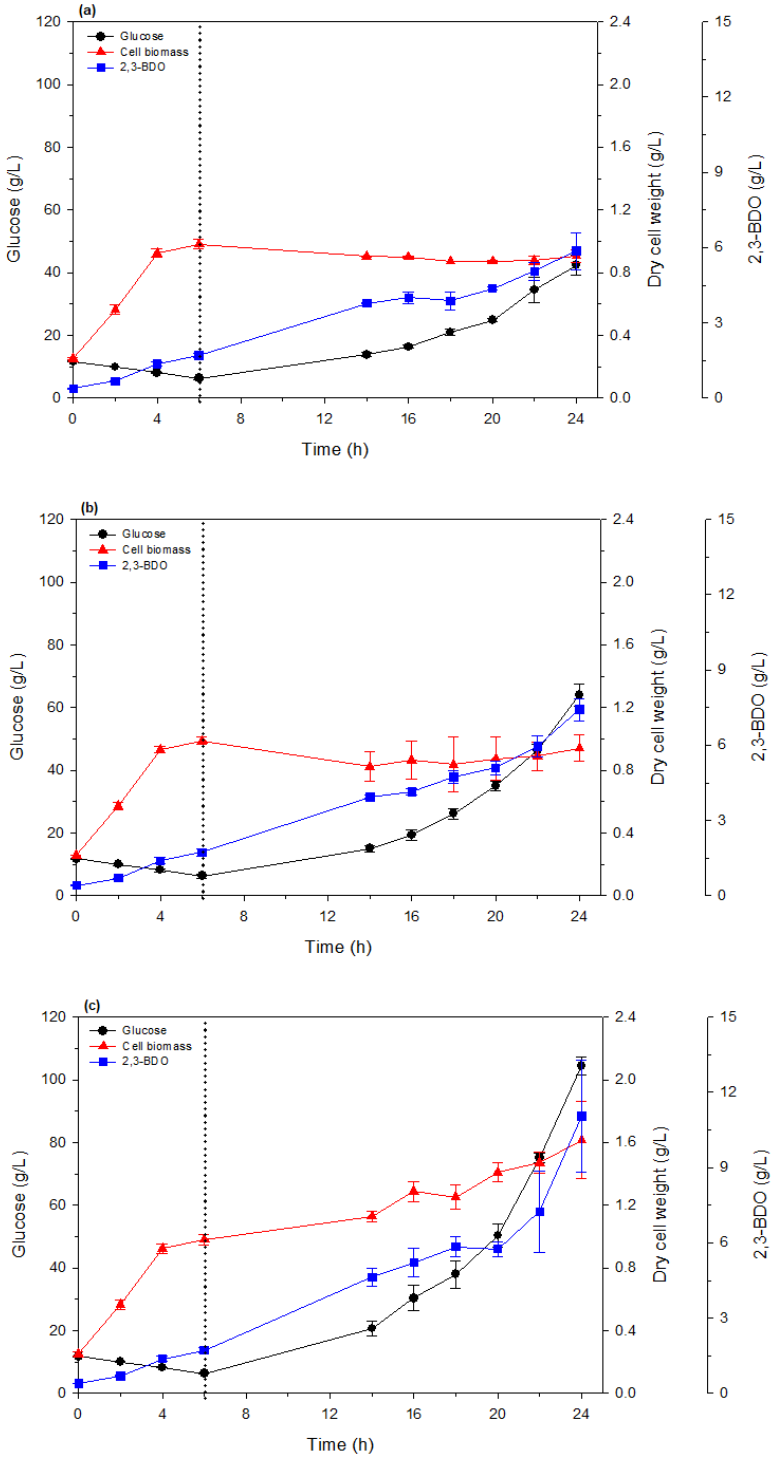

Figure 6.5: Time course of exponential fed-batch fermentation for 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and NH_4Cl at $C/N_{initial}$=8.5 g/g. Exponential flow was based on: (a) μ= 0.11 h^{-1}; (b) μ= 0.14 h^{-1}; (c) μ= 0.17 h^{-1}.

Although μ was preprogrammed to remain constant and equal to 0.11, 0.14, and 0.17 h^{-1} throughout exponential feeding, the actual cell growth was much lower, reaching rates around 0.01, 0.03, and 0.04 h^{-1}, respectively. As a result, the $Y_{X/S}$ was affected, staying below 0.134 g/g, the value adopted in the calculation of exponential feed rates (Table S1 – Supplementary material). The actual cell yields were 0.0005, 0.012, and 0.030 g/g, respectively. These changes made the 2,3-BDO titer on average equal to 62.3% of the expected for each μ evaluated. Table 6.2 summarizes the actual and expected values of μ, $Y_{X/S}$, and 2,3-BDO titer for the conditions investigated.

Table 6.2: Preprogrammed and real values of specific cell growth rate, cell yield, and 2,3-BDO production by *P. peoriae* NRRL BD-62 in exponential fed-batch fermentation.

μ (h^{-1})		**$Y_{X/S}$ (g/g)**		**2,3-BDO (g/L)**	
Set	**Real**	**Set**	**Real**	**Expected**	**Real**
0.11	0.01 ± 0.01	0.134	0.0005 ± 0.000	9.72	5.49 ± 0.77
0.14	0.03 ± 0.01	0.134	0.012 ± 0.004	11.60	7.01 ± 0.45
0.17	0.04 ± 0.01	0.134	0.030 ± 0.018	15.24	10.65 ± 2.24

μ= specific cell growth rate; $Y_{X/S}$= dry cell weight/glucose yield; 2,3-BDO= titer.

Previous studies have reported lower $Y_{X/S}$ in cultures at μ lower than the preprogrammed values in fed-batch fermentation at exponential feeding, responsible for compromising cell growth in the feeding phase (COS, RESINA, *et al.*, 2005, GORDILLO, SANZ, *et al.*, 1998). According to COS, RESINA, *et al.* (2005), the main exponential feeding disadvantage is the non-compensation in the face of a perturbation in the system, causing the microorganism to grow outside the preprogrammed μ. As a result, bioproduct production is affected, decreasing the bioprocess yield. Another critical exponential feeding limitation is the substrate accumulation in the fermentation broth if overfeeding is performed since it is a cultivation strategy without feedback control (KIM, LEE, *et al.*, 2004).

The continuous and increasing change in C/N ratio during exponential feeding seems to have been the disturbance responsible for affecting the *P. peoriae* NRRL BD-62 cell growth. The nutritional limitation caused by the relative nitrogen reduction with the glucose addition in the culture medium was intensified by the NH_4Cl nature. While YE is a complex nitrogen source rich in amino acids, vitamins, minerals, and macro/micro-elements (NARISETTY, ZHANG, *et al.*, 2022), NH_4Cl is a simple and chemically defined inorganic source whose cell growth absence may have more strongly

affected the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62. This hypothesis aligns with the residual glucose accumulation along exponential feeding. By compromising cell growth, NH_4Cl contributed to the μ_{set} values exceeding the μ_{real} in the evaluated conditions, which resulted in glucose overfeeding. Other studies have also reported substrate accumulation in the 2,3-BDO production in fed-batch fermentation at exponential feeding because of the observed differences between μ_{set} and μ_{real} (KIM, LEE, *et al.*, 2004, MORCELLI, RECH, *et al.*, 2018).

Incomplete glucose metabolism resulted in a low microbial activity ($\mu_{real} < \mu_{set}$), whose cell plasticity was already limited by the low initial NH_4Cl amount of only 1.79 g/L used in this study. This concentration was set based on the initial glucose of 10 g/L and the initial C/N ratio of 8.5 g/g. Therefore, the insufficient nitrogen supply was mainly responsible for the deficient cell growth and glucose accumulation, which directly affected the 2,3-BDO production by *P. peoriae* NRRL BD-62 in fed-batch fermentation at exponential feeding.

3.2.3 One-pulse and exponential feeding comparison

Table 3 presents $Y_{P/S}$, $Y_{P/X,}$ and the 2,3-BDO selectivity calculated for each feeding profile investigated. The results in exponential feeding cultures were generally better than those in one-pulse fed-batch fermentation, although the t-test verified no statistically significant difference in the 2,3-BDO titer and yield (Table 6.2). Similar statistical results were reported by MORCELLI, RECH, *et al.* (2018). The highest 2,3-BDO production was observed at μ_{set}=0.17 h^{-1}, about 1.8-fold higher than the production in intermittent feeding. In turn, the $Y_{P/S}$ reached an average of 0.38 g/g for the assays at μ_{set}<0.24 h^{-1} against 0.32 g/g for μ_{set}=0.24 h^{-1}.

The $Y_{P/X}$ was reliably calculated only for the intermittent fed-batch assays, equal to 5.43 g/g, due to the low $Y_{X/S}$ observed in exponential feeding. Alternatively, the $Y_{P/X}$ calculated using the Luedeking-Piret-based model also showed limitations since the relationship between q_p and μ was non-linear. The heterogeneous cell population nature resulting from a pseudo-steady-state culture may explain this non-linear behavior observed among the fermentation parameters (DE MACEDO ROBERT, GARCIA-

ORTEGA, *et al.*, 2019). Therefore, a comparison between μ and q_p, $Y_{P/S}$, and $Y_{X/S}$ for investigated conditions was performed, as presented in Figure 6.

Table 6.3: Fed-batch fermentation parameters for 2,3-BDO production by *P. peoriae* NRRL BD-62 from glucose and NH_4Cl at $C/N_{initial}$=8.5 g/g in 24/48 h.

Parameters	One-pulse feeding*	Exponential feeding*		
	μ = 0.24 h^{-1} **	**μ_{set} = 0.11 h^{-1}**	**μ_{set} = 0.14 h^{-1}**	**μ_{set} = 0.17 h^{-1}**
2,3-BDO (g/L)	6.06 ± 0.24^{A}	5.49 ± 0.77^{aA}	7.01 ± 0.45^{aA}	10.65 ± 2.24^{aA}
DCW (g/L)	1.11 ± 0.03^{A}	0.66 ± 0.03^{aB}	0.68 ± 0.08^{aB}	1.37 ± 0.24^{aA}
$Y_{P/S}$ (g/g)	0.32 ± 0.02^{A}	0.40 ± 0.04^{aA}	0.36 ± 0.14^{aA}	0.39 ± 0.17^{aA}
$Y_{P/X}$ (g/g)	5.43 ± 0.09	ND	ND	ND
GC (g/L)	18.84 ± 1.99	10.82 ± 2.99	15.58 ± 3.02	21.83 ± 0.06
Selectivity (*levo*:*meso*:acetoin)	1:1.01:0	1.32:1:0	1.05:1:0	1:1.35:0

*Glucose feeding started at the 8 h inoculation time. One-pulse feeding was carried out for 48 h, while exponential feeding was carried out for 24 h.

**Corresponding to the batch fermentation step.

C/N ratio= carbon/nitrogen ratio; μ= specific cell growth rate; 2,3-BDO= 2,3-BDO titer (*meso*- + *levo*-isomer titers); DCW= dry cell weight (cell biomass); $Y_{P/S}$= 2,3-BDO/glucose yield; $Y_{P/X}$= 2,3-BDO/dry cell weight yield; GC= glucose consumption; ND= not determined.

The means followed by the same letter do not show significant differences by t-test (α = 0.05); The lowercase letters correspond to the comparison between the exponential fed-batch fermentation assays, while the capital letters correspond to the comparison between different feeding profiles.

While $Y_{P/S}$ and $Y_{X/S}$ were considered no μ-dependent, q_p decreased with increasing μ_{set}, from more than 7.9 $g_{2,3\text{-}BDO}/g_{DCW}/h$ to μ_{set}= 0.11 h^{-1} to approximately 0.56 $g_{2,3\text{-}BDO}/g_{DCW}/h$ at μ_{set}= 0.17 h^{-1}. Despite the significant reduction attributed to the deficient cell formation at μ_{set}= 0.11 h^{-1}, q_p in exponential feeding were higher than those observed in the one-pulse fed-batch phase, equal on average to 0.08 $g_{2,3\text{-}BDO}/g_{DCW}/h$. Therefore, the carbon flux seems to have been preferentially directed to the 2,3-BDO synthesis in fed-batch fermentation at exponential feeding, justifying the $Y_{P/S}$ close to 0.40 g/g.

The 2,3-BDO selectivity was characterized by complete acetoin conversion under-investigated conditions (Table 6.3). An optical purity of 57.5% *meso*-isomer and 42.7% *levo*-isomer was observed at μ_{set}= 0.17 h^{-1}, while *levo*-2,3-BDO was produced in higher proportion under the other conditions, reaching a peak of 57 % at μ_{set}= 0.11 h^{-1}. In contrast, the *levo*-isomer amount was matched with the *meso*-isomer amount in one-pulse fed-batch fermentation, equaling approximately 50%. An inverse relationship between μ_{set} and *levo*-2,3-BDO production was verified in exponential feeding. It was suggested

that there is an optical purity pattern in the 2,3-BDO production by *P. peoriae* NRRL BD-62 as a function of the growth rate adopted.

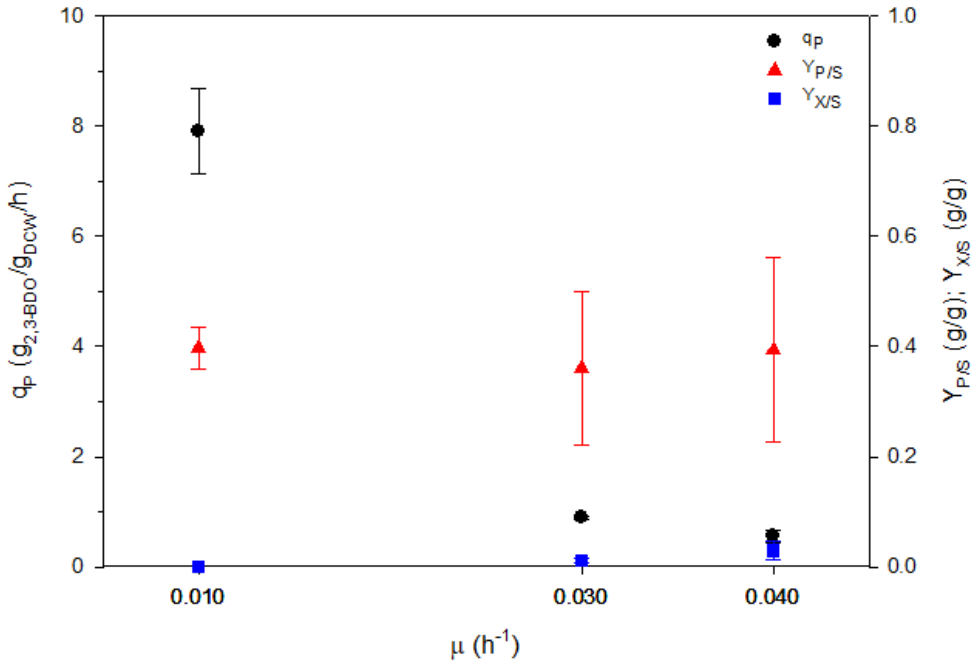

Figure 6.6: Correlation between fermentation parameters for exponential fed-batch cultures at μ_{set} equal to 0.11, 0.14, and 0.17 h^{-1}. $Y_{X/S}$= cell dry weight/glucose yield; $Y_{P/S}$= 2,3-BDO/glucose yield; q_P= specific production rate; and μ= specific cell growth rate.

Finally, the formation of 2,3-BDO metabolism byproducts was determined. Figure 6.7 compares the lactic acid, acetic acid, and ethanol production in the investigated condition for 24 h. Organic acid production was generally minimized, while ethanol synthesis was favored in fed-batch fermentation at exponential feeding. No net acetic acid production was verified in fed-batch assays. In contrast, a lactic acid titer lower than 0.5 g/L was observed in the evaluated conditions, while the net ethanol production was verified only in exponential feeding, equal to 2.2 g/L. Considering the statistical deviations, the organic acids and ethanol production in the fed-batch fermentation at exponential feeding did not vary significantly with the $\mu_{set.}$ Therefore, no strong production dependency relationship was suggested, as observed for $Y_{P/S}$ and $Y_{X/S}$.

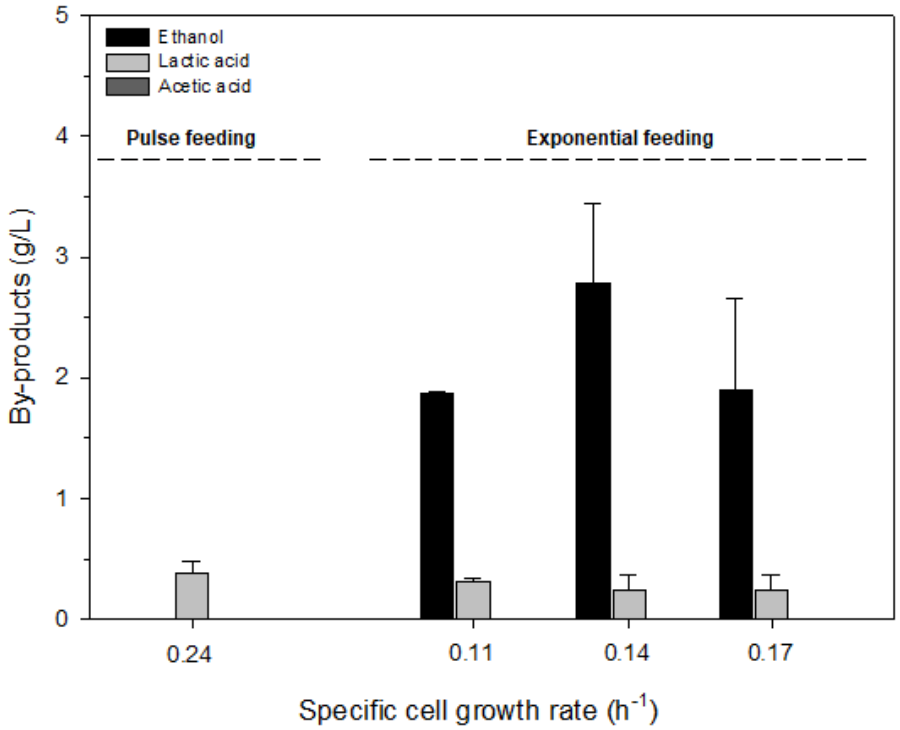

Figure 6.7: By-products formation of 2,3-BDO by *P. peoriae* NRRL BD-62 from glucose and NH_4Cl in one-pulse and exponential fed-batch at $C/N_{initial}$=8.5 g/g.

MORCELLI, RECH, *et al.* (2018) reported a similar byproduct production behavior when they investigated the 1,3-propanediol (1,3-PDO) production by *K. pneumoniae* BLh-1 in fed-batch fermentation at exponential feeding at three μ_{set} conditions: 0.035, 0.070 and 0.105 h^{-1}. No statistical difference was observed in the ethanol and lactic acid production among the evaluated conditions, although the highest titers were seen at μ_{set} of 0.070 and 0.105 h_{-1}. Unlike this study, net acetic acid production was observed in exponential feeding assays. While μ_{set} of 0.070 h^{-1} favored high production, μ_{set} of 0.105 h^{-1} led to the lowest reported acetic acid titer. Again, no statistically significant difference was seen in acetic acid production between the conditions evaluated.

Given the results, exponential feeding was considered inefficient to produce 2,3-BDO by *P. peoriae* NRRL BD-62 from glucose and NH_4Cl-based medium. The low nitrogen supply by NH_4Cl was considered the main limiting factor of this feeding strategy, outweighing the savings obtained by replacing YE in the culture medium since the 2,3-BDO production was below expectations. Furthermore, residual glucose and

byproduct accumulation in the fermentation broth were observed with the exponential feeding advancement, which could cause cell activity loss and the 2,3-BDO recovery increased costs in the long term. Therefore, for fed-batch fermentation at exponential feeding for high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62, it is first necessary to ensure an adequate nutrient supply, especially in the feeding phase.

WONG, Chiao Ling, YEN, *et al.* (2014) investigated the effects of adding the limiting nutrient with substrate feeding in fed-batch fermentation for 2,3-BDO production by *Klebsiella* sp. ZMD30. An increase in 2,3-BDO yield from 81% using glucose feeding without nitrogen source to 93% using glucose feeding supplemented with urea at 10% of its concentration in batch-phase was verified. In contrast, MORCELLI, RECH, *et al.* (2018) reported a nearly 60% reduction in 1,3-PDO yield when replacing the crude glycerol feeding with a complex culture medium containing crude glycerol and other nutrients in anaerobic fed-batch fermentation at exponential feeding. The lower 1,3-PDO production was accompanied by an improvement in cell biomass formation, indicating a possible shift in microbial metabolism towards cell plasticity in complex nutritional media.

4. Conclusion

The relationship between the pulse, constant and exponential feeding strategies, and the nitrogen source nature in the 2,3-BDO production by *P. peoriae* NRRL BD-62 was evaluated in this study. Overall, the constant and exponential rates contributed to higher 2,3-BDO production than pulse feeding, regardless of the nitrogen source type used. The best results were obtained in fed-batch fermentation at constant feeding at C/N= 8.5 g/g using the YE-based medium. The nutritional limitation represented by C/N= 18.5 g/g could not affect the 2,3-BDO production in one-pulse fed-batch assays but caused a 44% reduction in constant feeding. Replacement of YE by NH_4Cl in fed-batch cultures at exponential feeding compromised the q_p control by reducing μ_{real} and increasing glucose accumulation in the fermentation broth. As a result, the 2,3-BDO production was below the expected values investigated μ_{set} conditions. Despite the effects caused by the nutritional limitation on the 2,3-BDO titer, its yield was maintained at around 0.40 g/g, a value similar to that obtained in batch fermentation, indicating that glucose feeding strategies were unable to affect the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62.

Therefore, the nitrogen supply was considered the main limiting factor in the high-yield 2,3-BDO production in fed-batch fermentation and should be better studied and efficiently controlled for the bio-based 2,3-BDO process, mainly on a large scale.

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CHAPTER 7

Contextualization

In this chapter, the 2,3-BDO production by *P. peoriae* NRRL BD-62 from different substrates was investigated. As a result, sugarcane molasses was used to replace commercial glucose in the culture medium. The nitrogen source was also evaluated. Corn steep liquor (CSL) and ammonium chloride (NH_4Cl) were used to replace commercial yeast extract (YE) in a molasses-based medium. The effects of alternative carbon and nitrogen sources on 2,3-BDO yield and selectivity were evaluated for final culture medium selection. A cost calculation based on free-on-board (FOB) and commercial prices of the inputs used was performed to determine the savings achieved with the new culture medium. Finally, fed-batch cultures at a constant feeding rate, previously investigated in Chapter 3, were conducted to improve the 2,3-BDO titer and yield.

Therefore, the specific objectives of this chapter were:

- Investigate the *P. peoriae* NRRL BD-62 capacity to metabolize different commercial carbon sources and their effects on the 2,3-BDO production;
- Investigate the 2,3-BDO production by *P. peoriae* NRRL BD-62 from sugarcane molasses;
- Replace YE with alternative and inexpensive nitrogen sources, such as CSL and NH_4Cl, and determine their effects on 2,3-BDO production by *P. peoriae* NRRL BD-62 in a molasses-based medium;
- Determine the savings achieved with the culture medium by replacing glucose with molasses and YE with CSL and NH_4Cl;
- Investigate the 2,3-BDO production using the selected medium in fed-batch fermentation at constant feeding.

Relevance

Through the investigation carried out in this chapter, it was possible to understand:

- The effects of alternative carbon and nitrogen sources (commercial and residual) on the 2,3-BDO production and isomeric optical purity by a newly *P. peoriae* NRRL BD-62 in batch and fed-batch fermentation;
- The effects on the culture medium costs for the 2,3-BDO production by *P. peoriae* NRRL BD from commercial glucose and YE replacement for inexpensive carbon and nitrogen sources, based on FOB prices and those adopted by the international input market.

PRODUCTION OF OPTICALLY PURE (R,R)-2,3-BUTANEDIOL FROM MOLASSES USING LOW-COST SALT MEDIUM BY *Paenibacillus peoriae* NRRL BD-62

Abstract

The industrial bio-based 2,3-butanediol (2,3-BDO)production, a versatile green platform chemical used in different industrial segments, is strongly affected by the culture medium costs, mainly carbon and nitrogen sources, responsible for a sizeable final bioprocess costs portion. In this study, different compounds were investigated to replace commercial glucose and yeast extract for cheaper 2,3-BDO production by *Paenibacillus peoriae* NRRL BD-62, a newly GRAS (generally recognized as safe) strain. From preliminary results, residual biomasses such as corn steep liquor and sugarcane molasses were investigated. A savings of 75.2% were achieved using sugarcane molasses and NH_4Cl-based medium, whose final cost was equal to 4.74 US$/$kg_{2.3\text{-}BDO}$, considering the inputs' current FOB and market prices used. An absolute *levo*-2,3-BDO formation marked the 2,3-BDO isomeric optical ratio with a yield of 0.35 g/g in batch fermentation. The selectivity was maintained above 90% in fed-batch fermentation at constant feeding. In contrast, the yield showed a 20% reduction, indicating that this feeding strategy was less efficient than batch cultures. To the best of our knowledge, this is the first time *levo*-2,3-BDO with high optical purity has been produced by a safe and wild-type *P. peoriae* strain from molasses in a low-cost salt medium.

Keywords: *Levo*-2,3-BDO, sugarcane residue, corn residue, cheap fermentation medium, GRAS strain.

1. Introduction

Bio-based 2,3-butanediol (2,3-BDO) is a non-cytotoxic liquid fuel and bulk green chemical found in three distinct isomeric forms with particular physicochemical properties that give them different industrial applications such as food additives, cosmetics, moisturizing, and softening agents, polymers, solvent, printing inks, and plasticizers (DAI, ZHAO, *et al.*, 2015, ERIAN, GIBISCH, *et al.*, 2018, TINÔCO, BORSCHIVER, *et al.*, 2020). The optically active *levo*-isomer can be used as an antifreeze agent due to its low melting point of -60 °C and ability to interact with water through hydrogen bonds of its hydroxyls (TINÔCO, DE CASTRO, *et al.*, 2021, YANG, Zhiliang, ZHANG, 2018). The 2,3-BDO production with high optical purity depends on the microorganism and the fermentation conditions adopted (BIAŁKOWSKA, 2016).

Paenibacillus peoriae NRRL BD-62 is a GRAS (generally recognized as safe) microorganism, phylogenetically related to *P. polymyxa* strain for the 2,3-BDO production (TINÔCO, PATERAKI, *et al.*, 2021). Based on initial observations, *P. peoriae* NRRL BD-62 can produce the optically active *levo*-2,3-BDO with high purity from glucose under low oxygen supply (kLa ~5 h^{-1}), the temperature in the range of 32-37 °C, and slightly acidic pH around 5 (Chapter 1). Recently isolated, this strain has not yet been reported for bio-based 2,3-BDO production and is one of the first studies performed.

The culture medium is intrinsically related to the 2,3-BDO metabolism, which is responsible for its yield and production economy, especially on a large scale (PSAKI, MAINA, *et al.*, 2019, WONG, HUANG, *et al.*, 2012). Different nutrients can compose the culture medium, including carbon and nitrogen sources, trace metals, vitamins, and minerals (CELIŃSKA, GRAJEK, 2009). These compounds are usually used in cell plasticity, endogenous microbial maintenance, and metabolite synthesis (JI, HUANG, *et al.*, 2011).

Carbon and nitrogen are considered limiting nutrients in the culture medium. Besides affecting the 2,3-BDO formed, their high costs can make the bioprocess economically unfeasible since they account for a large portion of the final production costs. Different raw materials have been investigated for low-cost 2,3-BDO production

to overcome this limitation (DAI, ZHAO, *et al.*, 2015, JUNG, Moo Young, PARK, *et al.*, 2013). Residual biomasses such as raw inulin, cassava, crude glycerol, lignocellulosic hydrolysates, and CO (CAO, Can, ZHANG, *et al.*, 2017, FERNÁNDEZ-GUTIERREZ, VEILLETTE, *et al.*, 2020, GAO, JIANG, *et al.*, 2019, GHADERMAZI, RE, *et al.*, 2022, GUO, WANG, *et al.*, 2017, MA, HE, *et al.*, 2018, MOON, CHUNG, *et al.*, 2021, YANG, Tao-Wei, RAO, *et al.*, 2013b) have been used for carbon supply. In turn, nitrogen can be supplied from organic and inorganic compounds, commercial or residual, such as corn steep liquor, soybean meal, cotton seed meal, rice dry distillers' grain with solubles, urea, and ammonium salts (DESHMUKH, MISTRY, *et al.*, 2015, SONG, RATHNASINGH, *et al.*, 2018, TSIGORIYNA, GANCHEV, *et al.*, 2021, WONG, HUANG, *et al.*, 2012, XIAO, Zijun, WANG, *et al.*, 2012, YANG, Tao-Wei, RAO, *et al.*, 2013a).

Sugarcane molasses is a feedstock rich in sugars and highly cost-effective (YANG, Yuling, DENG, *et al.*, 2022). Found as a dark syrupy byproduct obtained from the sugarcane juice after sugar extraction (DESHMUKH, MISTRY, *et al.*, 2015), sugarcane molasses is composed of a large sucrose amount, besides glucose and fructose in equal proportions, nitrogenous substances, vitamins (nicotinic acid as a NAD^+ precursor), and mineral salts (mainly calcium and potassium) (CAO, Weifeng, WANG, *et al.*, 2018, SIKORA, KUBIK, *et al.*, 2016). In 2019 alone, approximately 63.8 Mtonnes of molasses were produced worldwide, of which 19.3% were produced by Brazil (FAO, 2019). The molasses market price is currently equal to 0.44 US$/kg, approximately 2-fold lower than the commercial glucose commonly used for the 2,3-BDO production (COMEXSTAT, 2022). Therefore, molasses is a powerful nutrient source with high availability, low cost, and great potential for application in circular economy-based biorefineries.

Ammonium salts are low-cost and chemically defined inorganic compounds capable of replacing traditional complex nitrogen sources or supplementing fermentation media to stimulate cell growth and bioproduct production (BEAULIEU, BEAULIEU, *et al.*, 1995). Several studies have investigated ammonium salts instead of yeast extract (YE) for the 2,3-BDO production (BERBERT-MOLINA, SATO, *et al.*, 2001, SATTAYASAMITSATHIT, PRASERTSAN, *et al.*, 2011, WANG, Dexin, OH, *et al.*, 2021). YE is a processed yeast product consisting of essential amino acids, vitamins,

minerals, and macro/micro-elements (NARISETTY, ZHANG, *et al.*, 2022, WANG, Dexin, OH, *et al.*, 2021, YANG, Taowei, ZHANG, *et al.*, 2012, ZAREI, DASTMALCHI, *et al.*, 2016). Although highly nutritious, industrial YE use is avoided due to high market costs and the complicated downstream processing for bioproduct recovery (YU, O'HAIR, *et al.*, 2022). YE has been marketed at 6.0 US$/kg (ANGEL YEAST, 2022), one of the bioprocess's most expensive inputs. The YE replacement with ammonium salts is an exciting culture strategy that enables 2,3-BDO production, especially on a large scale.

This study aimed to reduce the culture medium costs for the optically pure *levo*-2,3-BDO production by a newly *P. peoriae* NRRL BD-62 by replacing commercial glucose and YE with inexpensive, renewable, and no impact on the food chain for humans carbon and nitrogen sources. The higher culture medium economy was achieved using sugarcane molasses and ammonium chloride, which maintained high *levo*-isomer optical purity. To the best of our knowledge, this is the first time that a safe and wild-type *P. peoriae* strain has been used to produce optically pure *levo*-2,3-BDO from a low-cost molasses and NH_4Cl-based medium.

2. Materials and methods

2.1 Bacterial strain maintenance

A newly *Paenibacillus peoriae* NRRL BD-62 has been isolated from the maize rhizosphere (CSM36 genotype), planted in Cerrado soil (EMBRAPA-CNPMS, Sete Lagoas, MG) (VON DER WEID, DUARTE, *et al.*, 2002). It was deposited in the culture collection of the Microbial Genetics Laboratory of the Microbiology Institute of the Federal University of Rio de Janeiro, where it is maintained in Tryptic Soy Broth – TSB medium (17 g/L tryptone, 3 g/L soytone, 5 g/L NaCl, 2.5 g/L K_2HPO_4, and 2.5 g/L glucose) supplemented with 25% (v/v) glycerol, at -80°C.

2.2 Culture media

The seed medium was composed of (g/L): 10 yeast extract, 20 peptone, and 20 glucose, while the fermentation medium contained (g/L): 0.5 KH_2PO_4, 2.0 K_2HPO_4, 0.0225 $MnSO_4$, and 0.3 KCl (ADLAKHA, YAZDANI, 2015). The carbon and nitrogen amounts in the fermentation medium were defined based on an initial C/N ratio of 8.5

g/g, considering an initial concentration of 20-30 and 30 g/L of the substrate in batch and fed-batch fermentation, respectively. The media were sterilized by autoclaving at 121°C for 15 min. Carbohydrates were sterilized separately from the other nutrients. Antifoam 204 (Sigma-Aldrich, USA) was added to the fermentation media when needed.

2.3 Carbon and nitrogen sources

Glucose, fructose, sucrose, xylose, and glycerol were investigated as substrates, while yeast extract was used as a complex nitrogen source. All these compounds were purchased commercially from Sigma-Aldrich (San Luis, Missouri, USA). Residual biomasses were also investigated to reduce the culture medium costs. Sugarcane molasses was composed of approximately 50% (w/w) sugars (mainly sucrose, glucose, and fructose), TKN, protein, free amino nitrogen (FAN), and total phenolic content (TPC) expressed as g gallic acid equivalents per 100 g molasses (PAPADAKI, PAPAPOSTOLOU, *et al.*, 2018). It was kindly provided by CENPES/Petrobras, which obtained it from a sugar industry in São Paulo, Brazil. Corn steep liquor (CSL) was composed of approximately 50% (v/v) water and dry matter, organic acids, fatty acids, sulfate, and free amino acids (ZHOU, YU, *et al.*, 2022). It was kindly provided by the Microbial Biotechnology Laboratory (LaBiM) of the Chemistry Institute of the Federal University of Rio de Janeiro, Brazil. Sugarcane molasses and CSL were not previously treated and used in the conditions in which they were acquired. Ammonium chloride (NH_4Cl) was also investigated as an alternative nitrogen source, commercially obtained by Sigma-Aldrich (San Luis, Missouri, USA).

2.4 Fermentation assays

Initially, the fermentation inoculum was prepared by transferring 0.2 mL glycerol stock solution to 250 mL shake flasks containing 50 mL TSB medium for cell reactivation at 32 °C, 200 rpm for 24 h, and followed by transfer of 1% (v/v) reactivated cells to 1 L shake flasks containing 350 mL seed medium at 32 °C, 200 rpm for 16 h. A fermentation inoculum of 10% (v/v) was used in batch and fed-batch fermentations at 32 °C, pH=5, 400 rpm, and 0.1 vvm in DASbox® Mini Bioreactor – 300 mL (Eppendorf, Germany), designed as a 4-fold system with 16 parallel bioreactors, using a starting volume of 200 mL. Fermentation conditions were previously optimized. The pH was controlled by

adding 2 M NaOH or 1 M H_2SO_4. The oxygen supply was based on submerged aeration via an L-sparger (4 mm compression fitting) through which air was injected into a sterile filter (0.22 μm) and agitation using two Rushton-type impellers 3 cm apart, rotating clockwise for radial and axial flows. Fed-batch fermentation was used to validate the 2,3-BDO production from sugarcane molasses in an NH_4Cl-based medium. A sugarcane molasses solution of about 60 g/L containing 30 g/L of sugars was fed continuously at 1.3 mL/h throughout 75.5-144 h. All fermentation assays were performed in biological duplicate.

2.5 Analytical methods

Cell biomass was determined by dry cell weight (DCW) analysis, which was correlated to an optical density at 600 nm ($OD_{600\ nm}$). Cells were filtered on 0.22 μm cellulose acetate membrane (Sartorius, Germany) and dried in an oven at 60 °C until constant weight, while $OD_{600\ nm}$ was measured in a UV–visible spectrophotometer (Biospectro SP-22, Brazil). This procedure was performed for the investigated carbon and nitrogen sources. The relationship between $OD_{600\ nm}$ and DCW established in the fermentation media is shown in Table S1 (Supplementary material).

The substrate concentrations (glucose, fructose, sucrose, xylose, and glycerol) and fermentation products (*levo*- and *meso*-2,3-BDO, acetoin, ethanol, and lactic and acetic acids) were analyzed using a high-performance liquid chromatography system (HPLC; Agilent, USA) equipped with an HPX-87H Aminex column (300 × 7.8 mm, Bio-Rad, USA). Sulfuric acid at 5 mM was used as an eluent at 0.6 mL/min, and the column temperature was controlled at 45 °C. All compounds were detected in a Refractive Index Detector (RID; Agilent, USA) after sample treatment along the fermentation. This treatment was based on centrifugation at 10,000 x *g*, at 25 °C for 10 min, in a microtube centrifuge (MiniSpin®, Eppendorf, Germany), followed by 10-fold dilution with Milli-Q water of the supernatant, which was then filtered through a 0.22 μm cellulose membrane acetate (Minisart® NML Syringe Filters, Sartorius, Germany). Calibration standard substance curves (Merck KgaA, Darmstadt, Germany) were used to determine the substrate and fermentation product concentrations. The dilution promoted by fed-batch fermentation at constant feeding was considered in the nutrient quantification. The

standard deviation was calculated for samples from all fermentation assays performed here.

2.6 Fermentation parameters

The *P. peoriae* NRRL BD-62 growth was evaluated by determining the cell growth specific rate (μ; h^{-1}), defined as the slope of the biomass concentration semi-logarithmic plot versus time in the exponential growth phase. In turn, 2,3-BDO production was evaluated by $Y_{P/S}$ (g/g) and $Y_{P/X}$ (g/g), defined as the ratio between 2,3-BDO production and glucose consumption, 2,3-BDO production, and cell biomass, respectively. The 2,3-BDO selectivity was calculated based on the molar ratio between *levo*-2,3-BDO, *meso*-2,3-BDO, and acetoin (C4-compounds). All fermentation parameters were calculated considering the maximum 2,3-BDO production in batch and fed-batch fermentation.

3. Results and discussion

3.1 Production of 2,3-BDO from different pure carbon sources

Figure 7.1 presents the 2,3-BDO production by *P. peoriae* NRRL BD-62 from the five substrates investigated: glucose, fructose, sucrose, xylose, and glycerol. While *P. peoriae* NRRL BD-62 could metabolize the first four sugars in 49 h, glycerol was wholly consumed in 71 h. Fructose was the least consumed carbon source, around 92.4%. An average 2,3-BDO production of 7.7 g/L was observed for the five substrates, with the highest 2,3-BDO titer of 8.5-8.6 g/L achieved using glucose and sucrose (Fig. 7.1a).

The cell biomass formation was also similar among the investigated substrates, averaging approximately 2.6 g/L (Fig. 7.1b). Again, the medium containing sucrose was responsible for the highest DCW amount, equal to 2.9 g/L, followed by glycerol with 2.8 g/L. In turn, the specific cell growth rate (μ) was about 20% higher using glucose and sucrose, reaching an average of 0.30 h^{-1}.

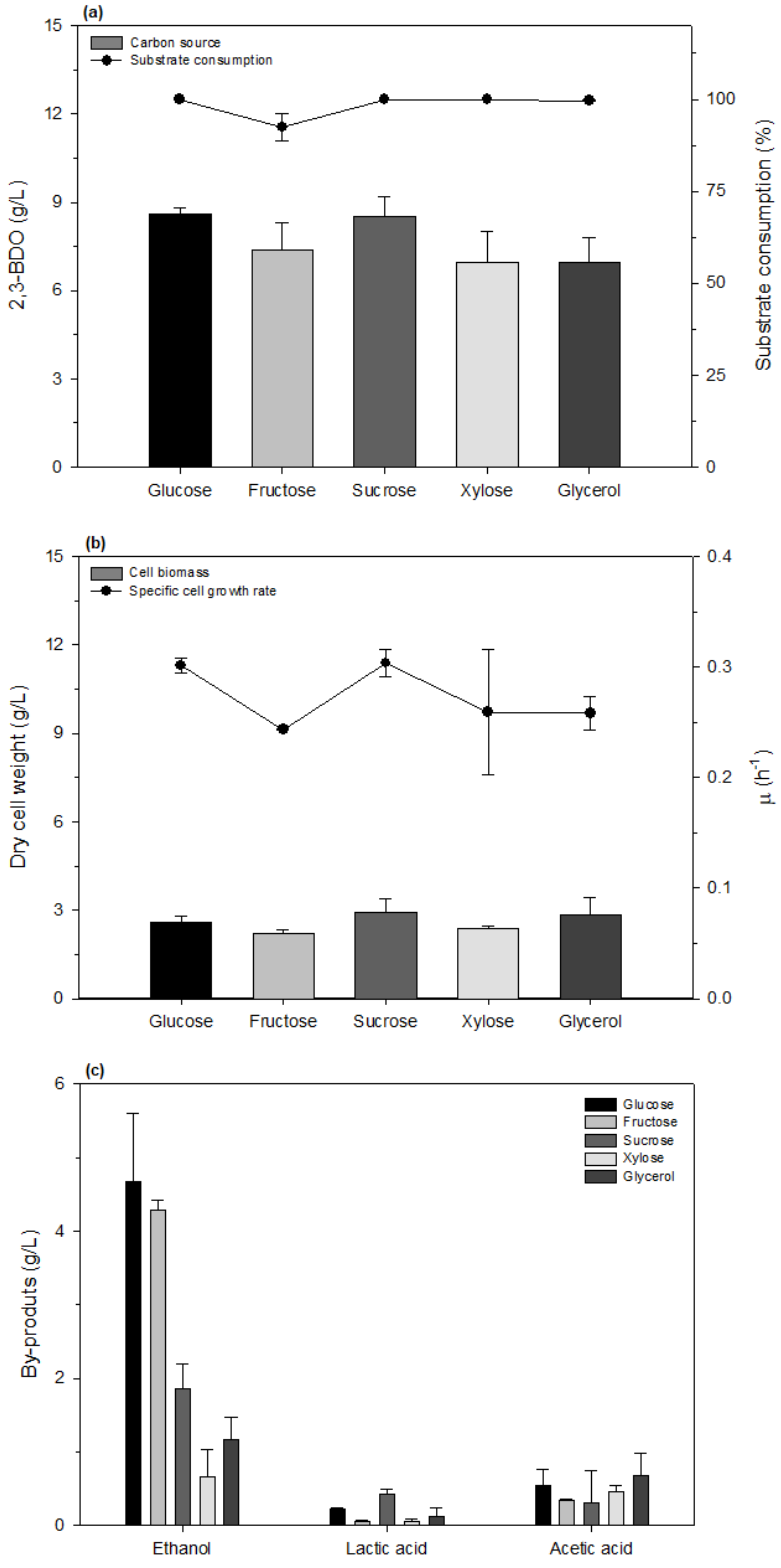

Figure 7.1: Effects of different pure substrates on 2,3-BDO production by *P. peoriae* NRRL BD-62 in batch fermentation at initial C/N= 8.5 g/g. (a) 2,3-BDO titer and substrate consumption. (b) Cell biomass and specific cell growth rate (μ). (c) By-products titers.

The main 2,3-BDO metabolism byproducts by *P. peoriae* NRRL BD-62 were ethanol, lactic acid, and acetic acid (Fig. 7.1c). Acetoin was not detected from any carbon source investigated. Ethanol was the second highest titer product formed after 2,3-BDO, reaching about 4.7 g/L from glucose and 4.3 g/L from fructose. Only 0.66 g/L of ethanol was produced using xylose as a substrate. In turn, the organic acids production was, on average equal to 0.17 and 0.46 g/L of lactic acid and acetic acid, respectively. The highest lactic acid production of 0.42 g/L was observed using sucrose, while the highest acetic acid production of 0.67 g/L was achieved using glycerol. On the other hand, the lowest lactic acid and acetic acid titers were equal to 0.05 and 0.31 g/L from xylose and sucrose, respectively.

Table 7.1 presents the fermentation parameters calculated for each substrate. The $Y_{P/S}$ and $Y_{P/X}$ were similar for all substrates, on average equal to 0.37 and 3.01 g/g, respectively, considering statistical deviations. The highest $Y_{P/S}$ of 0.39 g/g was verified using glucose, while the highest $Y_{P/X}$ of 3.36 g/g was obtained from fructose. Despite the higher initial sucrose concentration of approximately 24.6 g/L and its total metabolization in 49 h, a $Y_{P/S}$ of only 0.35 g/g was achieved. In turn, the high cell biomass formation observed using xylose contributed to the lowest $Y_{P/X}$ of 2.49 g/g.

Table 7.1: Fermentation parameters of different carbon sources used for the 2,3-BDO production by *P. peoriae* NRRL BD-62 in batch assays at $C/N_{initial}$= 8.5 g/g.

Carbon source	**$Y_{P/S}$ (g/g)**	**$Y_{P/X}$ (g/g)**	**Selectivity (*levo*:*meso*:acetoin)**	**Total initial substrate (g/L)**	**Time (h)***
Glucose	0.39 ± 0.01	3.35 ± 0.36	1:0:0	22.31 ± 0.24	49
Fructose	0.38 ± 0.07	3.36 ± 0.59	1:0:0	20.88 ± 0.41	
Sucrose	0.35 ± 0.04	2.95 ± 0.27	1:0:0	24.63 ± 0.76	
Xylose	0.36 ± 0.07	2.91 ± 0.52	1:0:0	19.15 ± 0.69	
Glycerol	0.36 ± 0.04	2.49 ± 0.24	0.145:1:0	19.67 ± 0.04	71

*maximum 2,3-BDO production.
C/N ratio= carbon/nitrogen ratio; $Y_{P/S}$= 2,3-BDO/substrate yield; $Y_{P/X}$= 2,3-BDO/dry cell weight yield.

The (R,R)-2,3-BDO production by *P. peoriae* NRRL BD-62 achieved absolute optical purity from all investigated sugars. In contrast, only 12.6% of optically active 2,3-BDO was produced from glycerol, while 87.4% corresponded to the *meso*-isomer (Table 7.1). Despite the racemic mixture observed using glycerol, the optically inactive 2,3-BDO was considered high and promising for a lower-cost downstream step.

ERIAN, GIBISCH, *et al.* (2018) investigated different sugars, usually contained in lignocellulosic hydrolysates and molasses, as an alternative to commercial glucose for the diol production (2,3-BDO + acetoin) by an engineered *Escherichia coli* W strain using a salt medium in shake flasks. Diol yields similar to those obtained from glucose of 0.40 g/g were verified from fructose, arabinose, and mannose, while an average yield of approximately 66% lower was observed from xylose and sucrose. Except for xylose and arabinose, all other sugars favored a significant acetoin production in 48 h. An incomplete substrate consumption was verified only in xylose and sucrose-based media. WANG, Ailong, WANG, *et al.* (2010) evaluated the use of glucose, xylose, and arabinose in corncob molasses for the 2,3-BDO production by *Klebsiella pneumoniae* SDM. The 2,3-BDO yields were 0.34, 0.34, and 0.36, respectively. The highest μ of 0.31 h^{-1} was achieved using arabinose. However, the cell biomass formed was lower than that obtained from glucose and xylose. The 2,3-BDO metabolism was best favored by using glucose as a substrate. YANG, Yuling, DENG, *et al.* (2022) investigated the assimilation mechanism of sugars present in sugarcane molasses to produce 2,3-BDO by a nonpathogenic *Clostridium ljungdahlii* DSM 13528. Initial concentrations of 5 g/L of glucose, fructose, and sucrose were used. The highest 2,3-BDO titer of 1.94 g/L was obtained from sucrose, while the highest cell biomass formation, on average equal to 1.49 g/L, was achieved using glucose and fructose. Ethanol and acetic acid were the main byproducts detected. Ethanol was similarly produced from all three substrates, and the highest acetic acid production of 1.1 g/L was verified from glucose.

3.2 Production of 2,3-BDO from sugarcane molasses

Sucrose showed the best results for producing 2,3-BDO by *P. peoriae* NRRL BD-62. Therefore, sugarcane molasses was investigated as a low-cost sucrose source. The time courses of sugar consumption, cell biomass formation, and the 2,3-BDO production and its byproducts using a molasses-based medium are presented in Figure 7.2.

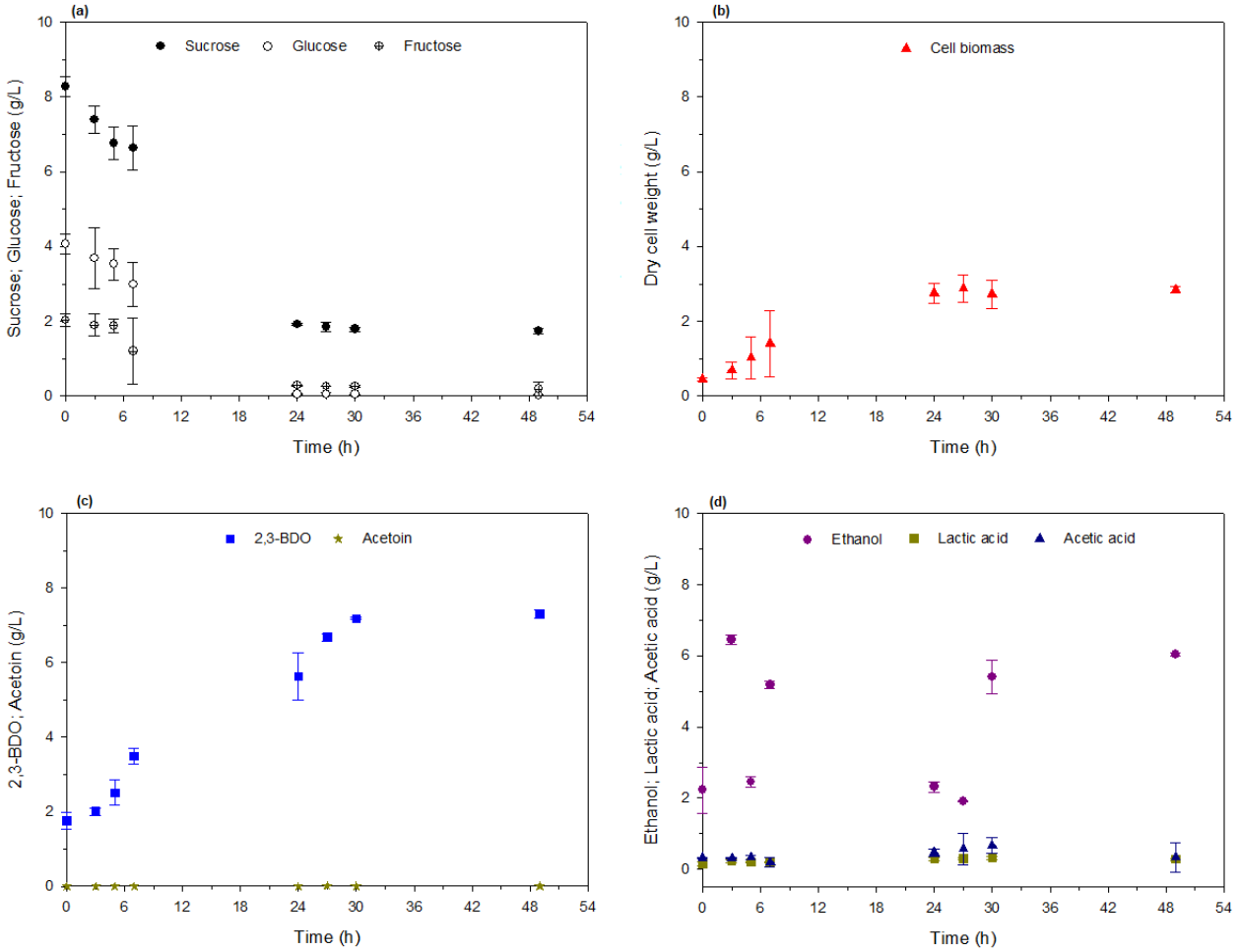

Figure 7.2: Time courses of 2,3-BDO production by *P. peoriae* NRRL BD-62 from molasses in batch fermentation at initial C/N= 8.5 g/g. (a) Sugar consumption. (b) Cell biomass. (c) 2,3-BDO and acetoin titers. (d) By-products titers.

An initial sugarcane molasses concentration of 20 g/L, composed of approximately 50% sugars, including sucrose, glucose, and fructose, was used (Fig. 7.2a). Sucrose and fructose consumption at the beginning of fermentation (0-6 h) was not affected by the glucose presence. It was suggested that an absence of the catabolic carbon repression, commonly reported in culture media containing a mixture of substrates and glucose (MA, HE, *et al.*, 2018, NIE, JI, *et al.*, 2011). However, while glucose and fructose were practically exhausted in 24 h, a sucrose accumulation of approximately 1.7 g/L was verified in 24-49 h. Therefore, only 86.5% sugarcane molasses was consumed at the final fermentation. The lower sugarcane molasses consumption by *P. peoriae* NRRL BD-62 can be explained by the complex interactions between the pathways of its constituent sugars. As a result, carbon metabolism can be compromised, leading to differential substrate consumption (JUNG, Moo-Young, JUNG, *et al.*, 2015).

Exponential cell growth was observed at a μ of 0.15 h^{-1} during the first 24 h when the stationary phase was reached (Fig. 7.2b). The biomass (DCW) was kept constant at around 2.8 g/L until the culture end, resulting in a cell yield of 0.19 g/g. About 5.5 g/L of 2,3-BDO was produced in 49 h (Fig. 7.2c). An acetoin accumulation of only 0.02 g/L was detected in 27-49 h and was considered negligible. The 2,3-BDO-isomers were produced throughout the fermentation, with $Y_{P/S}$ and $Y_{P/X}$ of 0.44 and 2.31 g/g, respectively. The optical purity reached about 97.3% of *levo*-2,3-BDO and 2.7% of *meso*-2,3-BDO, similar to those obtained by *P. polymyxa* strains (HÄßLER, SCHIEDER, *et al.*, 2012, TINÔCO, PATERAKI, *et al.*, 2021). Compared to the production from pure sucrose, the 2,3-BDO ratio in the products was reduced, suggesting that the total sugar concentration in sugarcane molasses can affect the 2,3-BDO production (YANG, Yuling, DENG, *et al.*, 2022).

Ethanol, lactic acid, and acetic acid were also formed during the fermentation (Fig. 7.2d). Ethanol showed a very varied production profile, forming and converting into other compounds during the first 27 h. Subsequently, an accumulation of 3.8 g/L was observed. On the other hand, organic acid showed similar and practically constant production profiles. An accumulation of only 0.13 and 0.14 g/L of lactic acid and acetic acid was detected at the culture end, respectively.

JANTAMA, POLYIAM, *et al.* (2015) used a salt medium without complex nitrogen sources to produce 2,3-BDO by an engineered *K. oxytoca* KMS005-73T from sugarcane molasses. A 2,3-BDO yield of 0.42 g/g was achieved in shake flasks after the sugar consumption of 46.1 g/L. Only acetate was detected as a byproduct, whose titer was approximately 4.6 g/L. JUNG, Moo Young, PARK, *et al.* (2013) reported the acetoin, ethanol, lactate, and succinate production as 2,3-BDO metabolism byproducts by an engineered *Enterobacter aerogenes* EMY-68 from sugarcane molasses in batch fermentation. The highest titers were verified for ethanol and succinate, which reached approximately 9.3 and 3.4 g/L, respectively. About 28.9 g/L of 2,3-BDO was produced from the sugar consumption of 82.1 g/L, which resulted in a yield of 0.35 g/g. PALAIOGEORGOU, PAPANIKOLAOU, *et al.* (2019) investigated the 2,3-BDO production by a newly isolated *Enterobacter* sp. strain (FMCC-208) from sugarcane molasses in Duran bottles under anaerobic conditions. An average 2,3-BDO yield of 0.40 g/g was achieved. However, the authors found improved 2,3-BDO production under aerobic cultivation conditions, and the assays were carried out in shaken flasks. As a result, a yield of 0.44 g/g was verified, considering the 2,3-BDO + acetoin production. Ethanol and succinate were produced as byproducts, reaching titers of 0.8 and 1.4 g/L from 32.5 g/L of consumed sugar.

3.3 Nitrogen source effects on 2,3-BDO production from a molasses-based medium

Alternatively to YE, CSL and NH_4Cl were investigated as nitrogen sources in a molasses-based medium at initial C/N= 8.5 g/g. Nitrogen can affect cell growth and 2,3-BDO metabolism (WONG, HUANG, et al., 2012). Therefore, the CSL and NH_4Cl effects on 2,3-BDO production and its byproducts, sugarcane molasses consumption, and cell biomass formation at 71 h were investigated. The results found are presented in Figure 7.3.

All fermentation assays were started with approximately 30 g/L of sugars (sucrose, glucose, and fructose), obtained from 60 g/L of sugarcane molasses (Table 7.2). A 2,3-BDO titer of 12.7 g/L was verified using YE, about 1.4-fold higher than 9.4 and 9.0 g/L achieved using CSL and NH_4Cl, respectively. A reduced sugarcane molasses consumption was verified by the YE replacement with CSL, decreasing from 96% to almost 85% while remaining around 95% for the NH_4Cl-based medium (Fig. 7.3a).

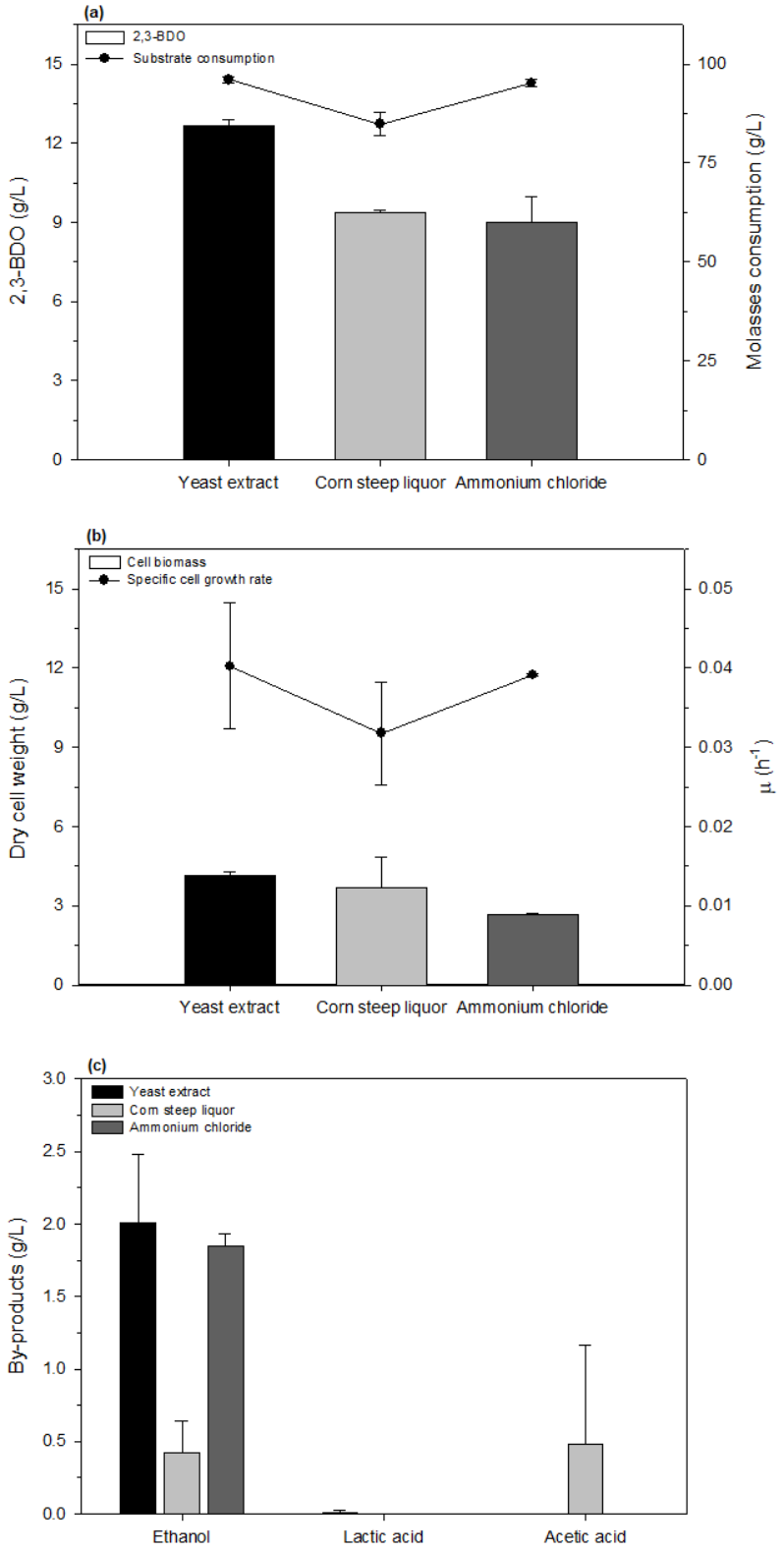

Figure 7.3: Effects of nitrogen source on 2,3-BDO production by *P. peoriae* NRRL BD-62 from a molasses-based medium in batch fermentation at initial C/N= 8.5 g/g. (a) 2,3-BDO titer and molasses consumption. (b) Cell biomass and specific cell growth rate (μ). (c) By-products titers.

Cell growth was similar for the media containing YE and CSL, with a DCW of 4.13 and 3.67 g/L, respectively. The YE replacement with NH_4Cl reduced the cell biomass formation, which reached only 2.67 g/L at the final fermentation. In contrast, the μ was reduced to 0.03 h^{-1} in the CSL-based medium, while it remained equal to 0.04 h^{-1} in the NH_4Cl-based medium. Unlike complex nitrogen sources, NH_4Cl favored a more uniform *P. peoriae* NRRL BD-62growth, with a low standard deviation between assays (Fig. 7.3b).

Again, ethanol was the main 2,3-BDO metabolism byproduct of *P. peoriae* NRRL BD-62 from sugarcane molasses, although it was drastically reduced in the CSL-based medium. While YE and NH_4Cl contributed to the ethanol production of 1.85-2.0 g/L, CSL reduced its synthesis by 4.6-fold, reaching around 0.42 g/L. Except for CSL, which showed a small acetic acid accumulation of 0.48 g/L, the organic acid production from the other nitrogen sources was low or negligible, considering both lactic acid and acetic acid (Fig. 7.3c).

The lower 2,3-BDO production observed using CSL and NH_4Cl can be justified by the nutritional limitation resulting from these compounds' nature and chemical composition, although a C/N ratio of 8.5 g/g was initially maintained in all fermentation assays. CSL is a byproduct of the wet milling process rich in amino acids, vitamins, minerals, reducing sugars, organic acids, and enzymes. It may also contain phenolic compounds and other cell growth-limiting agents (ZHOU, YU, *et al.*, 2022). Furthermore, inhibitory agents in the CSL-based media used here may have been responsible for incomplete glucose consumption, which reduced the carbon available for the 2,3-BDO synthesis.

In contrast, the lower cell biomass and the low 2,3-BDO production seen in the NH_4Cl-based medium may have been caused by the absence of growth factors responsible for affecting the 2,3-BDO metabolic activity by *P. peoriae* NRRL BD-62. Furthermore, the effects of varying C/N ratios throughout fermentation appear to have been more pronounced in NH_4Cl-based media due to the nutritional limitation imposed by its chemically defined nature. Finally, the 2,3-BDO production seems to have been unaffected by chloride released in the NH_4Cl-based medium, as previously verified by YANG, Yuling, DENG, *et al.* (2022). Therefore, the CSL and NH_4Cl nutritional

characteristics, mainly concerning nitrogen supply, were the main limiting factors for the 2,3-BDO production by *P. peoriae* NRRL BD-62 and should be considered for the culture medium selection.

The CSL and NH_4Cl effects were also observed on the $Y_{P/S}$, which reduced approximately 36% from 0.47 g/g (YE) to 0.34 g/g (CSL) and 0.35 g/g (NH_4Cl), as presented in Table 7.2. The $Y_{P/X}$ remained on average equal to 3.03 g/g, a result similar to that obtained with YE. The (R,R)-2,3-BDO optical purity was absolute for all nitrogen sources investigated, and there was no *meso*-isomer production. A small acetoin accumulation of 0.26% and 2.61% for YE and CSL was verified, respectively. The medium containing NH_4Cl did not show acetoin accumulation, contributing to an exclusive 2,3-BDO selectivity as a *levo*-isomer.

Table 7.2: Nitrogen source effects on 2,3-BDO production by *P. peoriae* NRRL BD-62 from molasses in 71h of batch fermentation at $C/N_{initial}$= 8.5 g/g.

Carbon source	**$Y_{P/S}$ (g/g)**	**$Y_{P/X}$ (g/g)**	**Selectivity (*levo*:*meso*:acetoin)**	**Total initial sugar (g/L)**
Yeast extract	0.47 ± 0.02	3.07 ± 0.16	1:0:0.003	27.93 ± 1.37
Corn steep liquor	0.34 ± 0.01	2.69 ± 0.84	1:0:0.03	32.33 ± 0.09
Ammonium chloride	0.35 ± 0.04	3.38 ± 0.43	1:0:0	27.26 ± 0.06

C/N ratio= carbon/nitrogen ratio; $Y_{P/S}$= 2,3-BDO/substrate yield; $Y_{P/X}$= 2,3-BDO/dry cell weight yield.

DAI, ZHAO, *et al.* (2015) optimized the culture medium composition for the 2,3-BDO production by *E. cloacae* (CGMCC 6053) from sugarcane molasses. Urea, CSL, and mineral salts were considered critical nutrients of the culture medium. However, CSL was discarded from the final composition after it was found that its absence did not compromise 2,3-BDO synthesis. A 2,3-BDO:acetoin ratio of approximately 5.3:1 was verified in batch fermentation. PSAKI, MAINA, *et al.* (2019) investigated different nitrogen sources to improve the 2,3-BDO production efficiency from molasses by an *E. ludwigii* strain. The 2,3-BDO yields of around 0.40 g/g were achieved using ammonium sulfate, urea, YE, and YE combined with CSL. The treated and untreated CSL alone, and untreated CSL combined with YE, resulted in yields around 0.35 g/g. WANG, Dexin, OH, *et al.* (2021) reported a high 2,3-BDO yield of 0.48 g/g with optical purity of approximately 90% *meso*-isomer by *Bacillus subtilis* CS13 from the sucrose in aerobic

batch fermentation. Ammonium citrate-based medium was selected as the sole nitrogen source after optimizing nutrient concentration.

Since the best results were obtained for YE, fermentation assays using a lower nitrogen concentration were carried out to minimize the culture medium costs. The newly investigated C/N ratio was equal to 11 g/g. A comparison of the results found for 2,3-BDO production, cell growth, sugarcane molasses consumption, and fermentation parameters for C/N= 8.5 and 11 g/g assays in 48 h are presented in Figure 7.4.

Overall, 2,3-BDO production, cell biomass formation, and sugarcane molasses consumption were reduced with an increasing C/N ratio to 11 g/g (Fig. 7.4a). Approximately 8.5 g/L of 2,3-BDO was obtained from consuming 89% of the nearly 30 g/L of sugars initially present in sugarcane molasses, which was also used for a small cell accumulation of 2 g/L. These results were about 1.2-fold, 2-fold, and 1.05-fold lower than those obtained at C/N= 8.5 g/g.

Except for $Y_{P/S}$, μ and $Y_{P/X}$ increased with increasing C/N ratio (Fig. 7.4b). While μ increased from 0.04 to 0.07 h^{-1}, $Y_{P/X}$ almost doubled, reaching about 4.26 g/g. The improved $Y_{P/X}$ suggested that the carbon flux was preferentially directed to the 2,3-BDO synthesis rather than cell biomass, representing a productive advantage. However, the $Y_{P/S}$ decreased by almost 15%, from 0.39 to 0.34 g/g. This result was similar to those obtained using CSL and NH_4Cl in 71 h.

The selectivity of 4C-compounds was also altered with an increasing C/N ratio (Fig. 7.4c). The (R,R)-2,3-BDO selectivity decreased from 86.9% to 74.8%, while that of *meso*-isomer increased from 12.9% to 25.2%. Despite the decrease in optical purity, no acetoin accumulation was detected in the medium at C/N= 11 g/g, indicating complete conversion to 2,3-BDO. The ethanol and acetic acid production were also reduced from 2.22 and 0.28 g/L to only 0.41 and 0.0 g/L, respectively. Lactic acid was not detected in any of the investigated conditions.

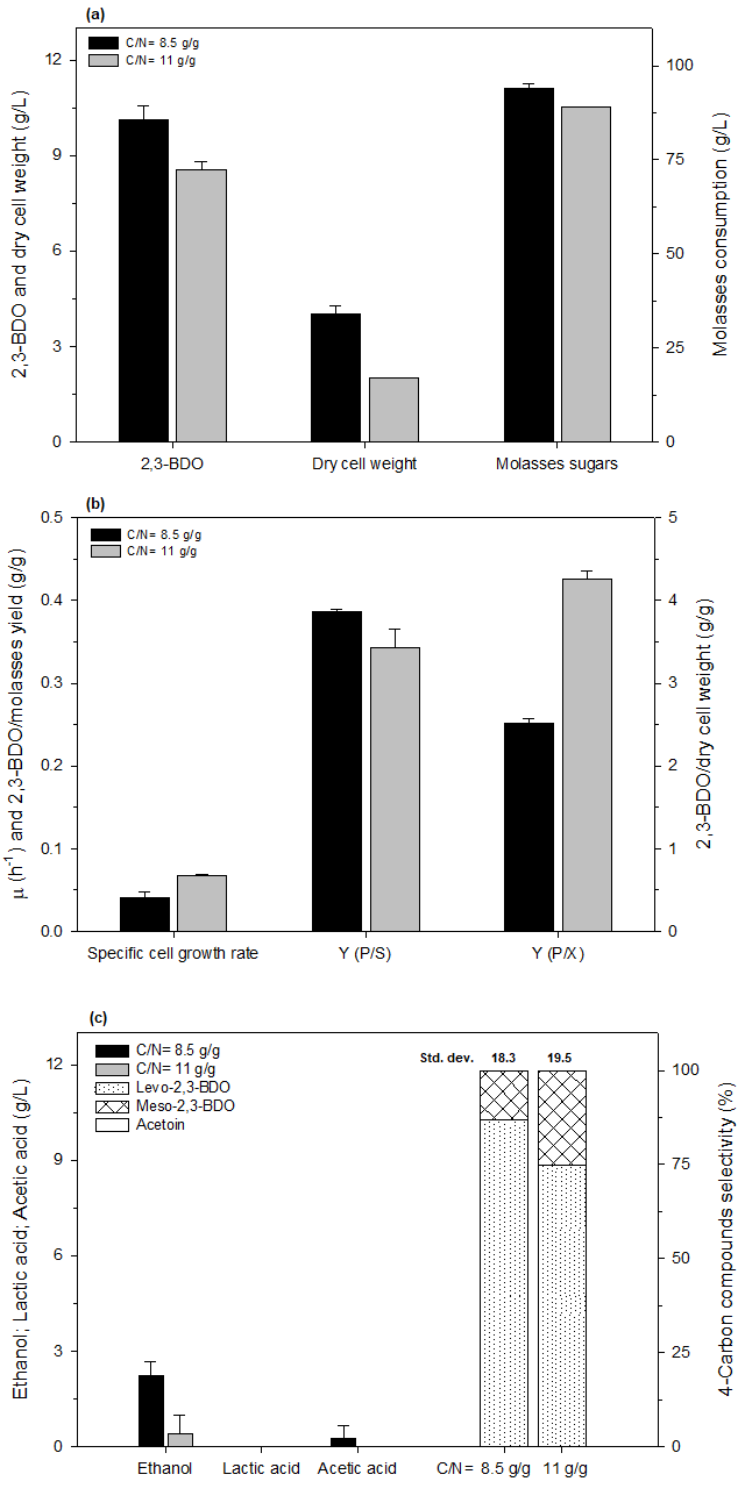

Figure 7.4: Effects of different initial C/N ratios on 2,3-BDO production by *P. peoriae* NRRL BD-62 from molasses and yeast extract-based medium in batch fermentation in 48 h. (a) 2,3-BDO titer, cell biomass, and molasses consumption. (b) Specific cell growth rate (μ), 2,3-BDO yield ($Y_{P/S}$), and specific 2,3-BDO yield ($Y_{P/X}$). (c) Selectivity of 4-Carbon compounds.

3.4 Preliminary cost evaluation of culture medium

The investigated culture medium costs were compared with the control medium cost using YE and glucose as nitrogen and carbon sources, respectively. Table 7.3 presents the results based on the free-on-board (FOB) prices adopted by MERCOSUR and the prices of industrial inputs sold by specialized biotechnology companies in 2021-2022. Tables S2 and S3 (Supplementary material) show the nutrient cost details.

Table 7.3: Culture medium economy by replacing commercial glucose and yeast extract for the 2,3-BDO production by *P. peoriae* NRRL BD-62 in batch fermentation at $C/N_{initial}$= 8.5 g/g.

Culture medium	Composition	Total culture medium cost (US$/m³)[a]	Cost reduction (%)
Control	Salts + yeast extract + glucose	171.87	-
M1	Salts + yeast extract + sucrose	157.88	8.1
M2	Salts + yeast extract + molasses*	172.95	-0.6
M3	Salts + yeast extract + molasses* (C/N=11 g/g)	127.95	25.6
M4	Salts + corn steep liquor + molasses*	99.16	42.3
M5	Salts + ammonium chloride + molasses*	42.69	75.2

[a]Based on an initial 30 g/L substrate to maintain C/N= 8.5 g/g. Nutrient costs are presented in the Supplementary Material (Tables S1 and S2).
*Molasses composition: 50% (w/w) sugar.
C/N ratio= carbon/nitrogen ratio.

The highest cost corresponded to the control medium, whose value was 171.87 US$/m³. With the glucose replacement with sucrose, a reduction of just over 8% was verified. However, when sugarcane molasses was used as a cheap carbon source, the total culture medium cost increased by 0.6%. This result was attributed to the current FOB prices of 0.44 US$/kg associated with the 50% (w/w) sugarcane molasses composition. Therefore, a higher initial sugarcane molasses amount was required to ensure C/N= 8.5 g/g, making its final cost higher than that of pure glucose.

Given these results, the nitrogen source was considered the limiting factor for the culture medium costs. The saving strategies were to increase the C/N ratio to 11 g/g and the YE replacement with cheap nitrogen sources. A reduction of almost 26% was verified when using a lower initial YE concentration in the sugarcane molasses-based medium, while the CSL and NH_4Cl use contributed to a final cost lower than 80 US$/m³. A savings

of 42.3% was achieved using CSL, with a final cost of 99.16 US$/m³. In turn, NH_4Cl contributed to the highest culture medium savings of 75.2%, which resulted in a final value of only 42.69 US$/m³.

The NH_4Cl-based medium costs without the carbon source were lower than those reported by KHUNNONKWAO, JANTAMA, *et al.* (2021), who used a medium with little trace metals and about 4 g/L ammonium salts. While less than 0.012 US$/L was spent here, the authors reported about 0.43 US$/L. In contrast, the culture medium cost per kg of 2,3-BDO produced from cassava starch was lower, equal to 3.12 US$/kg, against about 4.74 US$/kg from sugarcane molasses in this study. However, it is worth noting that the cassava starch price of 0.63 US$/kg for the base year 2021 is higher than the value adopted by the authors in 2020, which was equal to only 0.40 US$/kg. CHAN, JANTAMA, *et al.* (2016) estimated the fermentation medium cost for producing 2,3-BDO from maltodextrin at 2.04 US$/kg. The medium comprised ammonium salts and maltodextrin from cassava starch, whose adopted costs were 0.43 and 0.69 US$/kg, respectively. Currently, the maltodextrin price is approximately 0.81 US$/kg (COMEXSTAT, 2022).

KOUTINAS, YEPEZ, *et al.* (2016), using the 2,3-BDO production efficiency reported by JUNG, Moo Young, PARK, *et al.* (2013), obtained a molasses-based medium cost of 2.12 US$/$kg_{2.3\text{-}BDO}$ for an annual production capacity of 10000t. The culture medium consisted of salts, organic acids, YE, and molasses. PSAKI, MAINA, *et al.* (2019), based on the calculations by KOUTINAS, YEPEZ, *et al.* (2016), estimated the cost of the raw materials to be US$17,761 million/year, which was equivalent to US$1.78/$kg_{2.3\text{-}BDO}$ for an annual production capacity of 10000t, assuming sugarcane molasses market price of 0.20 US$/kg. The culture medium was optimized and formed by ammonium salts, complex nitrogen sources, trace elements, and molasses as carbon source. REBECCHI, PINELLI, *et al.* (2018) reported a low culture medium cost of only 1.20 US$/$kg_{2,3\text{-}BDO}$. This low value was attributed to the use of only molasses and chicken meat and bone meal (CMBM) as medium nutrients, whose market prices adopted by the authors for the period 2017-2018 were equal to 0.07 and 0.25 US$/kg, respectively. Today, the prices of these feedstocks are in the range of 0.40-0.45 US$/kg, which significantly increases the final culture medium cost (COMEXSTAT, 2022).

In almost all previous studies, the sugarcane molasses price was lower than the FOB price adopted as a reference, whose current value is 0.44 US$/kg (Table S1 – Supplementary material). Therefore, the culture medium costs per kg of 2,3-BDO found here can be considered promising to achieve the bioprocess economic viability, although a detailed analysis considering other production costs is needed to reach more reliable conclusions.

3.5 Fed-batch fermentation

Based on the culture medium economy and the 2,3-BDO yield and selectivity by *P. peoriae* NRRL BD-62, the M5 medium was selected. Fed-batch fermentation assays were performed to improve the 2,3-BDO production from sugarcane molasses and NH_4Cl-based medium at an initial C/N= 8.5 g/g. Figure 7.5 presents the time courses of 2,3-BDO and its byproducts production, cell growth, and sugar consumption from sugarcane molasses in 168 h.

An initial sugarcane molasses concentration corresponding to approximately 30 g/L of sugars was used in the batch fermentation phase for 75.5 h. After that, sugarcane molasses was fed continuously into the culture medium until 144 h, and fermentation was carried out again in batch cultures for another 24 h. Sucrose, glucose, and fructose were metabolized simultaneously throughout the fermentation period, being almost wholly consumed in 168 h (Fig. 7.5a). This sugar consumption pattern was different from that reported in previous studies for substrate mixtures. A sequential metabolism profile resulting from the catabolic carbon repression effects has been commonly observed in many bacteria (JUNG, Moo Young, PARK, *et al.*, 2013).

Small glucose and fructose accumulation were seen during the constant feeding phase. Two possible explanations for this accumulation are: (a) natural sucrose interconversion into glucose and fructose throughout fermentation; (b) stress generated from repeated catabolic changes between sugars consumed, resulting from the sugarcane molasses feeding, capable of altering sucrose catabolism (JUNG, Moo-Young, JUNG, *et al.*, 2015). JANTAMA, POLYIAM, *et al.* (2015) suggested that spontaneous mutations generated during metabolic evolution were responsible for the scr operon derepression caused by the scrR gene, a transcriptional scr regulon repressor for sucrose catabolism.

As a result, preferential sucrose consumption by *K.oxytoca* KMS005-73T was observed. JUNG, Moo-Young, JUNG, *et al.* (2015) attributed preferential sucrose consumption to the overexpression of the gene encoding catabolite repressor/activator (Cra), responsible for regulating the scrAB operon in *E. aerogenes*. The authors also verified a glucose accumulation after a series of sugarcane molasses feeding pulses using the mutant *E. aerogenes* EMY-70S.

Cell biomass reached the microbial growth stationary phase in 74 h during the batch fermentation step and remained constant throughout the feeding (Fig. 7.5b). At the end, a net cell biomass formation of 10.1 g/L was verified. Gradual nutrient feeding avoids the cellular inhibition effects of the excess substrate resulting from failed water activity (SIKORA, KUBIK, *et al.*, 2016). In this study, the cellular activity of *P. peoriae* NRRL BD-62 appears to have been unaffected by constant sugarcane molasses feeding. Previous studies have suggested that untreated molasses contains nutrients such as crude protein and vitamins that may stimulate cell growth (WANG, Dexin, OH, *et al.*, 2021, ZHANG, FENG, *et al.*, 2012).

However, a lower 2,3-BDO titer of approximately 8.6 g/L was obtained with a small acetoin accumulation (Fig. 7.5c), suggesting that constant molasses feeding inhibited the 2,3-BDO formation (PSAKI, MAINA, *et al.*, 2019). The compromised 2,3-BDO metabolism was mainly a result of the impurities introduced into the fermentation broth (DAI, ZHAO, *et al.*, 2015), and the consequent increase in the culture medium viscosity responsible for affecting the initial oxygen transfer rates (YANG, Jeongmo, KIM, *et al.*, 2015), since the molasses used had not been previously treated. Furthermore, the salt medium used contained minimal nutrients essential for cell growth, which may have compromised the 2,3-BDO synthesis since the C/N ratio was modified with the culture medium feeding (CHAN, JANTAMA, *et al.*, 2016).

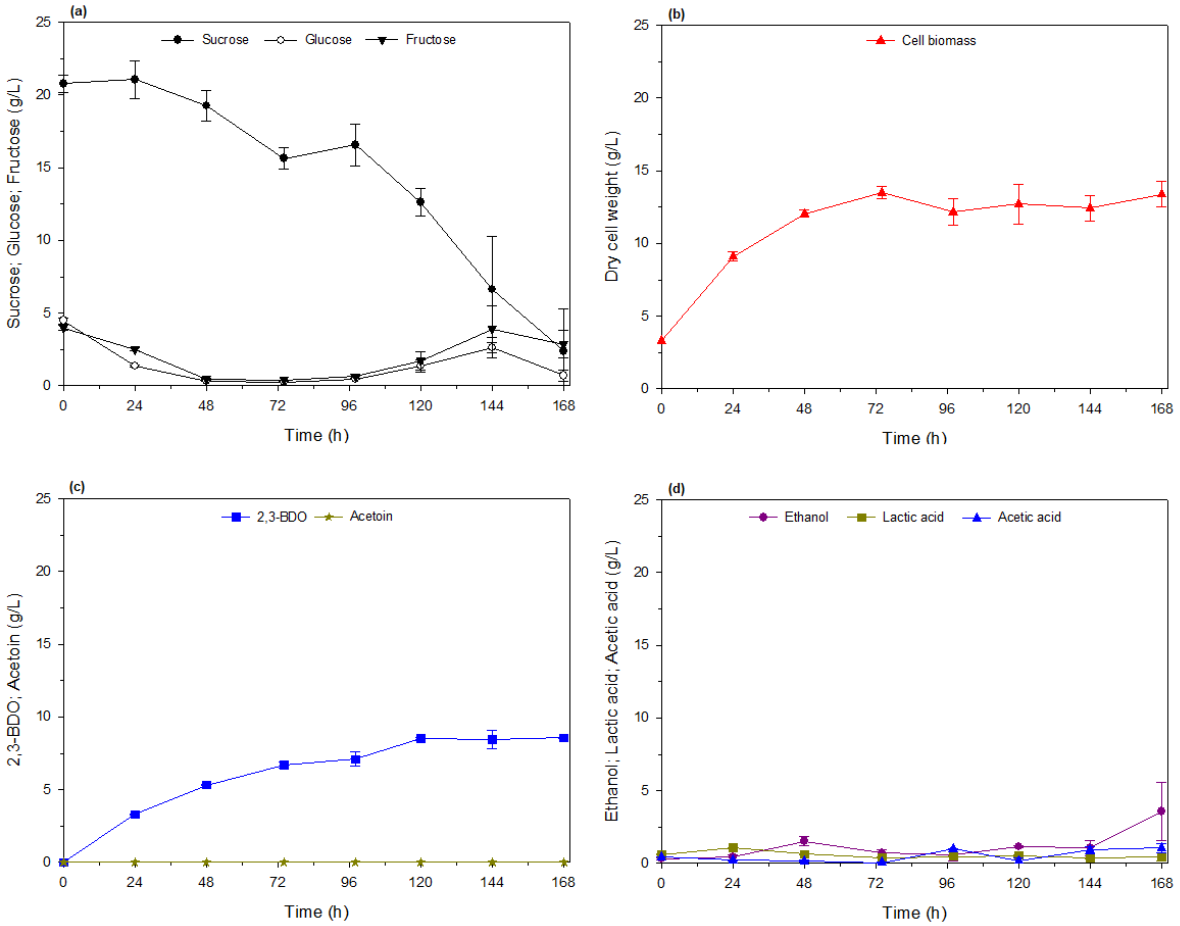

Figure 7.5: Time courses of 2,3-BDO production by *P. peoriae* NRRL BD-62 from molasses and ammonium chloride-based medium in fed-batch fermentation at constant feeding at initial C/N= 8.5 g/g. (a) Sugar consumption. (b) Cell biomass. (c) 2,3-BDO and acetoin titers. (d) By-products titers. Constant feeding was performed in the period 75.5-144 h.

Similar behavior was observed by YANG, Yuling, DENG, *et al.* (2022), who reported a reduction in sugar consumption and 2,3-BDO production when feeding the culture medium with untreated molasses used as the sole carbon source. The authors concluded that molasses-based batch cultures at initial total sugar of 35 g/L were the most suitable culture condition for the 2,3-BDO production. WANG, Dexin, OH, *et al.* (2021) observed a decrease in 2,3-BDO volumetric productivity along with a gradual decrease in cell biomass after 30 h of feeding untreated molasses in the culture medium. The authors suggested that the added metal ions and ash exceeded the threshold tolerance of *B. subtilis* CS13, which compromised the 2,3-BDO synthesis. ERIAN, GIBISCH, *et al.* (2018) also attributed the enrichment potentially substances inhibitory contained in untreated beet molasses as the cause of the reduction in substrate absorption rates and 2,3-BDO formation with the fed-batch fermentation advance.

The molasses acid pretreatment effects on 2,3-BDO and acetoin production have been previously investigated (DAI, ZHAO, *et al.*, 2015, JUNG, Moo Young, PARK, *et al.*, 2013, XIAO, Z. J., LIU, *et al.*, 2007). Furthermore, the synthesis of other bioproducts from treated molasses has been reported. PAPADAKI, PAPAPOSTOLOU, *et al.* (2018) found differences in the fumaric acid yield from treated and untreated sugarcane molasses. The authors employed identical molasses samples in this study and obtained a fumaric acid yield of only 0.18 g/g. When the molasses was treated with sulfuric acid followed by resin treatment, the yield reached 0.32 g/g, suggesting that the total phenolic content (TPC) present in the molasses may have affected the fumaric acid metabolism by *Rhizopus arrhizus* NRRL 2582.

The compromised 2,3-BDO production from sugarcane molasses in fed-batch fermentation at constant feeding was confirmed by the low $Y_{P/S}$ and $Y_{P/X}$ (Table 7.4). A 20% reduction in the 2,3-BDO yield was verified in fed-batch cultures, from 0.35 g/g to 0.28 g/g, while the 2,3-BDO specific yield was almost 4-fold lower than the verified in batch cultures, reaching only 0.85 g/g in 168 h. Despite the yield loss, the *levo*-2,3-BDO selectivity was maintained above 90% because of the microaerobic condition reproduced in fed-batch fermentations. Similar behavior was reported by PSAKI, MAINA, *et al.* (2019). The authors obtained a 93-97% *meso*-2,3-BDO production in fed-batch fermentation using synthetic medium and diluted molasses as feed solution at the 2,3-

BDO yield expense, which reached only 0.31 g/ g. In contrast, WANG, Dexin, OH, *et al.* (2021) reported a high 2,3-BDO yield of around 0.42 g/g when replacing sucrose with untreated sugarcane molasses. However, the selectivity was marked by the 4C-compounds mixture, formed by 33.9, 56.7, and 9.4% of *levo*-2,3-BDO, *meso*-2,3-BDO, and acetoin, respectively. Acetoin accumulation was attributed to the culture medium's dissolved oxygen (DO) conditions.

Table 7.4: Fed-batch fermentation at constant feeding for 2,3-BDO production by *P. peoriae* NRRL BD-62 from molasses in a salt medium using ammonium chloride at $C/N_{initial}$= 8.5 g/g.

Fermentation parameters	Units	Values
2,3-BDO	g/L	8.56 ± 0.01
Cell biomass (DCW)	g/L	10.07 ± 0.88
Total sugar consumption	g/L	31.15 ± 1.32
$Y_{P/S}$	g/g	0.28 ± 0.01
$Y_{P/X}$	g/g	0.85 ± 0.08
Selectivity	*levo*:*meso*:acetoin	10.9:1:0

C/N ratio= carbon/nitrogen ratio; 2,3-BDO= 2,3-BDO titer (*meso*- + *levo*-isomer titers); $Y_{P/S}$= 2,3-BDO/substrate yield; $Y_{P/X}$= 2,3-BDO/dry cell weight (DCW) yield.

Again, the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 was marked by the ethanol, lactic acid, and acetic acid formation, which remained below 2.5 g/L in 144 h (Fig. 7.5d). A small ethanol accumulation below 5 g/L was observed after sugarcane molasses constant feeding was stopped. Possibly, the limited DO caused by the high culture medium viscosity in 168 h shifted the carbon flux from 2,3-BDO to ethanol. Regardless of the byproduct formed, the 2,3-BDO production is compromised by diverting the total carbon flux (NARISETTY, NARISETTY, *et al.*, 2022). PALAIOGEORGOU, PAPANIKOLAOU, *et al.* (2019) reported a lactic acid accumulation of around 33 g/L from approximately 230 g/L of sugars derived from sugarcane molasses, used as the sole carbon source. The high lactic acid production compromised the 2,3-BDO + acetoin yield, which reached only 0.26 g/g in fed-batch fermentation. According to the authors, lactate dehydrogenase activity was possibly favored, while α-acetolactate synthase activity was compromised by the continuous addition of inhibitors in untreated sugarcane molasses such as phenolic compounds and

melanodins. As a result, the metabolic flux was diverted to lactic acid synthesis instead of 2,3-BDO.

Given the results, batch culture was considered the most suitable fermentation strategy for the 2,3-BDO production by *P. peoriae* NRRL BD-62 from sugarcane molasses since continuous feeding possibly increased the viscosity and impurities in the fermentation broth throughout the bioprocess. This conclusion is in line with the studies of SIKORA, KUBIK, *et al.* (2016), who reported higher cost-effectiveness when conducting three batch fermentation assays rather than a single fed-batch fermentation using glucose and sucrose as feed substrate.

Unlike this study, previous fed-batch fermentations based on intermittent sugarcane molasses feeding contributed to improved 2,3-BDO production. ERIAN, GIBISCH, *et al.* (2018) applied the 2-step cultivation strategy for 2,3-BDO production from beet molasses-derived sucrose in a mineral medium. In the first step, the cell biomass formation under aerobic conditions was prioritized, while in the second step, the 2,3-BDO synthesis was stimulated by NADH/NAD^+ recycling through low oxygen supply. A 2,3-BDO yield of 0.44 g/g was achieved after three feeding pulses. No acetoin formation was detected. The 2,3-BDO yield from residual sucrose was higher than that obtained using pure sucrose, which the authors attributed to the amino acids and trace elements in beet molasses. BIAŁKOWSKA, JĘDRZEJCZAK-KRZEPKOWSKA, *et al.* (2016) performed four glucose feeding pulses in a molasses-containing medium equivalent to an initial concentration of 68.3 g/L sucrose and observed a 7-fold increase in 2,3-BDO titer compared to that obtained in batch fermentation. The 2,3-BDO yield increased 2-fold, reaching about 0.31 g/g of reducing sugars at the bioprocess end. LEE, OH, *et al.* (2013) reported a 2,3-BDO yield of 0.39 g/g by mutant *K. pneumoniae* ΔldhA after a series of sugarcane molasses feeding pulses containing mainly sucrose, glucose, and fructose. These sugars were consumed simultaneously under optimized fermentation conditions, in which CSL was used as a nitrogen source.

4. Conclusion

The culture medium costs for the 2,3-BDO production by *P. peoriae* NRRL BD-62 were significantly reduced by replacing commercial glucose and YE with inexpensive

and highly available carbon and nitrogen sources. The capacity of *P. peoriae* NRRL BD-62 to metabolize different sugars, including glucose, fructose, and sucrose, made it possible to use sugarcane molasses as a low-cost, renewable feedstock and no impact on the food chain for humans. The good results observed for NH_4Cl in previous studies were confirmed in molasses-based medium, mainly concerning their market costs and the fermentation broth processing ease in the downstream step. The final culture medium cost per kg of 2,3-BDO was comparable to previous studies considering the current inputs' current prices. The high *levo*-2,3-BDO optical purity was maintained in fed-batch fermentation at constant feeding, although the 2,3-BDO yield was decreased, indicating that this production strategy was unsuitable for improved 2,3-BDO production. Therefore, the low-cost salt medium containing sugarcane molasses selected here was considered productively and economically promising for the *levo*-2,3-BDO production by *P. peoriae* NRRL BD-62, mainly in industrial biorefinery batch fermentation.

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CHAPTER 8

Contextualization

In this chapter, the 2,3-BDO production scale-up was performed in a pilot bioreactor with an initial volume of 70 L. Keeping the oxygen transfer rate (OTR) constant was used as the scale-up strategy to partially meet the geometric similarity criterion in three different systems: mini (350 mL), benchtop (3 L), and pilot (200 L) bioreactors. The adopted cultivation conditions considered the high 2,3-BDO yield of about 0.40 g/g, commonly reported for risk group 2 pathogens, and the instrumental limitations of the pilot plant: glucose and yeast extract-based medium at initial C/N= 8.5 g/g; low oxygen supply ($kLa \leq 7.5\ h^{-1}$), microbial pH autoregulation (~5), and temperature of 32 °C. Fermentations were performed in batch and pulse-fed batch cultures, and results were compared between the different scales. A brewer's yeast extract was used to replace traditional yeast extract to reduce the culture medium costs.

Therefore, the specific objectives of this chapter were:

- Verify the constant OTR efficiency as a scale-up strategy for high-yield 2,3-BDO production in three different systems (initial volume): mini (200 mL), benchtop (1 L), and pilot (70 L) bioreactors;
- Assess the fermentation mode effects on 2,3-BDO production on a pilot scale.

Relevance

Through the investigation carried out in this chapter, it was possible to understand:

- The effects of the constant OTR-based strategy on 2.3-BDO production at larger scales;
- The relationship between fermentation mode, fermentation scale, and high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62.

SCALE-UP OF 2,3-BUTANEDIOL PRODUCTION BY *Paenibacillus peoriae* NRRL BD-62 USING CONSTANT OXYGEN TRANSFER RATE-BASED STRATEGY

Abstract

The production of bio-based 2,3-butanediol (2,3-BDO), an important platform chemical used in different industrial applications, is still limited to the lab scale, although it has advantages over the industrial chemical route, such as the optically pure 2,3-BDO production and low energy demand. Few previous studies have investigated the bio-based 2,3-BDO production scale-up, and information is quite scarce. In this study, the 2,3-BDO production scale-up by a newly *Paenibacillus peoriae* NRRL BD-62 was investigated. Keeping the oxygen transfer rate (OTR) constant by controlling the volumetric oxygen transfer coefficient (kLa) was used to partially meet the geometric similarity criterion required in scale-up processes while maintaining microaerobic conditions for 2,3-BDO synthesis. Three different production scales were investigated in batch and fed-batch cultures (initial volume): mini (200 mL), benchtop (1 L), and pilot (70 L) bioreactors. Despite the high energy dissipated by the gassed power/volume ratio and impeller tip speed, the best results for yield, productivity, and selectivity of 2,3-BDO were obtained in batch fermentation in a pilot bioreactor, being equal to 0.40 g/g, 0.90 g/L/h, and 98% *levo*-isomer, respectively. To the best of our knowledge, this is the first time that 2,3-BDO has been produced with high yield, good productivity, and high optical purity by a wild-type *P. peoriae* strain in a pilot bioreactor. Therefore, this study provides relevant information on the OTR-based strategy for safe and efficient microbial 2,3-BDO production on a large scale.

Keywords: *Levo*-2,3-BDO, microaerobic fermentation, kLa, pilot scale, industrial production.

1. Introduction

Bio-based processes have great potential to replace traditional chemical processes to generate commercial interest products. They require less energy, do not require expensive catalysts (WONG, HUANG, *et al.*, 2012), and can be eco-friendly based on the circular economy principles and integrated biorefineries (GE, LI, *et al.*, 2016, PRIYA, LAL, 2019). Despite these advantages, few studies have investigated large-scale bioconversion processes (SCHWEDE, THORIN, *et al.*, 2017). One example is the bio-based 2,3-butanediol (2,3-BDO) production, an important platform chemical used for the synthesis of different products such as rubber, high-value drugs, antiseptics, humectants, pest control agents, aviation fuel, resins, paints, solvents, flavorings, and antifreeze, which is still limited to lab-scale (TINÔCO, PATERAKI, *et al.*, 2021).

Bio-based 2,3-BDO production depends on metabolic and operational factors, which may affect its techno-economic feasibility and, therefore, its large-scale implementation. The main limiting factors include low bioproduct yield and productivity, by-products formation, change in selectivity and isomeric optical purity, high culture media costs, production efficiency linked to unsafe microbial producers, costly downstream processing, and significant investment in scale-up processes (KOUTINAS, VLYSIDIS, *et al.*, 2014, TINÔCO, BORSCHIVER, 2020, YANG, RAO, *et al.*, 2017). Therefore, for successful industrial bio-based 2,3-BDO production, controlling the bioprocess critical factors, mainly the operational variables, is crucial to ensure the cell physiological reproducibility for the biochemical conversion processes. This control allows optimal bioprocess environmental conditions to be maintained at different scales, allowing efficient 2,3-BDO production (YANG, ZHANG, *et al.*, 2012).

Specific operating parameters such as area/volume ratio and hydrostatic pressure change with scaling-up, regardless of maintaining the optimal fermentation conditions. These modifications can accentuate the culture medium homogeneity problems resulting from deficient mixing verified in large-volume bioreactors, which compromises the cellular metabolic performance and reduces bioproduct yield (VILLADSEN, NIELSEN, *et al.*, 2011). In this case, the similarity principle and dimensional analysis must be used to ensure as little change between the different scales as possible (XIA, WANG, *et al.*, 2015). Four similarity criteria can be considered: geometric (linear dimensions),

mechanics (statics – equilibrium; kinematics - reaction fluid velocity; and dynamics - applied forces), thermal (temperature), and chemical (chemical composition of the reaction medium) (BLOCK, 2005). Among these criteria, the geometric similarity is considered a prerequisite for the scale-up relationships commonly applied by industries in bioreactor projects, although it is rarely fully met in practice (GARCIA-OCHOA, GOMEZ, *et al.*, 2010).

The main scale-up strategies are based on the constant maintenance of operational properties, which can partially meet geometric similarity and dimensional analysis between systems (BÖHM, HOHL, *et al.*, 2019, SCHMIDT, 2005). About 30% of industries use maintaining input power per unit useful volume (P/V) extrapolation and aeration extrapolation from constant volumetric oxygen transfer coefficient (kLa) in bioreactor projects (GARCIA-OCHOA, GOMEZ, 2009). A smaller portion, about 20% of the industries, also uses constant impeller tip speed (ITS) as a scale-up strategy. However, its application is less common in bacterial and yeast fermentations because it causes a reduction in P/V and thus compromises aeration and homogeneity of the reaction medium (GARCIA-OCHOA, GOMEZ, 2009, JUNKER, 2004).

An offshoot of the strategy based on constant kLa is the constant oxygen transfer rate (OTR) maintenance. Despite the consensus lack on its application as a scaling-up criterion for microaerobic processes (BYUN, ZENG, *et al.*, 1994, GARCIA-OCHOA, GOMEZ, 2009), it is known that OTR fine-tuning can maintain the intracellular $NADH/NAD^+$ balance under suitable conditions for the acetoin conversion to 2,3-BDO, consequently maximizing its yield (REBECCHI, PINELLI, *et al.*, 2018). Furthermore, the dissolved oxygen (DO) amount in the fermentation broth can be controlled by OTR. As a result, the formation of by-products such as organic acids and ethanol resulting from the carbon flux shift to pathways parallel to 2,3-BDO can be reduced, as well as the 2,3-BDO-isomer optical purity from the selective activation of key enzymes in its pathway can be favored (HÄßLER, SCHIEDER, *et al.*, 2012, RODRIGUEZ, RIPOLL, *et al.*, 2017). Alternatively, previous studies have investigated maintaining the respiration coefficient (RQ), oxygen uptake rate (OUR), and specific oxygen uptake rate (q_{O2}) as control parameters for 2,3-BDO scale-up under microaerobic conditions (BYUN, ZENG,

et al., 1994, CONVERTI, PEREGO, *et al.*, 2003, REBECCHI, PINELLI, *et al.*, 2018, ZENG, BYUN, *et al.*, 1994).

Therefore, this study aimed to scale up the 2,3-BDO production by a newly *Paenibacillus peoriae* NRRL BD-62 by maintaining constant OTR in three different systems: mini bioreactor (350 mL), benchtop bioreactor (3 L), and pilot bioreactor (200 L). High yield, high *levo*-2,3-BDO optical purity, and good productivity were achieved on a pilot scale in batch fermentation. To the best of our knowledge, this is the first time that a wild-type *P. peoriae* strain has been used for the 2,3-BDO production with high yield, good productivity, and high selectivity in a pilot bioreactor. Therefore, the results showed that constant OTR was an efficient scale-up strategy and that *P. peoriae* NRRL BD-62 is a potential candidate for large-scale 2,3-BDO production under microaerobic conditions.

2. Materials and methods

2.1 Microorganism, inoculum preparation, and media

Paenibacillus peoriae NRRL BD-62 has been isolated from the maize rhizosphere (CSM36 genotype), planted in Cerrado soil (EMBRAPA-CNPMS, Sete Lagoas, MG), and deposited in the culture collection of the Microbial Genetics Laboratory of the Microbiology Institute of the Federal University of Rio de Janeiro (UFRJ) (VON DER WEID, DUARTE, *et al.*, 2002), where it is maintained in Tryptic Soy Broth – TSB medium (17 g/L tryptone, 3 g/L soytone, 5 g/ L NaCl, 2.5 g/L K_2HPO_4, and 2.5 g/L glucose) supplemented with 25% (v/v) glycerol, at -80°C.

Initially, cells were reactivated by transferring 0.2 mL glycerol stock solution to 250 mL shake flasks containing 50 mL TSB medium and cultured at 32 °C, 200 rpm, for 24 h. For the lab-scale assays, the pre-inoculum was obtained by transferring 1% (v/v) reactivated cells to 1 L shake flasks containing 350 mL YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose), used as seed medium. The cells were cultured at 32 °C, 200 rpm, for 16 h. For the pilot-scale assay, pre-inoculum was obtained by transferring 1% (v/v) reactivated cells to a 2 L shaken flask containing 700 mL YPD medium, which was then transferred to a 10 L bioreactor containing 6.3 L YPD. The cells

were cultured at 32 °C, 200 rpm, for 16 h in shake flasks, at 32 °C, 150 rpm, and 1 vvm in a 10 L bioreactor. An inoculum of 10% (v/v) was used in the fermentation assays.

The fermentation medium used was proposed by ADLAKHA, YAZDANI (2015) and was modified to initially maintain a C/N ratio of 8.5 g/g from commercial glucose and yeast extract (YE). The fermentation medium contained (g/L): 0.5 KH_2PO_4, 2.0 K_2HPO_4, 0.0225 $MnSO_4$, and 0.3 KCl. The fermentation media were sterilized by autoclaving at 121 °C for 15 min, separating glucose from the other nutrients. Antifoam 204 (Sigma-Aldrich, USA) was used when needed.

The pilot unit was sterilized using clean-in-place (CIP) and steam-in-place (SIP) processes. Initially, 0.5% (w/v) NaOH was pumped and recirculated into the vessel, tubes, and valves and then neutralized with micro-filtered water. Next, steam at 0.5 barg into the pilot unit for 1 h. At the process end, 63 L fermentation medium was prepared in the preparation tank and pumped to the main vessel, passing through a sterile filter (0.22 μm).

2.2 Scale-up procedure

2.2.1 Bioreactors

Fermentation assays were investigated in three different reaction systems: mini bioreactor (350 mL), benchtop bioreactor (3 L), and pilot bioreactor (200 L).

DASbox® Mini Bioreactor System – 300 mL vessel (Eppendorf, Germany), designed as a 4-fold system, was equipped with temperature, pH, and dissolved oxygen (DO) sensors. The temperature was controlled by a liquid-free heating and cooling system (Peltier). The pH control was performed by peristaltic pumps responsible for adding 2 M NaOH or 1 M H_2SO_4 to the culture medium. The agitation system consisted of two Rushton-type impellers 3 cm apart, rotating clockwise, which allowed both radial and axial flows, while the aeration system was based on a submerged gas supply via an L-sparger (4 mm compression fitting) through which air was injected into a sterile filter (0.22 μm).

Bioflo®/CelliGen® 310 System – 3 L vessel (New Brunswick Scientific, Eppendorf, USA) was equipped with temperature, pH, and DO sensors. The temperature

was controlled by the bioreactor control software from a resistance temperature detector (RTD) submerged in the thermowell. The pH was controlled by a P&I (proportional & integral) controller, which operated two peristaltic pumps assigned to acid and base addition ports. The agitation system consists of one Rushton 6-plate-impeller, which provides turbulent mixing of the culture broth. And aeration system was based on a submerged gas supply via a ring sparger at the bottom of the vessel through which air was injected into a sterile filter (0.22 μm).

Pilot Plant System – 200 L vessel (Microbial Biotechnology Laboratory – LaBiM, UFRJ, Brazil) was equipped with temperature and DO sensors. The temperature was controlled by the bioreactor control software from a resistance temperature detector (RTD) submerged in the thermowell. The agitation system consists of one Rushton 4-plate-impeller, which provides turbulent mixing of the culture broth, while the aeration system was based on an overlay gas supply via aeration tubes connected to sterile filters (0.22 μm) through which air was injected.

2.2.2 Oxygen transfer rate (OTR)

The oxygen supply of the culture medium was characterized by the transfer of air bubbles to the liquid phase, where the gas was dissolved to be sent to the oxidative phosphorylation site inside the cell, considered a solid particle. Oxygen mass transfer is controlled by the liquid film resistance around the bubbles and is commonly modeled according to Whitman's two-film theory (GARCIA-OCHOA, GOMEZ, 2009). From the oxygen mass balance (Equation 1), two parameters can be defined: oxygen transfer rate (OTR) and oxygen uptake rate (OUR). While OTR corresponds to oxygen supplied to the cells, OUR corresponds to the DO consumed by the cells throughout the fermentation. Often used as a scale-up strategy by aeration extrapolation, OTR is determined based on the volumetric oxygen transfer coefficient (kLa) (Equation 2), which in turn is defined as the product between the local mass transport coefficient (kL) and the interfacial gas-liquid area (a). This parameter is usually determined experimentally by different methods. In this study, the dynamic method based on the DO measurement in the cell-free culture medium (OUR=0) by absorption and desorption of oxygen was used (Equation 3) (GARCIA-OCHOA, GOMEZ, 2009). The optimal kLa value was previously defined and

kept constant at the beginning of each scale-up assay for the 2,3-BDO production by *P. peoriae* NRRL BD-62.

$$\frac{dC}{dt} = kLa\ (Cs\text{-}C) - q_{O2}.X \qquad (1)$$

$$OTR = kLa\ (Cs\text{-}C) \qquad (2)$$

$$\ln \frac{(Cs\text{-}C_1)}{(Cs\text{-}C_0)} = -kLa\ (t_1 - t_0) \qquad (3)$$

Where C is the DO concentration in the culture medium (g/L) at two instants: initial (t_0) and final (t_1) times within the linear region of the DO curve, C_S is the saturated DO concentration at 32 °C (g/L), X is dry cell weight (g/L), and q_{O2}= oxygen uptake specific rate ($g_{O2}/g_{DCW}/h$). The oxygen parameter units are OTR and OUR=g_{O2}/L/h, and kLa=h^{-1}.

2.2.3 Batch and fed-batch fermentation

Batch fermentations were carried out in benchtop and pilot bioreactors at 32 °C, without pH control and at kLa in the range of 2-7.5 h^{-1}, with initial volumes of 1 and 70 L, respectively. The initial C/N ratio was maintained at 8.5 g/g, considering an initial concentration of 50 g/L glucose and 37.5 g/L YE. The assays were conducted once until glucose exhaustion.

Fed-batch fermentations were carried out in mini and pilot bioreactors at 32 °C, without pH control and at kLa in the range of 2-7.5 h^{-1}, with initial volumes of 200 mL and 70 L, respectively. The initial C/N ratio was maintained at 8.5 g/g, considering an initial concentration of 50 g/L glucose and 37.5 g/LYE, which in this case was replaced by a YE brewer to reduce the culture medium costs. Glucose solutions of about 500 g/L were fed by a single pulse at 72 h (mini bioreactor) and 54 h (pilot bioreactor) to restore their concentration to around 50 g/L. The assays were carried out in biological duplicates in a mini bioreactor and once in a pilot bioreactor until glucose exhaustion.

2.3 Analytical methods

The biomass concentration was determined by optical density at 600 nm ($OD_{600\ nm}$) measured in a UV–visible spectrophotometer (Biospectro SP-22, Brazil). Glucose and fermentation products (*levo*- and *meso*-2,3-BDO, acetoin, ethanol, and lactic and acetic acids) were analyzed by high-performance liquid chromatography (HPLC; Agilent, USA) equipped with an HPX-87H Aminex column (300×7.8 mm, Bio-Rad, USA), and a Refractive Index Detector (RID; Agilent, USA). The mobile phase consisted of 5 mM H_2SO_4 and HPLC-grade water at 0.6 mL/min. The column temperature was controlled at 45°C. The collected samples were initially treated before HPLC analysis by centrifugation at 10,000 x *g*, at 25 °C for 10 min, in a microtube centrifuge (MiniSpin® - Eppendorf, Germany). The supernatant was diluted 10-fold with Milli-Q water and filtered through a 0.22 μm cellulose acetate membrane (Minisart® NML Syringe Filters – Sartorius, Germany). HPLC grade standards of glucose, 2,3-BDO, acetoin, ethanol, and lactic and acetic acids (>99.5% purity) were used to quantify the substrate and fermentation products. The standard deviation was calculated for the assays performed in biological duplicates, while the error range for fermentations that were not repeated was estimated by the coefficient of variation (CV) based on fed-batch assays in mini bioreactors.

2.4 Fermentation and operational parameters

The 2,3-BDO production was evaluated by $Y_{P/S}$ yield (2,3-BDO production/glucose consumption ratio, g/g) and the selectivity of *levo*-2,3-BDO, *meso*-2,3-BDO, and acetoin (C4-compounds), calculated as percentage composition. Besides kLa and OTR, the operational parameters Pg/V (gassed power/culture volume ratio) and ITS (impeller tip speed) were calculated according to Equations 4 and 5.

$$P_g/V = 0.8N_p.\rho.N^3.D_i^5/V \qquad (4)$$

$$ITS = \pi N D_i \qquad (5)$$

Where 0.8 represents a 20% reduction due to a small air amount supplied (EPPENDORF, 2014), N_p is the impeller power number (dimensionless), ρ is the water-specific mass (1

kg/m³), N is the agitation rate (rpm), D_i is the outer impeller diameter (m), and π is the constant Pi (3.14159). The operational parameter units are $P_g/V=W/m^3$ and ITS=m/s.

3. Results and discussion

3.1 Constant OTR-based scale-up criterion

Maintaining constant OTR was defined as a scale-up criterion based on the geometric parameters of the bioreactors and the metabolic characteristics of 2,3-BDO production by *P. peoriae* NRRL BD-62. Due to the differences in the geometric relationships observed between the mini/benchtop systems and the pilot plant, and the limiting condition of bio-based 2,3-BDO production linked to a low oxygen supply, the constant kLa control was considered suitable for partially meeting the geometric similarity and dimensional analysis between the systems. Thus, the aeration extrapolation was based on the adjustment of agitation rate (N) and aeration rate (Q_{ar}), which allowed for maintaining the initial OTR in the initial range of 0.45-1.68 $mmolO_2/L/h$. Under these conditions, a microaerobic environment was established in all three scales. REBECCHI, PINELLI, *et al.* (2018) reported an optimal OTR range equal to 7-15 $mmolO_2/L/h$ for the high-yield 2,3-BDO production by *Bacillus licheniformis* ATCC9789.

Table 8.1 presents the operational parameters used in each production scale to keep the initial OTR value constant. N and Q_{ar} values were adjusted according to kLa, whose range of 2-7.5 h^{-1} was previously set as the optimal condition for the high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62. The oxygen saturation concentration was equal to approximately 0.23 $mmolO_2/L$, while the actual oxygen concentration in the culture medium corresponded to 0-5% of the saturation value until total glucose consumption. The oxygen solubility in water at 32 °C was used as a reference.

The air supply profile influenced N and Q_{ar} values in each system. Submerged-type aeration via L- and ring-sparger was used in mini and benchtop bioreactors, respectively (Table S1 – Supplementary material). The oxygen supply through the reaction solution required low N and Q_{ar} values, which remained close to the lower equipment operating limit since an efficient mass oxygen transfer was achieved by the forced air contact with the culture medium, a typical stirred tank bioreactor behavior

(SEIDEL, MASCHKE, *et al.*, 2021). On the other hand, the overlay aeration in a pilot bioreactor (Table S1 – Supplementary material) required a high N value, around the maximum safe equipment operating limit (~260 rpm), to promote oxygen diffusion in the reaction medium.

Table 8.1: Operational parameters of the different 2,3-BDO production scales by *P. peoriae* NRRL BD-62.

System	Working volume (L)	N (rpm)	Q_{ar} (vvm)	kLa (h^{-1})	OTR* ($mmolO_2/L/h$)	P_g/V** (W/m^3)	ITS (m/s)
Mini bioreactor	0.2	400	0.10	~7.5	~1.68	157.6	0.628
Benchtop bioreactor	1.0	200	0.40	5.0-7.5	1.12-1.68	126.1	0.628
Pilot bioreactor	70.0	260	0.14	2.0-7.5	0.45-1.68	270.5	2.041

*At 32 °C

**20% reduction due to a small air amount supplied (EPPENDORF, 2014), Np= 5.5 (mini and benchtop bioreactors) and 0.7Np (pilot bioreactor) (POST MIXING, 2022), $\rho_{medium} \sim \rho_{H2O\ (32\ °C)} = 995.1\ kg/m^3$

N= agitation rate; Q_{ar}= aeration rate; kLa= volumetric oxygen transfer coefficient; OTR= oxygen transfer rate; P_g/V= power input/volume ratio (agitated and aerated system); ITS= impeller tip speed; vvm= $L_{air}/L_{medium}/min$

Overlay aeration is characterized by a negligible force between the liquid and gas phases, in which the coalescence phenomena and decay of air bubbles are minimized (SEIDEL, MASCHKE, *et al.*, 2021). Despite these advantages, the overlay aeration adopted here was responsible for a higher energy demand resulting from the high N value at the pilot scale. A different behavior than expected in scale-up processes was observed for the adequate power (P) applied to the culture medium (Table 8.1). In industrial practice, the useful bioreactor volume (V) increase is accompanied by a decrease in the P/V ratio when keeping kLa constant (BÖHM, HOHL, *et al.*, 2019, EINSELE, 1976). However, this study observed a direct relationship when production scale-up from benchtop to pilot unit. The same behavior was verified for the ratio between gassed power and the useful bioreactor volume (P_g/V), whose 20% reduction concerning the P value was attributed to the small air amount used near the mechanically agitated system, responsible for turbulent flows of the reaction medium (EPPENDORF, 2014, JUNKER, 2004). The production scale-up from mini to benchtop bioreactor followed the expected

trend for P_g/V since both systems had the same oxygenation system and vessels with proportionally similar dimensions.

The increase of P_g/V was also attributed to the impeller diameter (D_i) used in the pilot bioreactor, which did not follow the geometric similarity criterion compared to the other scales. Its value was higher than that predicted by the MICHEL, MILLER (1962) model for agitated and aerated systems (Table S1 – Supplementary material). According to this model, the P_g/V value is affected by $D_i^{3.5}$, which indicates that the D_i oversizing can significantly increase the energy demanded agitation of the aerated system. In this study, D_i=0.15 m contributed to a 1.9-fold increase in the average P_g/V value of 141.85 W/m³ observed for the other 2,3-BDO production scales. Therefore, the scale economy was impaired here, which could increase the final bioprocess operating costs.

Changing the impeller type from six-bladed Rushton to four-bladed Rushton in the pilot bioreactor was not able to significantly affect the P_g/V value, although the power number (N_p) was reduced by approximately 30% (POST MIXING, 2022) from 5.5 to 3.85, respectively (Table S1). The N_p effect on P_g/V is considered small compared to the Di effect since a $P_g/V \propto N_p$ relationship is verified (BÖHM, HOHL, *et al.*, 2019). Therefore, the P_g reduction caused by N_p in this study was overcome by the increase generated by the Di oversizing, which contributed to the high P_g/V value of 270.5 W/m³ observed at the pilot scale (Table 8.1).

Another critical operational parameter affected by D_i oversizing was ITS. ITS increased about 3.25-fold in the pilot bioreactor, reaching a value of approximately 2 m/s against 0.63 m/s for the other scales (Table 8.1). The constant ITS maintenance in mini and benchtop bioreactors was attributed to the geometric similarity established between the scales, while its increase in pilot bioreactors resulted from the high N value of 260 rpm and D_i=0.15 m adopted in this system. Since ITS is related to the dissipated energy (HÄßLER, SCHIEDER, *et al.*, 2012), its increase is aligned with the high P_g/V value in the pilot scale. Nevertheless, ITS= 2 m/s was below the value typically seen in industrial production fermenters of about 3-7 m/s (JUNKER, 2004).

Like P_g/V, ITS was not kept constant for 2,3-BDO production in the pilot bioreactor (Table 1). This behavior was observed from the OTR constant used since it is

impossible to ensure similarity in all operational parameters during the scale-up process (BÖHM, HOHL, *et al.*, 2019, GARCIA-OCHOA, GOMEZ, 2009). In industrial practice, changes in the fluid dynamics and the heat and mass exchange are verified with the increase of the useful bioreactor volume, which can affect the shear rates and the Reynolds number of the systems (GARCIA-OCHOA, GOMEZ, *et al.*, 2010). Consequently, the culture medium homogeneity may be compromised, significantly reducing cell performance (LARA, GALINDO, *et al.*, 2006). Therefore, fluid-dynamic conditions are considered crucial factors in the development and scaling-up of bioprocesses and should be controlled as much as possible (RODRIGUEZ, RIPOLL, *et al.*, 2017).

Despite operational limitations, the constant OTR followed the basic rule of maintaining the optimal conditions for 2,3-BDO production in the different scales, mainly concerning low oxygen supply. Under microaerobic conditions, cell physiological activity could be maintained, which contributed to the reproducibility of the glucose biochemical transformation into 2,3-BDO by *P. peoriae* NRRL-BD-62 at the pilot scale, as will be discussed in the following sections.

LIU, ZHANG, *et al.* (2007) demonstrated that controlling N at 120 rpm and Q_{ar} at 0.04 vvm was sufficient to establish a microaerobic environment for 1,3-propanediol (1,3-PDO) production by *K. pneumoniae* DSM 2026 at pilot scale. PARK, RATHNASINGH, *et al.* (2017) maintained constant OD by adjusting N at 70 rpm and reproducing Q_{ar} at 0.5 vvm in a 5 m³ bioreactor for the 1,3-PDO and 2,3-BDO production by a mutant *K. pneumoniae* ΔldhA Δmdh. Despite a slight reduction in the fermentation parameters, the fermentation conditions were suitable for diol production scale-up. In contrast, BYUN, ZENG, *et al.* (1994) concluded that the constant OTR criterion was not applicable for the 2,3-BDO production scale-up by *E. aerogenes* DSM 3005 since the fluid dynamics of large bioreactors under microaerobic conditions did not favor a homogeneous oxygen supply throughout the reaction volume. As a result, the growth and metabolism of *E. aerogenes* DSM 3005 were affected by a more prolonged circulation and mixing time, which compromised the maximum 2,3-BDO production at an OTR of 35 $mmol_{O2}$/L/h.

3.2 Scale-up of microaerobic batch fermentation

Microaerobic batch fermentations were carried out in benchtop and pilot bioreactors for 168 h and 54 h, respectively. The time courses of glucose consumption, 2,3-BDO production and by-products, and cell biomass formation for each system are shown in Figure 8.1.

On the benchtop scale, maximum 2,3-BDO production of 22.5 g/L was reached in 145 h after *P. peoriae* NRRL BD-62 entered a cell growth stationary phase (Fig. 8.1a). Although the carbon source was nearly exhausted by 120 h, with a consumption of approximately 54 g/L of glucose, 2,3-BDO continued to be produced until 145 h. During this interval, the ethanol titer decreased from 3.4 to 2.2 g/L (Fig. 8.1b), whereas 2,3-BDO increased from 24 to 24.9 g/L (Fig. 8.1a). In the same period, the cells showed a slight decrease in their $OD_{600\ nm}$, reaching a net value of 4 in 145 h (Fig. 8.1a). These results suggested that the ethanol consumed was used exclusively for the 2,3-BDO production in the glucose absence since no maintenance of the cell biomass was observed in this period. A small acetoin accumulation of only 0.11 g/L was also detected in 145 h (Fig. 8.1a), along with the formation of 1.8 and 0.7 g/L of lactic acid and acetic acid, respectively (Fig. 8.1b).

Unlike the benchtop scale, maximum 2,3-BDO production was reached in a shorter time in the pilot bioreactor, considering an equal glucose consumption of approximately 54 g/L (Fig. 8.1c). A 2,3-BDO titer of 21.5 g/L was achieved in 28 h from the complete glucose metabolization at 1.9 g/L/h, about 4.5-fold higher than that seen in benchtop bioreactor of only 0.42 g/L/h. The 2,3-BDO production rate was also higher in the pilot bioreactor, around 5-fold, reaching a value of 0.76 g/L/h against 0.15 g/L/h in the benchtop bioreactor. The final 2,3-BDO titer was slightly lower at a higher working volume, a result similar to that observed by YANG, ZHANG, *et al.* (2012) when investigating the 2,3-BDO production scale-up by *B. amyloliquefaciens* B10-127.

The rapid 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 observed in the pilot bioreactor led to high cell biomass formation with net $OD_{600\ nm}$ equal to 21.2 in 28 h. This result differed from YANG, ZHANG, *et al.* (2012) for 2,3-BDO production in a 30 L bioreactor and LIU, ZHANG, *et al.* (2007) for 1,3-PDO production in a 1 m^3 bioreactor,

in which the scale-up led to slow cell growth. Again, 2,3-BDO production occurred in the cell growth stationary phase. The microbial biomass was kept practically constant until the fermentation ended due to the 2,3-BDO consumption by *P. peoriae* NRRL BD-62 in the glucose absence. No acetoin accumulation was detected in the pilot scale (Fig. 1c), while 0.8 and 5.7 g/L of ethanol and acetic acid were produced as 2,3-BDO by-products, respectively (Fig. 8.1d).

The difference in the time to maximum 2,3-BDO production between benchtop and pilot scales was attributed to the inoculum used in each system. While cells were grown with N control in shake flasks for lab-scale assays, an N and Q_{ar}-controlled benchtop bioreactor were used for large-scale applied inoculum formation. As a result, a higher initial microbial biomass equal to $OD_{600\ nm}$ of 2.3 was verified in the pilot bioreactor (70 L) versus 1.2 in the benchtop bioreactor (1 L). Differential cell activation contributed to the 5.6-fold increase in 2,3-BDO productivity from 0.16 g/L/h to almost 0.90 g/L/h at the pilot scale. This result was considered unexpected since the 2,3-BDO yield is usually favored at the expense of its productivity under low oxygen supply (ERIAN, GIBISCH, *et al.*, 2018). Therefore, better-activated inoculum use was considered an efficient strategy to overcome the low 2,3-BDO productivity by *P. peoriae* NRRL BD-62 in microaerobic fermentation. CHENG, ZHANG, *et al.* (2007) also verified the inoculum development effects at early steps of scale-up processes on the 1,3-PDO production by *K. pneumoniae* M5al from crude glycerol in a 5 L bioreactor. The reported results are also predicted on a large scale, highlighting the inoculum importance for efficient industrial bio-based 1,3-PDO production.

The 2,3-BDO productivity found here was considered economically promising since values above 0.5 g/L/h contribute to the achievement of a unit production cost of less than 1.0 $/kg bioproduct (KOUTINAS, VLYSIDIS, *et al.*, 2014). Furthermore, the increase in 2,3-BDO productivity to 0.90 g/L/h led to the product-related energy efficiency of 3.27, a value higher than that reported by BYUN, ZENG, *et al.* (1994) in a 1.5 m³ airlift reactor equal to 2.54. Even with the impaired scale economy previously identified by the high P_g/V value, E_p was considered favorable. However, this result can be further improved by applying culture medium feeding strategies to increase the 2,3-BDO volumetric productivity and the correct bioreactor design to minimize the energy

dissipated by P_g/V. With this, yields in the range of 2.5-3.0 g/L/h could be achieved, making the 2,3-BDO production costs reasonably more competitive (KOUTINAS, VLYSIDIS, *et al.*, 2014).

The acetic acid production of 5.7 g/L in the pilot bioreactor resulted from the metabolic synergy established with the 2,3-BDO pathway. Under microaerobic conditions, 2,3-BDO production was favored at both scales. However, the low oxygen supply was better established at the pilot scale due to the kLa decrease as fermentation progressed (JUNKER, 2004, RODRIGUEZ, RIPOLL, *et al.*, 2017), the DO gradient formation across the reaction medium (BLOCK, 2005, ZENG, BYUN, *et al.*, 1994), and the higher hydrostatic pressure responsible for increasing the driving force of cellular oxygen consumption (BÖHM, HOHL, *et al.*, 2019). In contrast to observations by BYUN, ZENG, *et al.* (1994), the lower adequate oxygen supply in large volumes contributed to a high $NADH/NAD^+$ ratio necessary for the acetoin conversion into 2,3-BDO. The NAD^+ released in this reaction was possibly used to initiate the acetic acid production pathway from the pyruvate conversion by the pyruvate dehydrogenase complex action (TINÔCO, PATERAKI, *et al.*, 2021). The carbon flux was then divided between the pathways, resulting in the coproduction of 2,3-BDO and acetic acid at the pilot scale.

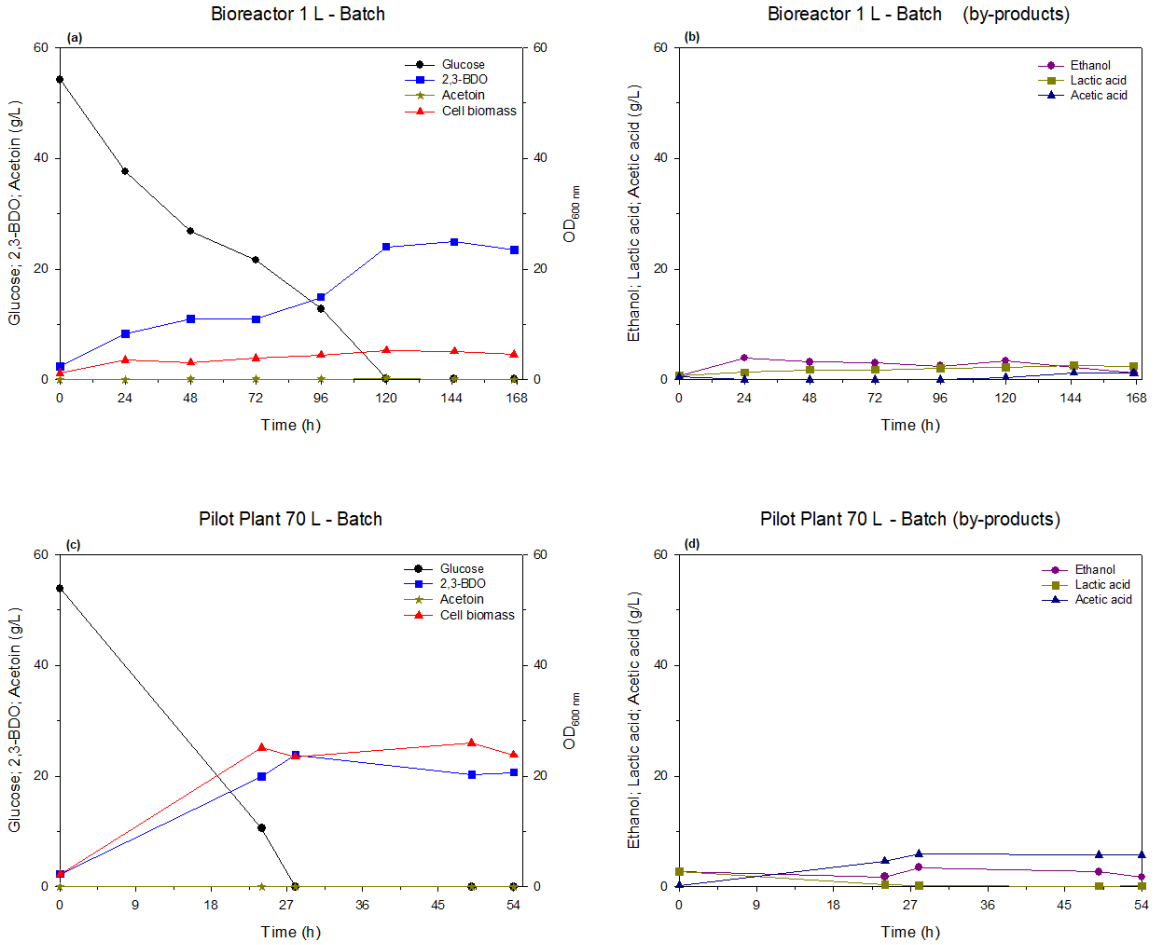

Figure 8.1: Time courses comparison of the batch fermentation for 2,3-BDO production by *P. peoriae* NRRL BD-62 in bioreactor 1 L (a, b) and pilot plant 70 L (c, d). (a) and (c) Glucose consumption, 2,3-BDO and acetoin production, and cellular optical density at 600 nm; (b) and (d) By-products: ethanol, lactic acid, acetic acid production.

The fermentation parameters obtained in each system were compared to evaluate the efficiency of the scale-up criterion adopted. Figure 8.2 presents the results found. A slight $Y_{P/S}$ reduction was observed with the change from benchtop to pilot bioreactor, which went from 0.42 to 0.40 g/g, respectively (Fig. 8.2c). This result was in line with the previously discussed coproduction of 2,3-BDO and acetic acid, in which the carbon flux shift to organic acid formation was realized at the 2,3-BDO yield expense. Despite this reduction, the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 does not appear to have been compromised by the scale-up process. Instead, its yield was maintained at around 0.40 g/g. Therefore, constant OTR was considered efficient to maintain the optimal conditions for the high-yield 2,3-BDO production by *P. peoriae* NRRL BD-62 in pilot-scale batch fermentation. MAINA, SCHNEIDER, *et al.* (2021) could replicate the experimental results obtained in a 5 L bioreactor in a 50 L bioreactor by maintaining kLa. A 2,3-BDO yield of 0.36 g/g was achieved by *B. amyloliquefaciens* 18.025 (BCCM-LMG) from bread waste hydrolysate-based glucose in batch fermentation.

Low oxygen supply in the pilot bioreactor also favored the *levo*-isomer optical purity and the 2,3-BDO selectivity (HÄßLER, SCHIEDER, *et al.*, 2012), which reached approximately 98 and 100%, respectively, without acetoin accumulation (Fig. 8.2d). Differently, the selectivity of the 4-Carbon compound was characterized by 78.3, 21.3 and 0.4% of *levo*-2,3-BDO, *meso*-2,3-BDO, and acetoin in benchtop bioreactor, respectively, suggesting that the supplied oxygen was better transferred to the cells from the submerged type aeration. GE, LI, *et al.* (2016) reported higher optical purity in the 2,3-BDO production on a larger scale. The cell growth of the mutant *B. licheniformis* MW3 (Δgdh) was not affected, and the *meso*-2,3-BDO production was favored, achieving a high yield of 0.49 g/g and high selectivity of 99.6%, with the increase of the useful bioreactor volume from 5 to 50 L.

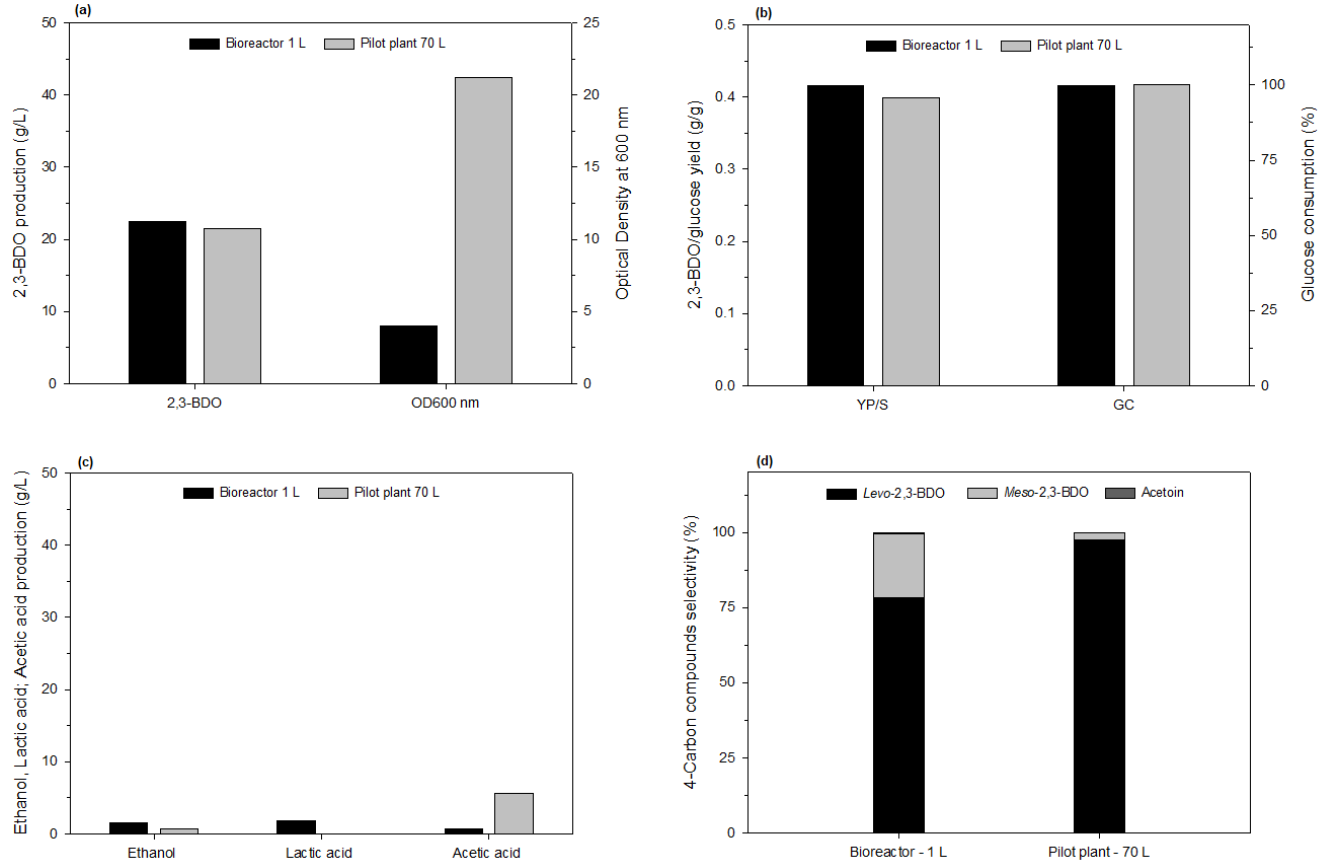

Figure 8.2: Fermentation parameters comparison of batch cultures for 2,3-BDO production by *P. peoriae* NRRL BD-62 in bioreactor 1 L and pilot plant 70 L. (a) 2,3-BDO production (CV=5.35%) and cellular optical density at 600 nm (CV=0.32%); (b) 2,3-BDO/glucose yield (CV=2.83%) and glucose consumption (CV=0.36%) (c) By-products: ethanol (CV=7.51%), lactic acid (CV=8.88%), acetic acid production (CV= 0%); (d) 4-Carbon compounds selectivity (CV_{levo}=0.61%, CV_{meso}=9.12%, $CV_{acetoin}$=0%). CV= coefficient of variation.

3.3 Scale-up of microaerobic one-pulse fed-batch fermentation

A glucose pulse feeding strategy was implemented to improve the 2,3-BDO titer without compromising its yield at the pilot scale. Initially, fed-batch fermentation was carried out in a mini bioreactor for 215 h to evaluate the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62. Then, the 2,3-BDO production was investigated in a pilot bioreactor for 119 h. The time courses of glucose consumption, 2,3-BDO production and its by-products, and cell biomass formation observed in each system are shown in Figure 8.3.

Maximum 2,3-BDO production of 37.6 g/L was achieved in 192 h in the mini bioreactor after the system was fed with pure glucose for 72 h (Fig. 8.3a). Substrate addition was performed at the time when the cells seemed to lose metabolic activity, with a drop in $OD_{600\ nm}$ from 7 to 4.5. Just over 32 g/L glucose was consumed by *P. peoriae* NRRL BD-62 at 0.43 g/L/h in 72 h. Glucose was fed to maintain its concentration of around 60 g/L. So, the cells grew again, reaching a stationary phase in approximately 120 h at an $OD_{600\ nm}$ of 9.9. Furthermore, glucose consumption increased 1.9-fold, showing a rate of 0.81 g/L/h in 72-144 h. The 2,3-BDO production rate remained around 0.20 g/L/h throughout the fermentation, except for192-215 h, when a small drop was observed. A net production of 5.4 and 2.5 g/L of ethanol and lactic acid was also verified in 192 h, respectively. No acetoin and acetic acid accumulation were detected in this period (Fig. 8.3b).

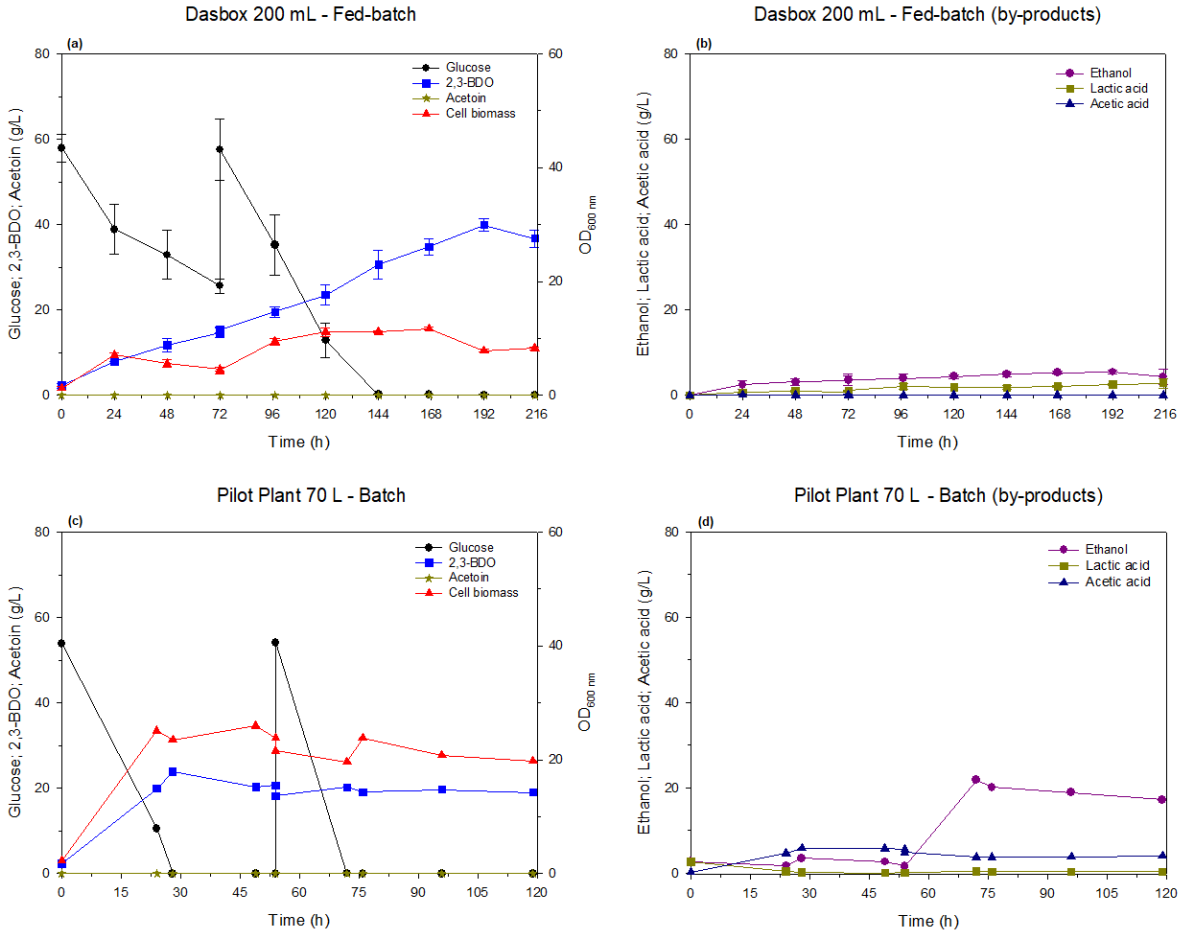

Figure 8.3: Time courses comparison of the one-pulse fed-batch fermentation for 2,3-BDO production by *P. peoriae* NRRL BD-62 in Dasbox system 200 mL (a, b) and pilot plant 70 L (c, d). (a) and (c) Glucose consumption, 2,3-BDO and acetoin production, and cellular optical density at 600 nm; (b) and (d) By-products: ethanol, lactic acid, acetic acid production.

Based on the results in the mini bioreactor, glucose was expected to be entirely metabolized by *P. peoriae* NRRL BD-62 after 72 h in the pilot bioreactor. However, glucose was exhausted in 28 h, as previously discussed, which forced substrate feeding to be brought forward to 54 h (Fig. 8.3c). This time was set due to limitations in treating the samples, whose results took at least 24 h to be ready. Therefore, the fermentation was maintained without food for 28-54 h, not preventing the bacterial cells from starvation (PRIYA, DUREJA, *et al.*, 2016). To maintain cell viability, 2,3-BDO was partially consumed by *P. peoriae* NRRL BD-62, which reduced its titer from 21.5 to 15.8 g/L. As a result, the $OD_{600\ nm}$ was maintained at around 22. The concentration of ethanol, lactic acid, and acetic acid was also reduced, suggesting a shift in carbon flux to cell maintenance processes rather than the 2,3-BDO-linked metabolite production pathways (Fig. 8.3d).

Only 2.1 g/L 2,3-BDO was produced in 72 h from approximately 54 g/L glucose feeding (Fig. 8.3c). In contrast, about 20.4 g/L of ethanol was found in the same period (Fig. 8.3d) from total glucose consumption by *P. peoriae* NRRL BD-62, whose cell biomass decreased slightly until 119 h (Fig. 3c). CHENG, ZHANG, *et al.* (2007) observed an ethanol increase using a glycerol-deficient pre-culture medium (<5 g/L) as substrate. The authors reported a lower 1,3-PDO production when ethanol-rich seed liquids were used in 5 L bioreactor fermentations.

In this study, ethanol production at the expense of 2,3-BDO was attributed to the highly restricted oxygen environment in the pilot bioreactor, whose supply was possibly lower than cellular demand (RODRIGUEZ, RIPOLL, *et al.*, 2017), especially in 28-54 h. During this interval, the multienzyme pyruvate dehydrogenase complex appears to have been activated by NAD^+ released upon acetoin conversion to 2,3-BDO at 28 h, as previously discussed. The difference is that the acetyl-CoA obtained from pyruvate conversion was used for acetaldehyde synthesis instead of acetyl phosphate due to the low ATP availability under microaerobic conditions (TINÔCO, PATERAKI, *et al.*, 2021). Acetaldehyde was converted to ethanol to maintain the $NADH/NAD^+$ balance, whose production competed with 2,3-BDO, resulting in the accumulation of both products in 72 h (REBECCHI, PINELLI, *et al.*, 2018). A small lactic acid amount of 0.3

g/L was verified in this period, and 5.7 g/L of acetic acid was already produced in 28 h. Again, no acetoin accumulation was detected throughout the fermentation (Fig. 8.3d).

A recent study proposed a coupling mechanism between 2,3-BDO synthesis and ethanol formation for eliminating the acetate accumulation produced from glucose (MENG, ZHANG, *et al.*, 2021). According to this proposal, NADH released in glycolysis and not used in the acetoin conversion to 2,3-BDO is provided for the bifunctional acetaldehyde/ethanol dehydrogenase (AdhE)-catalyzed ethanol production from acetyl-CoA, which in turn is obtained from ATP-consuming acetate conversion. Metabolic reactions are driven so that the coproduction of ethanol and 2,3-BDO replaces a toxic product accumulation in cells using this overflow strategy. Consequently, the fermentation broth acidification is avoided, favoring the industrial bioprocess application. Although the ability of *P. peoriae* NRRL BD-62 to perform this metabolic strategy is unknown, the acetic acid production absence, along with the ethanol accumulation and a small 2,3-BDO amount after delayed feeding of the culture medium, suggests that this strain used acetate overflow to balance the proteomic demands of cell biomass formation and energy generation. This hypothesis is supported by the high microbial growth verified in the pilot bioreactor.

The fermentation parameters obtained in each system were compared again, as shown in Figure 8.4. The 2,3-BDO yield was affected by the delay in feeding the pilot bioreactor, which was also responsible for changing the *levo*-2, 3-BDO optical purity (Fig. 8.4c). The 2,3-BDO yield was halved from 0.42 g/g in the mini bioreactor to 0.22 g/g in the pilot bioreactor, followed by the ethanol formation, whose yield was equal to 0.19 g/g. Although the 2,3-BDO production was affected in fed-batch fermentation in the pilot bioreactor, the glucose conversion to 2,3-BDO metabolism products by *P. peoriae* NRRL BD-62 was maintained at a yield of 0.41 g/g, considering the joint production of 2,3-BDO and ethanol. This result was interesting since it suggested a favorable outcome for 2,3-BDO synthesis at the pilot scale if feeding was performed immediately after glucose depletion. Without the idle interval verified in the pilot bioreactor, the cell performance could have been preserved, thus ensuring a high 2,3-BDO yield of around 0.40 g/g.

PRIYA, DUREJA, *et al.* (2016) investigated the 2,3-BDO production by *E. cloacae* TERI BD18 from glucose in a 150 L bioreactor. The authors reported a high 2,3-BDO yield of 0.48 g/g in fed-batch fermentation. The assays reproduced a pH and N-based strategy on a pilot scale, defined by a two-step pH (7.5 → 6.5) and a dual agitation rate (200 → 150 rpm) in 50 h. ERIAN, GIBISCH, *et al.* (2018) proposed the application of continuous bioprocessing for the 2,3-BDO production by a mutant *E. coli* W. By this strategy, the authors aimed to combine the good titer and yield results obtained in fed-batch fermentation under low oxygen supply with a high 2.3-BDO volumetric productivity to make the proposed bioprocess industrially relevant.

The selectivity of 4-C compounds was marked exclusively by the 2,3-BDO formation due to nil net acetoin production in both systems evaluated (Fig. 4d). However, a 5.2% reduction in *levo*-2,3-BDO optical purity in the pilot bioreactor was verified. Despite the increase in the relative *meso*-2,3-BDO amount in fed-batch fermentation, the *levo*-2,3-BDO selectivity was maintained at around 93%. Reproducing the optical purity observed on a lab scale during large-scale optically pure 2,3-BDO production is challenging. This difficulty is in line with the limited number of previous studies on the 2,3-BDO production scale-up (GE, LI et al., 2016). Therefore, the results provided relevant information for the industrial 2,3-BDO production by *P. peoriae* NRRL BD-62, mainly of its optically pure *levo*-isomer, presenting significant potential in future applications.

Table 8.2 summarizes the significant studies on bio-based 2,3-BDO production on the pilot scale. Information about the producing microorganisms, bioreactor volume, fermentation mode, and oxygen supply was correlated with the 2,3-BDO titer, yield, productivity, and selectivity. However, few studies investigating large-scale bio-based 2,3-BDO production have been reported to date.

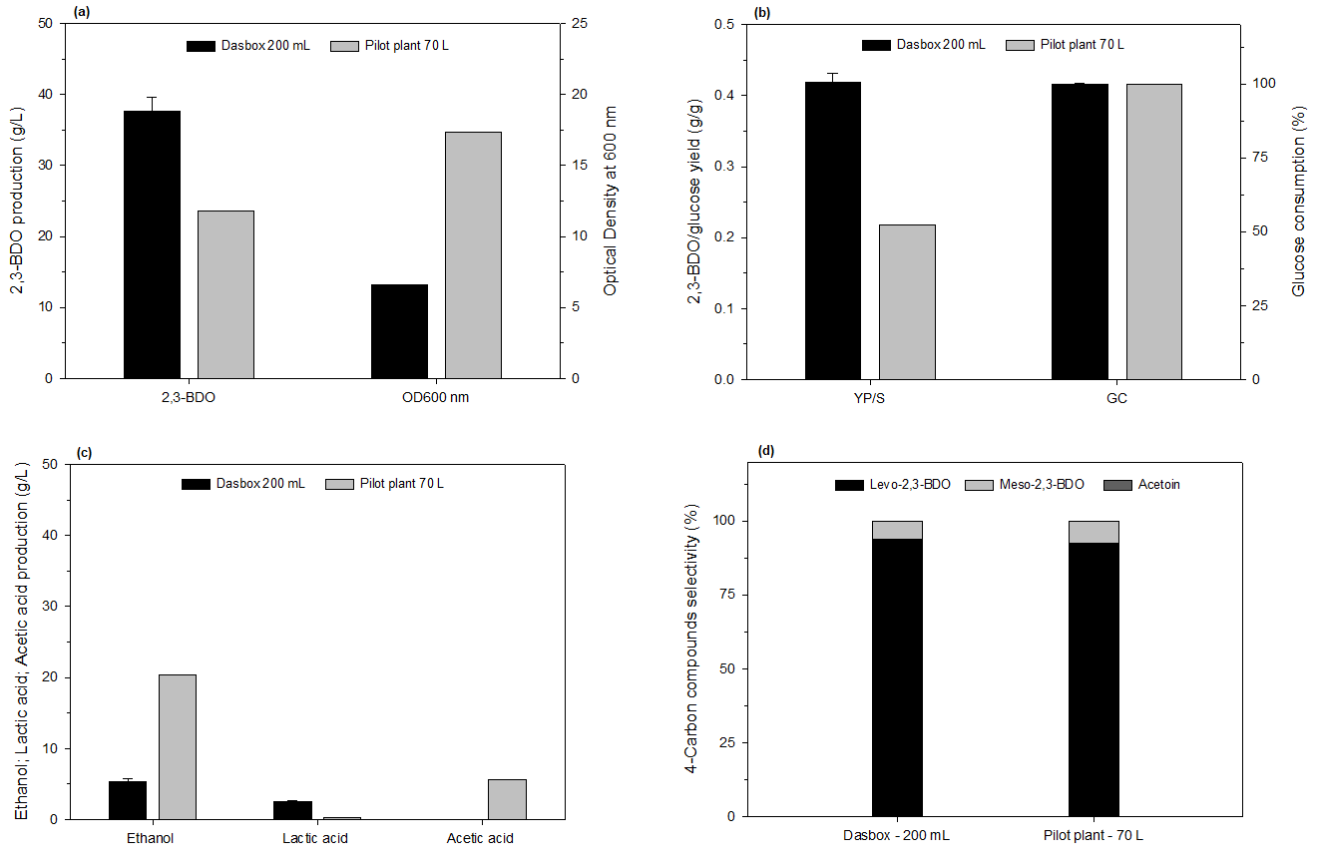

Figure 8.4: Fermentation parameters comparison of one-pulse fed-batch cultures for 2,3-BDO production by *P. peoriae* NRRL BD-62 in Dasbox system 200 mL and pilot plant 70 L. (a) 2,3-BDO production (CV=5.35%) and cellular optical density at 600 nm (CV=0.32%); (b) 2,3-BDO/glucose yield (CV=2.83%) and glucose consumption (CV=0.36%) (c) By-products: ethanol (CV=7.51%), lactic acid (CV=8.88%), acetic acid production (CV= 0%); (d) 4-Carbon compounds selectivity (CV_{levo}=0.61%, CV_{meso}=9.12%, $CV_{acetoin}$=0%). CV= coefficient of variation.

Table 8.2: Comparative analysis of pilot-scale bio-based 2,3-BDO production.

Strain	Risk factor	Bioreactor full-volume (L)	Fermentation mode	Oxygen supply	2,3-BDO (g/L)	$Y_{P/S}$ (g/g)	Qp (g/L/h)	Selectivity (%)	Reference
Enterobacter aerogenes DSM 30053 (wild strain)	2	1500	Batch	OTR = 35 mmolO_2/L/h	45.0*	0.37	2.44	-	BYUN, ZENG, *et al.* (1994)
E. aerogenes DSM 30053 (wild strain)	2	50	Batch	RQ = 4-4.5	~60.0*	~0.34*	~2.00*	-	ZENG, BYUN, *et al.* (1994)
Bacillus amyloliquefaciens B10-127 (wild strain)	1	30	Batch	300 rpm 0.66 vvm (submerged)	61.4	0.38	1.71	86% 2,3-BDO 14% acetoin	YANG, ZHANG, *et al.* (2012)
B. licheniformis MW3 (Δgdh) (mutant strain)	1	50	Fed-batch	500 rpm 1.0 vvm (submerged)	90.1	0.49	2.82	95.8% *levo*-2,3-BDO 0.4% *meso*-2,3-BDO 3.8% acetoin	GE, LI, *et al.* (2016)
E. cloacae TERI BD 18 (wild strain)	2	150	Fed-batch	200 → 150 rpm (two-stage)	85.9	0.48	1.73	99.1% 2,3-BDO 0.9% acetoin	PRIYA, DUREJA, *et al.* (2016)
B. amyloliquefaciens 18,025 (BCCM-LMG) (wild strain)	1	50	Batch	kLa = 116 h^{-1}	~42.0	0.36	1.64	~79% *levo*-2,3-BDO 21% acetoin	MAINA, SCHNEIDER, *et al.* (2021)
Paenibacillus peoriae NRRL BD-62 (wild strain)	1	200	Batch	OTR ≤ 1.68 mmolO_2/L/h	21.5	0.40	0.90	98% *levo*-2,3-BDO 2% *meso*-2,3-BDO	This study

*2,3-BDO + acetoin

2,3-BDO= 2,3-BDO titer (*meso*- + *levo*-isomer titers); $Y_{P/S}$= 2,3-BDO/glucose yield; Qp= 2,3-BDO productivity; Selectivity= *levo*-/*meso*-2,3-BDO/acetoin ratio; vvm= L_{air}/L_{medium}/min; RQ= respiratory coefficient; OTR= oxygen transfer rate

4. Conclusion

The application of the constant OTR maintenance strategy to increase the 2,3-BDO production by *P. peoriae* NRRL BD-62 was considered efficient in this study since the excellent results obtained in mini and benchtop bioreactors were reproduced in the pilot scale, especially the high yield 2,3-BDO around 0.40 g/g. A good 2,3-BDO volumetric productivity and a high *levo*-isomer selectivity were also verified in batch fermentation at the pilot scale due to the synergism established between an efficient cell activation of the inoculum used and a higher oxygen restriction typical of large equipment. Therefore, the partial fulfillment of the geometric similarity criterion and the establishment of microaerobic conditions necessary for the 2,3-BDO synthesis were validated on the pilot scale at constant OTR. To the best of our knowledge, this was the first time that 2,3-BDO was produced with high yield, good productivity, and high optical purity by a wild-type *P. peoriae* strain in batch fermentation in a pilot bioreactor.

Good results are also expected for the 2,3-BDO fermentation parameters in fed-batch cultures at the pilot scale, despite the productive limitations verified in this study. This hypothesis was based on the affected 2,3-BDO metabolism following a practical rather than technical problem of the fermentation method. The ethanol formation at the expense of the high 2,3-BDO yield was considered reversible since no carbon flux deviation to another pathway type was verified, only to a pathway directly related to 2,3-BDO metabolism. Furthermore, the *levo*-2,3-BDO selectivity remained high, above 90%, and the glucose consumption rate was similar to that observed in batch fermentation, even with the delayed feeding of the system. These observations suggested that the mass balance can be returned to 2,3-BDO synthesis if the feeding problem is overcome.

Therefore, the 2,3-BDO production by *P. peoriae* NRRL BD-62 in the pilot bioreactor was considered promising. The results provide relevant information on using new microbial producers and applying a constant OTR-based strategy for safe and well-performing industrial bio-based 2,3-BDO production.

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CHAPTER 9

Contextualization

In this chapter, notes of the upstream and mainstream steps of the proposed 2,3-BDO bioprocess have been made to better understand the scientific contributions obtained from this doctoral thesis. The main results and conclusions verified in each chapter were summarized.

Relevance

Through the investigation carried out in this doctoral thesis, it was possible to understand:

- The relationship between bioprocess steps (upstream and mainstream) and the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62;
- The effects of optimized fermentation conditions, culture medium composition, fermentation modes, and scale-up strategies on the 2,3-BDO yield, selectivity, and economy;
- Metabolic control as a tool for achieving a bio-based 2,3-BDO production that is more cost-effective, safe, eco-friendly, and based on circular economy and biorefinery principles.

1. Scientific contributions

The main scientific contributions provided by this doctoral thesis are highlighted below. The main results and conclusions obtained in each chapter have been summarized.

1.1 Chapter 2

- The techno-scientific prospect analysis showed that despite the advantages over the chemical route, the industrial bio-based 2,3-BDO production is not yet consolidated, being strongly limited to the lab scale;
- The main limiting factors for large-scale bio-based 2,3-BDO production were grouped into four drivers: production process, recovery process, product, and application. More than 50% of the documents evaluated addressed the 2,3-BDO production step, while less than 15% referred to the 2,3-BDO-isomer formed and its main industrial applications;
- The production process was the main focus in the short, medium, and long terms, while the 2,3-BDO applications were prioritized at the current stage due to some industries' bioprocess already commercially implemented. Furthermore, an increase in interest for 2,3-BDO recovery technologies was observed in the long term;
- The production process was represented by microorganisms (wild and mutants; safe and pathogenic), substrates (commercial and residual), nutritional supplementation (amino acids, vitamins, minerals, and other stimulatory compounds), and methods (fermentation modes, process integration, and cultivation conditions). Most of the documents showed that the microorganisms (wild and mutant-types of *Klebsiella, Enterobacter, Serratia, Bacillus, Paenibacillus, Clostridium, Raoultella, Zymobacter, Escherichia coli, Pichia pastoris,* and *Saccharomyces cerevisiae*) and substrate (artichoke biomass, vinegar, corn hydrolysate, xylose, whey, oil palm, cassava, and crude glycerol) had been researched over time, although production methods have gained prominence in the long term (simultaneous saccharification and fermentation of lignocellulosic biomass, fermentation conditions optimization, and culture medium composition, and fed-batch fermentation application);

- The recovery process was represented by distillation, membrane separation, solvent extraction, ion exchange, salting-out, and sugaring-out. Distillation was the leading technology used to recover bio-based 2,3-BDO from fermentation broth in the current stage, short and medium terms, while solvent extraction has been investigated in the long term;
- Product and applications were represented by the 2,3-BDO isomers and their industrial applications. *Levo*-2,3-BDO was primarily used as an antifreeze in the polymer industry; *meso*-2,3-BDO as an antiseptic, humectant, and emollience in the cosmetics industry; and *dextro*-2,3-BDO as an innate immunity enhancer, anti-inflammation, and biomarker in the pharmaceutical and food industries;
- The primary holders of the bio-based 2,3-BDO production technology were from China, responsible for almost 70% of registered patents and just over 30% of published scientific articles;
- In the current stage, the major players were LanzaTech, Orochem Technologies Inc., Global Bio-Chem Technology Group, Biosyncaucho S.L., Intrexon, Tokyo Chemical Industry Co. Ltd, Merck KGaA, GS Caltex Corporation, and Praj Industries Ltd., which focused on the new microorganisms development by recent isolation or genetic modification;
- In the short term, the leading players were academic research institutions linked mainly to Chinese and Korean universities, which focused on engineered microorganisms, residual biomass, and efficient fermentation methods;
- In the medium term, the leading players were companies, research centers, and academic research institutions (mainly Chinese universities) that focused on engineered microorganisms and residual biomass;
- In the long term, the leading players were academic research institutions (China and the Republic of Korea) that focused on distillation and solvent extraction methods;
- The same business and partnerships were identified as clusters. Partnerships primarily marked the bio-based 2,3-BDO process in at least 70% of clusters in the long term. Partnerships were formed between universities of the same nationality or from different countries, companies, universities and research centers, and universities and industries;

- The bio-based 2,3-BDO process technological trajectory showed that the leading players present over time were Yancheng Institute of Technology (YIT – China) and LanzaTech (USA). While YIT was identified in the short, medium, and long terms, focusing on the production process and product drivers, especially solid-state fermentation method (short term), amino acids supplementation (medium term), and engineered *P. polymyxa* strain development (long term), LanzaTech was present at all temporal stages, focusing on production and recovery process, and application drivers, especially using 2,3-BDO as a high-octane fuel (current stage), produced by *Clostridium* strain (short term), from carbon monoxide gas (medium term), and recovered by a membrane separation process (long term);
- Technology roadmaps were considered essential decision-making tools for planning and developing future research.

1.2 Chapter 3

- The wild-type *P. peoriae* strain has not yet been used for the 2,3-BDO production, although it is phylogenetically related to *P. polymyxa* and *P. brasilensis* strains for the 2,3-BDO and acetoin synthesis. Therefore, the bibliographic review highlighted the novelty degree of this doctoral thesis;
- Bio-based 2,3-BDO metabolism was related to the redox balance promoted by the NADH/NAD^+ ratio under low oxygen supply and by the activity of three key enzymes (ALS, ALDC, and BDH) regulated by the pH;
- The 2,3-BDO isomer formation was related to three gene types (*but*A, *but*B, and *but*C) and the BDH enzyme's stereospecificity. *P. peoriae* strain presented only the *but*B gene, which allowed the *levo*- and *meso*-2,3-BDO production by R-BDH coding;
- The oxygen supply was the most critical factor of the 2,3-BDO metabolism, as it can alter the 2,3-BDO yield and its isomeric optical purity. The microaerobic conditions required for the 2,3-BDO synthesis can be achieved by controlling the agitation and aeration of the system. The parameters OTR, OUR, kLa, q_{O2}, and RQ can be used for oxygen supply control;
- The average optimum temperature and pH range for the 2,3-BDO production by *Paenibacillus* strain was 30-39 °C and 5-6.5, respectively;

- The primary nutrients used to supplement the culture medium for the bio-based 2,3-BDO production were nitrogen sources such as amino acids and yeast extract, vitamins, minerals, and oxidizing agents such as vitamin C;
- Acetate, propionate, pyruvate, and succinate can be used to enhance the bio-based 2,3-BDO production;
- Different residual carbon sources can be metabolized by *Paenibacillus* strain, including corn stover, sugarcane bagasse, rice straw, oil palm front, kenaf core powder, sugar beet molasses, soy hull hydrolysate, and crude glycerol. Commercial sources are also commonly used, including glucose, xylose, arabinose (monosaccharides), sucrose, maltose, cellobiose (disaccharides), starch (polysaccharides), and glycerol;
- Metabolic engineering was a widely used tool for strain improvement for the bio-based 2,3-BDO production with a high titer, yield, and productivity. Other previously reported enhancement strategies were the application of the microorganism co-culture, solid-state fermentation, simultaneous saccharification and fermentation process, and cell recycling;
- The bio-based 2,3-BDO recovery was the most critical step in the bioprocess, responsible for the increased energy demand and costs. The main limiting factors for the bio-based 2,3-BDO recovery were the fermentation broth complexity (mixture of 2,3-BDO isomers, acetoin, by-products, organic material residues, cells, and water) and the low 2,3-BDO titer;
- The leading recovery technologies were distillation, solvent extraction, anionic extraction, salting-out, sugaring-out, and membrane separation. The integration of recovery methodologies such as precipitation and vacuum distillation, extraction-distillation of hybrids, extraction and salting-out, solvent extraction and pervaporation, multi-effect evaporation-assisted distillation, and reactive extraction and reactive distillation has also been investigated;
- The 2,3-BDO isomers e their derivatives have been used as latex, plastics, footwear, asphalt, modifiers and lubricating oil additives, aviation fuels, resins, paints and solvents, gasoline additives, fuel, drug and cosmetic precursors, thermoplastic polymer plasticizer, flavoring for aromas and essences, cardiovascular applications and other drugs.

- The information obtained in the literature review contributed to the planning and development of this doctoral research involving the wild-type *P. peoriae* strain for 2,3-BDO production with high yield, selectivity, and economy.

1.3 Chapter 4

- *P. peoriae* NRRL BD-62 was able to produce 2,3-BDO with high yield and selectivity, and low operational cost (oxygen supply);
- Culture conditions were efficiently optimized by the full factorial design of experiments (DoE) with the replicated center point, indicating a first-order interaction among temperature, pH, aeration, and agitation;
- The DoE assays were performed in a mini bioreactor in batch fermentation, which allowed the simultaneous optimization of the investigated variables, commonly not reported in previous studies due to the operational limitation imposed by the use of shake flasks;
- In this order, pH, aeration, and agitation were the statistically significant variables for 2,3-BDO production by *P. peoriae* NRRL BD-62. The temperature did not show relevant effects within the investigated range. And a synergism between pH and aeration was the only statistically significant interaction to improved *levo*-2,3-BDO yield selectivity;
- A higher acetoin accumulation was seen in the DoE assays at 400 rpm and 0.3 vvm, while the lowest acetoin accumulation was seen at 200 rpm and 0.1 vvm;
- All conditions investigated in the DoE showed kLa less than 30 h^{-1}, which led to an oxygen-limited environment. The low oxygen supply was confirmed by the linear profile of cell growth, glucose consumption, and 2,3-BDO production by *P. peoriae* NRRL BD-62 in the first 8 h of batch fermentation;
- The optimized culture conditions were 32 °C, pH=5, and kLa~7.5 h^{-1}, defined from the DoE interpretation, in which the economic and techno-operational factors were superimposed on the mathematical result predicted by the proposed first-order model;
- The optimized culture conditions allowed a 2,3-BDO production of 39.4 g/L, with a yield of 0.43 g/g, and a *levo*-/*meso*-2,3-BDO ratio of 1.9:1, without acetoin accumulation, in fed-batch fermentation;

- Although the final 2,3-BDO titer by *P. peoriae* NRRL BD-62 was low, its yield was comparable to that of non-GRAS microorganisms, representing an economic gain from the culture medium due to the reduced loss of unmetabolized glucose in the bioprocess;
- The non-acetoin accumulation represented an economic gain from the downstream step. The 2,3-BDO production at the expense of cell growth and by-products formation such as lactic acid and ethanol further reduced the costs of 2,3-BDO separation, recovery, and purification;
- A highly oxygen-limited environment was achieved at kLa~7.5 h^{-1}, the lowest condition e so far reported for bio-based 2,3-BDO production in a bioreactor;
- The cell metabolism control by optimizing fermentation conditions was considered an important production strategy, favoring the high 2,3-BDO yield and selectivity by a GRAS microorganism, without resorting to genetic modifications, often expensive to the bioprocess;

1.4 Chapter 5

- YE, commonly used as a nitrogen source for bio-based 2,3-BDO production, was efficiently replaced by lower-cost compounds, keeping a high 2,3-BDO yield and selectivity;
- The C/N ratio of 8.5 g/g was considered the most suitable condition for the 2,3-BDO production by *P. peoriae* NRRL BD-62, allowing a yield greater than 0.40 g/g, similar to that of non-GRAS microorganisms, and a high 2,3-BDO selectivity, with no acetoin accumulation;
- The best results for the 2,3-BDO yield and selectivity by *P. peoriae* NRRL BD-62 were achieved using CSL and NH_4Cl as nitrogen sources. The 2,3-BDO metabolism was compromised in a medium containing urea and $NaNO_3$ as nitrogen sources;
- In general, complex nitrogen sources (organic nitrogen) favored cell growth, while simple nitrogen sources (inorganic nitrogen) favored the 2,3-BDO production;
- The 2,3-BDO selectivity was marked by the *levo*- and *meso*-isomer formation, with no acetoin accumulation, for all nitrogen sources investigated. The largest

experimental deviations were verified using organic nitrogen, mainly from residual sources;

- The 2,3-BDO isomeric optical purity was affected by the fermentation broth viscosity from each investigated nitrogen source. *Levo*-2,3-BDO was preferentially formed from inorganic nitrogen sources, while *meso*-2,3-BDO from organic nitrogen sources;
- The adding Arg favored cell growth in the CSL-based medium due to the ARS activation, regulated by ADP under low oxygen supply. As a result, a greater NADH amount was released, favoring the preferential *levo*-2,3-BDO synthesis. On the contrary, adding Arg in the NH_4Cl-based medium affected the *P. peoriae* NRRL BD-62metabolic activity due to the inhibited ADP by excess NH_4^+. The AP was then activated, causing the urea accumulation. As a result, the *levo*-2,3-BDO yield and selectivity were reduced;
- The adding Asn in the CSL-based medium had little influence on the 2,3-BDO metabolism by *P. peoriae* NRRL BD-62, although it caused a reduced 2,3-BDO yield below 0.40 g/g. In the NH4Cl-based medium, adding Asn improved the 2,3-BDO titer, also keeping its yield high. The NAD biosynthesis pathway was activated, favoring the *levo*-2,3-BDO synthesis by adjusted $NADH/NAD^+$ balance;
- The Arg and Asn addition in the CSL-based medium did not change the by-product production pattern. However, in the NH_4Cl-based medium containing Arg and Asn, the ethanol production was reduced. Due to the catabolism via acetyl-CoA, a small acetic acid accumulation was observed with adding Asn in the NH_4Cl-based medium. The synergism between the 2,3-BDO and acetic acid pathways also contributed to their co-production;
- In the uncontrolled pH NH_4Cl-based medium, 2,3-BDO was produced by *P. peoriae* NRRL BD-62 due to an adaptive pH auto-control mechanism based on cell homeostasis. The 2,3-BDO yield and selectivity were kept high, being statistically similar to the medium at pH=5;
- In the uncontrolled pH NH_4Cl-based medium, adding Arg made cell metabolism more susceptible to pH lowering by inhibiting ADP by excess NH_4^+, which affected the ARS. Cell growth was compromised, reducing NADH release. As a

result, the 2,3-BDO yield and selectivity were decreased. In turn, adding Asn compromised cell activity by lowering the pH (pH<5), resulting from releasing NH_4^+ from its catabolism to Asp. The 2,3-BDO selectivity was also affected. Ethanol production was reduced with amino acid supplementation, while organic acids production remained similar to the pure medium;

- Although an enhanced 2,3-BDO metabolism by *P. peoriae* NRRL BD-62 was verified with nutritional supplementation by amino acids (Arg and Asn), no statistically significant difference was observed in the 2,3-BDO yield and selectivity in the CSL and NH_4Cl-based media, according to t-test. Furthermore, the high costs and cell viability loss observed from adding Arg and Asn in the uncontrolled pH NH_4Cl-based medium made its large-scale application unfeasible. Therefore, the nutritional supplementation strategy using amino acids was disregarded in the final culture medium;
- The NH_4Cl-based medium was defined as a promising replacement for the YE-based medium on a large scale due to the high 2,3-BDO yield, around 0.40 g/g, selectivity greater than 85% *levo*-isomer, and culture medium economy of at least 67%;
- The validation of the uncontrolled pH NH_4Cl-based medium using high initial glucose concentrations was limited by the ammonia and chloride excess required to maintain C/N= 8.5 g/g, and the pH gradient collapsed by acidity excess in the culture medium;
- The 2,3-BDO production from low initial glucose concentrations in the uncontrolled pH NH_4Cl-based medium was partially validated in batch fermentation. While the 2,3-BDO yield was maintained around 0.40 g/g, its selectivity was marked by an increased *meso*-2,3-BDO and a small acetoin accumulation due to the increased oxygen dissolved, responsible for decreased NADH in the late fermentation steps;
- The 2,3-BDO production from low initial glucose concentrations in the uncontrolled pH NH_4Cl-based medium has not been validated in fed-batch fermentation. The 2,3-BDO yield was affected by nutritional limitation caused by relative nitrogen decrease (C/N increase) after glucose feeding, while its selectivity was affected by the differences observed in the oxygenation systems,

although the oxygen supply was kept constant (kLa~ 5 h-1). The system had different agitator/aerator types and vessel geometries;

- The q_{O2} parameter was considered the main responsible for the high 2,3-BDO yield and selectivity on a large scale, overlapping the OTR parameter, mainly in future scale-up assays;
- Nitrogen source, pH, and oxygen supply were considered important control variables for the high-yield and high-selectivity 2,3-BDO production by *P. peoriae* NRRL BD-62 and should be evaluated in synergy on a large scale.

1.5 Chapter 6

- Overall, fed-batch fermentations did not compromise 2,3-BDO metabolism by *P. peoriae* NRRL BD-62. However, nitrogen supply was the main limiting factor for the bio-based 2,3-BDO production in this setup. While complex nitrogen sources such as YE contributed to an enhanced 2,3-BDO titer, with a yield above 0.40 g/g in fed-batch cultures at constant feeding, the nutritional limitation imposed by simple nitrogen sources such as NH_4Cl, and intensified in fed-batch cultures mainly at exponential feeding rates, compromised glucose metabolism, and bio-based 2,3-BDO production;
- Intermittent fed-batch fermentation was inadequate for complete and rapid glucose metabolism by *P. peoriae* NRRL BD-62. A residual glucose accumulation was verified in the culture medium in the final fermentation steps, regardless of the C/N ratio. No statistically significant differences between 2,3-BDO titers and yields for each evaluated C/N ratio were identified;
- Fed-batch fermentation at constant feeding was adequate for complete and relatively rapid glucose metabolism by *P. peoriae* NRRL BD-62 at an initial C/N of 8.5 g/g. However, a residual glucose accumulation was observed at C/N= 18.5 g/g, which affected the 2,3-BDO synthesis. Therefore, the 2,3-BDO titer in fed-batch fermentation at constant feeding depended on the nutritional culture medium composition. In contrast, the 2,3-BDO yield was maintained at around 0.40 g/g, regardless of the C/N ratio;
- Except for the 2,3-BDO titer, all other fermentation parameters were statistically similar in the assays at C/N= 8.5 g/g, regardless of the feeding strategy adopted.

Furthermore, no statistically significant differences were observed in the assays at C/N= 18.5 g/g, regardless of the feeding strategy adopted. Therefore, the feeding profile did not affect the 2,3-BDO production by *P. peoriae* NRRL BD-62, mainly under nutritional limitation, represented by a higher C/N ratio;

- The 2,3-BDO isomer optical purity was affected by the culture medium nutritional limitation (C/N ratio). However, no acetoin accumulation was detected in the investigated culture conditions and fermentation modes, which also showed the same by-product formation pattern, except for ethanol, which was detected only in fed-batch fermentation at constant feeding;
- The 2,3-BDO formation rate was reduced in the NH_4Cl-based medium compared to the YE-based medium in intermittent fed-batch fermentation at C/N= 8.5 g/g;
- Fed-batch fermentation at exponential feeding was found inefficient in producing 2,3-BDO by *P. peoriae* NRRL BD-62 in the NH_4Cl-based medium. As the fermentation progressed, a residual glucose accumulation was observed compared to intermittent fed-batch fermentation. The more significant *meso*-2,3-BDO formation was also verified, although no acetoin accumulation was detected;
- The NH_4Cl-based medium affected the μ_{real} values, which were far below μ_{set}, leading to an excess glucose feed. As a result, $Y_{X/S}$ was compromised, causing 2,3-BDO production to decline. Although the 2,3-BDO yield remained statistically around 0.40 g/g, its titer was lower than expected;
- The decreased relative nitrogen (increased C/N) after continuous glucose feeding at exponential rates in the NH_4Cl-based medium also compromised microbial activity ($\mu_{real}<\mu_{set}$), contributing to glucose accumulation and reduced 2, 3-BDO production;
- The 2,3-BDO titer and yield did not show statistically significant differences between the feeding strategies and the different μ conditions investigated. The $Y_{P/X}$ calculation for fed-batch fermentation at exponential feeding assays was limited by the low cell formation and the non-linear relationship between q_p and μ;
- The parameters $Y_{P/S}$ and $Y_{X/S}$ were independent of μ_{set}, while q_p decreased with increasing μ_{set}. The q_p values in fed-batch fermentation at exponential feeding were higher than in intermittent feeding. Therefore, the carbon flux was

preferentially directed to the 2,3-BDO synthesis in fed-batch fermentation at exponential feeding;

- An inverse relationship between *levo*-2,3-BDO production and μ_{set} was observed in fed-batch fermentation at exponential feeding. No relationship was observed between the organic acids and ethanol production and μ_{set}. No net acetic acid production was found in fed-batch culture assays, whereas only lactic acid was detected in intermittent fed-batch fermentation;
- The relationship between the feeding strategy and the nitrogen source in fed-batch fermentation was efficiently determined for the 2,3-BDO production by wild-type *P. peoriae* strain, indicating that the nitrogen supply is a limiting factor for the bioprocess success, mainly on a large scale, regardless of the fermentation production adopted.

1.6 Chapter 7

- A low-cost 2,3-BDO production by *P. peoriae* NRRL BD-62 was achieved by substituting commercial glucose for sugarcane molasses and YE for NH_4Cl, keeping the yield statistically close to 0.40 g/g, and the high 2,3-BDO selectivity, with no acetoin accumulation and with the absolute *levo*-isomer formation in batch fermentation;
- *P. peoriae* NRRL BD-62 produced 2,3-BDO from almost complete consumption of glucose, fructose, sucrose, xylose, and glycerol, with an average yield of 0.37 g/g, with no acetoin accumulation in batch fermentation. Furthermore, the organic acid production was less than 1 g/L for all investigated carbon sources, while ethanol exceeded 4 g/L in glucose and fructose-based media. The same average cell biomass was formed in all evaluated media;
- The best results were achieved using the sucrose-based medium. Therefore, sugarcane molasses was selected as a cheap sucrose source for 2,3-BDO production by *P. peoriae* NRRL BD-62. A 2,3-BDO yield of about 0.47 g/g and selectivity of just over 97% *levo*-isomer were achieved from sugarcane molasses in the YE-based medium. Again, the organic acids production was low, less than 0.2 g/L, while almost 4 g/L of ethanol was detected in batch fermentation;

- The 2,3-BDO titer and yield were reduced, while its selectivity did not show statistical differences with the YE replacement with CSL and NH_4Cl. A reduced molasses consumption, minimal acetoin accumulation, and ethanol and acetic acid production of less than 0.5 g/L were verified using CSL, while a lower cell biomass formation, ethanol accumulation of almost 2 g/L as the only by-product, and an exclusive *levo*-2,3-BDO production were observed using NH_4Cl;
- Increasing the C/N ratio in the culture medium containing YE was not considered an efficient production strategy, as the 2,3-BDO titer, yield, and *levo*-isomer optical purity were reduced, although the by-product formation was avoided. Furthermore, savings of only 26% were found by reducing the YE amount in the culture medium, almost 3-fold lower than the savings achieved using NH_4Cl;
- A reduction of more than 75% in the culture medium costs was verified using the sugarcane molasses-based medium containing NH_4Cl as a nitrogen source. The final cost of 4.74 US$/$kg_{2,3\text{-}BDO}$ was promising, considering the current FOB and market prices of the inputs and previous studies reported;
- Fed-batch fermentation at constant sugarcane molasses feeding in the NH_4Cl-based medium was inadequate to enhance the bio-based 2,3-BDO production, whose titer and yield were affected. Small glucose and residual fructose accumulation were observed in the late fermentation steps, although it did not affect the 2,3-BDO production by *P. peoriae* NRRL BR-62. The 2,3-BDO selectivity was maintained above 90% *levo*-isomer, with no acetoin accumulation. Organic acids were kept below 2.5 g/L, while ethanol accumulation of less than 5 g/L was detected after stopping the constant sugarcane molasses feeding;
- Batch fermentation using the sugarcane molasses-based medium containing NH_4Cl was considered the most suitable strategy for the 2,3-BDO production by *P. peoriae* NRRL BD-62 due to the problems generated with the previously untreated sugarcane molasses feeding in fed-batch fermentation;
- The culture medium costs for the bio-based 2,3-BDO production were reduced, contributing to the final bioprocess economy and its industrial viability. However, a tech-economic analysis is required to reach a realistic and adequate conclusion on the large-scale 2,3-BDO production.

1.7 Chapter 8

- The 2,3-BDO production scale-up by *P. peoriae* NRRL BD-62 was efficiently carried out using constant OTR to partially meet the geometric similarity criterion and the dimensional analysis between the systems and maintain the necessary microaerobic conditions for the 2,3-BDO synthesis. This was the first time that a wild-type *P. peoriae* strain was used for the 2,3-BDO production with high yield, good productivity, and high selectivity in a 200 L bioreactor;
- The extrapolated aeration was based on the N and Q_{ar} adjustment to keep the initial OTR within the range of 0.45-1.68 $mmol_{O2}$/L/h. The N and Q_{ar} adopted were defined according to the oxygen supply (aerator and agitator types, aeration profile, and geometrical characteristics);
- OTR was kept constant at the expense of P_g/V and ITS variation, as expected in scale-up processes;
- Due to the surface aeration adopted, P_g/V did not follow the expected behavior, increasing the pilot bioreactor. Under these conditions, a higher N was required, increasing the energy demand. The Di of the pilot bioreactor also contributed to the increased P_g/V since the impeller's geometric similarity criterion was not used. In contrast, the impeller type and its Np did not significantly affect the P_g/V;
- The highest ITS value was verified in the pilot bioreactor due to the high N and Di adopted;
- A high 2,3-BDO yield and selectivity were achieved in the three production scales investigated, although the best results were obtained in batch fermentation in a 200 L bioreactor, equal to 0.40 g/g, 0.90 g/L/h, and 98% *levo*-isomer, due to highly oxygen-limited environment;
- Batch-fermentations carried out in benchtop and pilot bioreactors showed a 2,3-BDO yield of around 0.40 g/g on both scales. A small acetoin accumulation was detected in the benchtop bioreactor, while about 98% *levo*-2,3-BDO was formed in the pilot bioreactor. Except for acetic acid in the pilot bioreactor, whose concentration was equal to approximately 6 g/L, the other by-products were below 2.2 g/L in both production scales;
- The difference in time fermentation to maximum 2.3-BDO production between benchtop and pilot scales was attributed to the inoculum activation used in each

system. The cells used in the pilot bioreactor were grown under controlled N and Q_{ar}, while the cells used in the benchtop bioreactor were grown only by N control. As a result, glucose was rapidly consumed on the pilot scale, showing a higher cell biomass formation;

- Fed-batch fermentations carried out in mini and pilot bioreactors showed different 2,3-BDO production profiles due to the feed types of the systems. The highest 2,3-BDO titer of 37.6 g/L, with a yield of 0.42 g/g, was achieved in the mini bioreactor, whose feeding pulse was performed in a culture medium containing non-metabolized glucose. In contrast, 2,3-BDO titer and yield were affected in the pilot bioreactor due to the delay in pulse feeding, which occurred 26 h after glucose was depleted, not preventing the bacterial cells from starvation. As a result, the added glucose was converted to ethanol instead of 2,3-BDO;
- *Levo*-2,3-BDO formation was maintained above 90% and with no acetoin accumulation in fed-batch fermentation assays. Furthermore, lactic acid production was low in all systems, while almost 6 g/L of acetic acid was verified in the pilot bioreactor;
- Although the ethanol had been produced at the 2,3-BDO expense in the pilot bioreactor, the results suggested that the 2,3-BDO yield would be maintained at around 0.40 g/g if the system feeding had not been delayed. Therefore, the 2,3-BDO production by *P. peoriae* NRRL BD-62 in a 200 L bioreactor was considered promising, regardless of the fermentation mode used;
- The information on the scale-up strategy based on the constant OTR obtained in this research can be used for a safe and well-performing bio-based 2,3-BDO industrial production, serving as a significant scientific contribution to the limited number of previous studies investigating the bio-based 2,3-BDO production on a large scale.

CHAPTER 10

1. Conclusion

The development of the upstream and mainstream 2,3-BDO bioprocess steps using *P. peoriae* NRRL BD-62 for potential large-scale application was investigated in this doctoral thesis. Critical aspects of bio-based 2,3-BDO production were identified and correlated with the bioprocess technological maturity level. The optimized fermentation conditions, culture medium composition, fermentation modes, and investigated scale-up strategies were used to enhance 2,3-BDO formation, minimize bioprocess costs, and make *P. peoriae* NRRL BD-62 an efficient industrial 2,3-BDO producer. Therefore, the bio-based 2,3-BDO metabolism control was considered a crucial factor for the bioprocess with high yield, selectivity, and economy. The hypothesis of a safe, efficient, and potentially applicable large-scale 2,3-BDO production by a wild-type GRAS strain as an alternative to traditional microbial producers was validated. As a result, a cost-effective 2,3-BDO bioprocess, based on circular economy and biorefinery principles and without the need for genetic engineering, can be industrially applied in the future, contributing to the growing bio-based 2,3-BDO production market.

2. Future studies and perspectives

To ensure a 2,3-BDO bioprocess industrial application, the following suggestions for future studies are:

- Investigate the q_{O2} effects on the bio-based 2,3-BDO production on a lab scale and its application as a scale-up strategy in a pilot bioreactor;
- Carry out a techno-economical assessment (TEA) and a life cycle analysis (LCA) of the 2,3-BDO production from sugarcane molasses in a medium containing NH_4Cl or CSL;
- Carry out more fermentation assays in the pilot bioreactor to control the culture medium feeding and increase the 2,3-BDO productivity.

APPENDICES

Supplementary material

Chapter 4

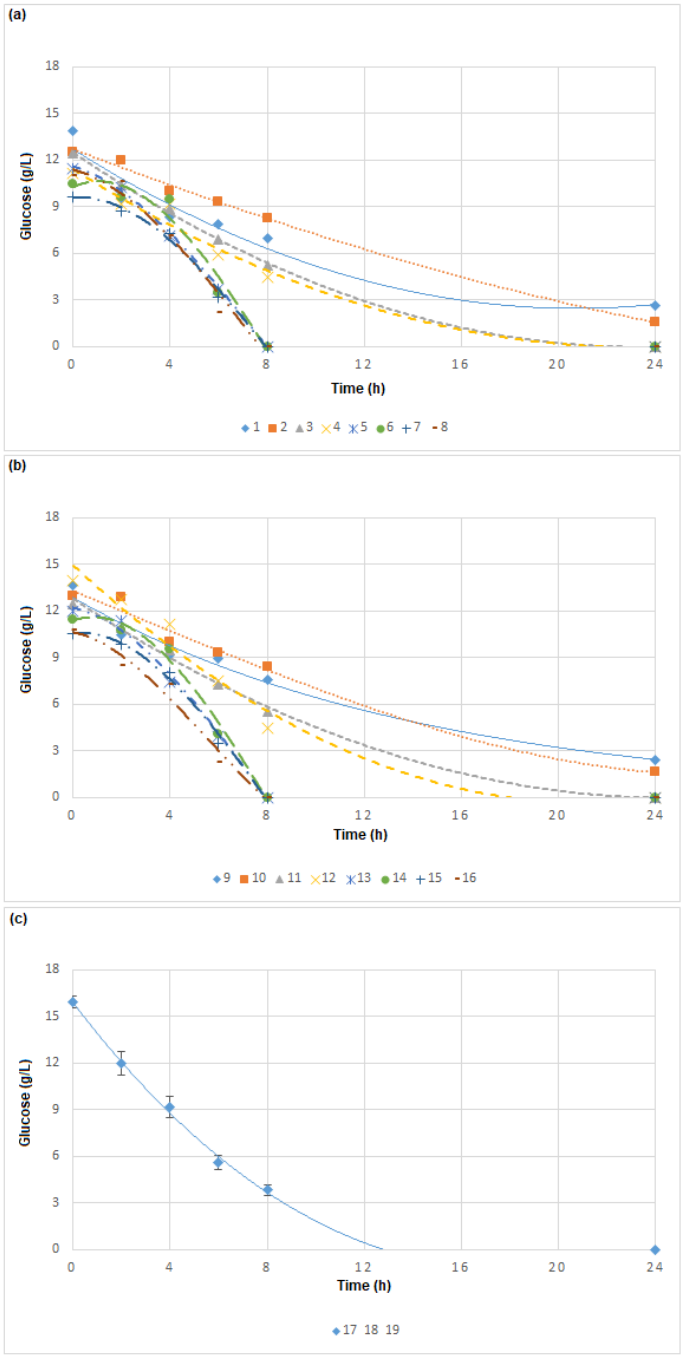

Figure S1: Time course of glucose consumption by *P. peoriae* NRRL BD-62 for experimental design (2^4+3) for 24 h fermentation: (a) 1-8 runs; (b) 9-16 runs; (c) central point triplicate.

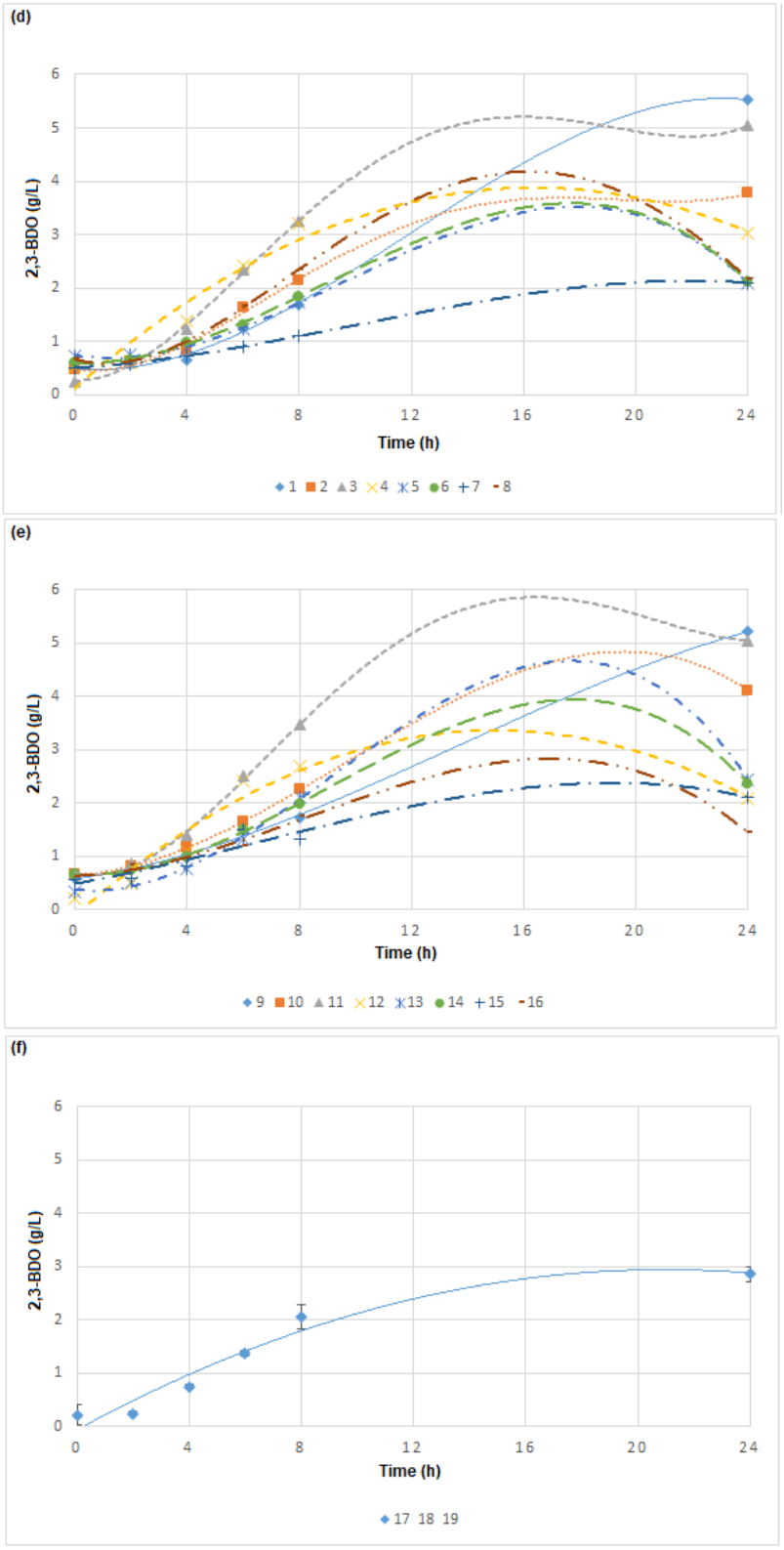

Figure S2: Time course of 2,3-BDO production by *P. peoriae* NRRL BD-62 for experimental design (2^4+3) for 24 h fermentation: (a) 1-8 runs; (b) 9-16 runs; (c) central point triplicate.

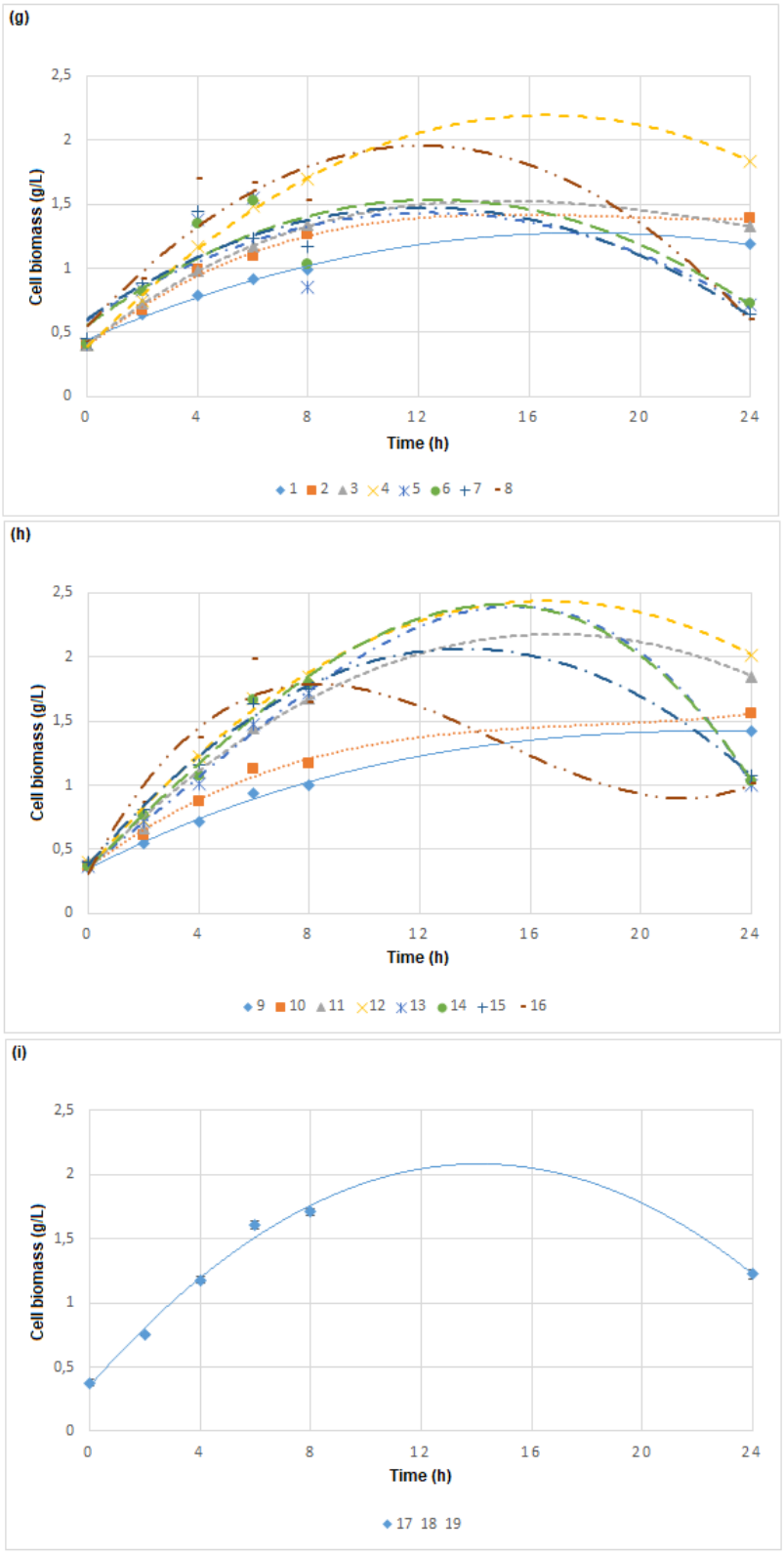

Figure S3: Time course of cell biomass of *P. peoriae* NRRL BD-62 for experimental design (2^4+3) for 24 h fermentation: (a) 1-8 runs; (b) 9-16 runs; (c) central point triplicate.

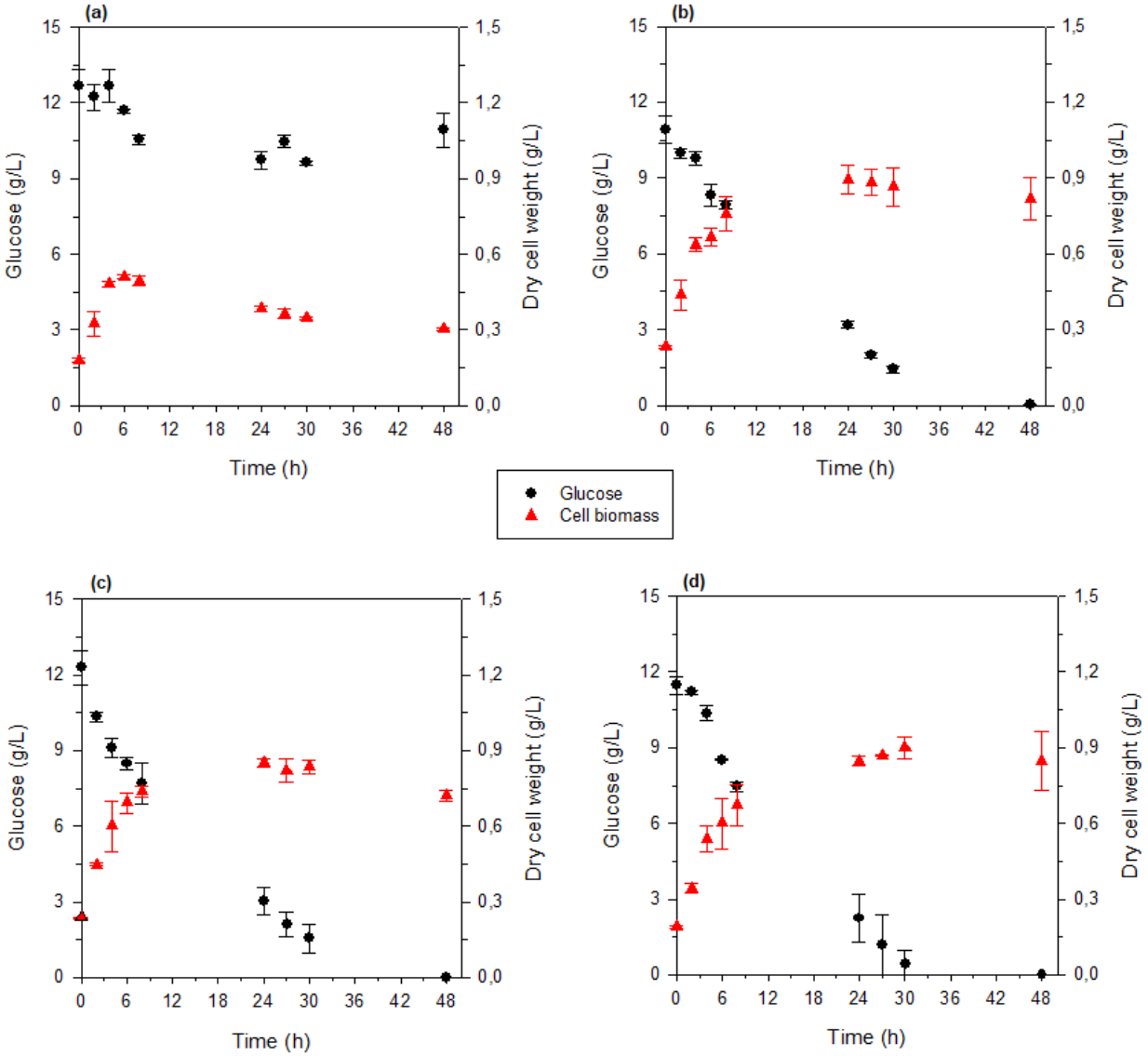

Figure S1: Time courses of glucose consumption and cell biomass formation of *P. peoriae* NRRL BD-62 using different organic nitrogen sources in batch fermentation at C/N= 8.5 g/g: (a) Yeast extract Senai; (b) Yeast extract brewer; (c) Corn steep liquor; (d) Urea.

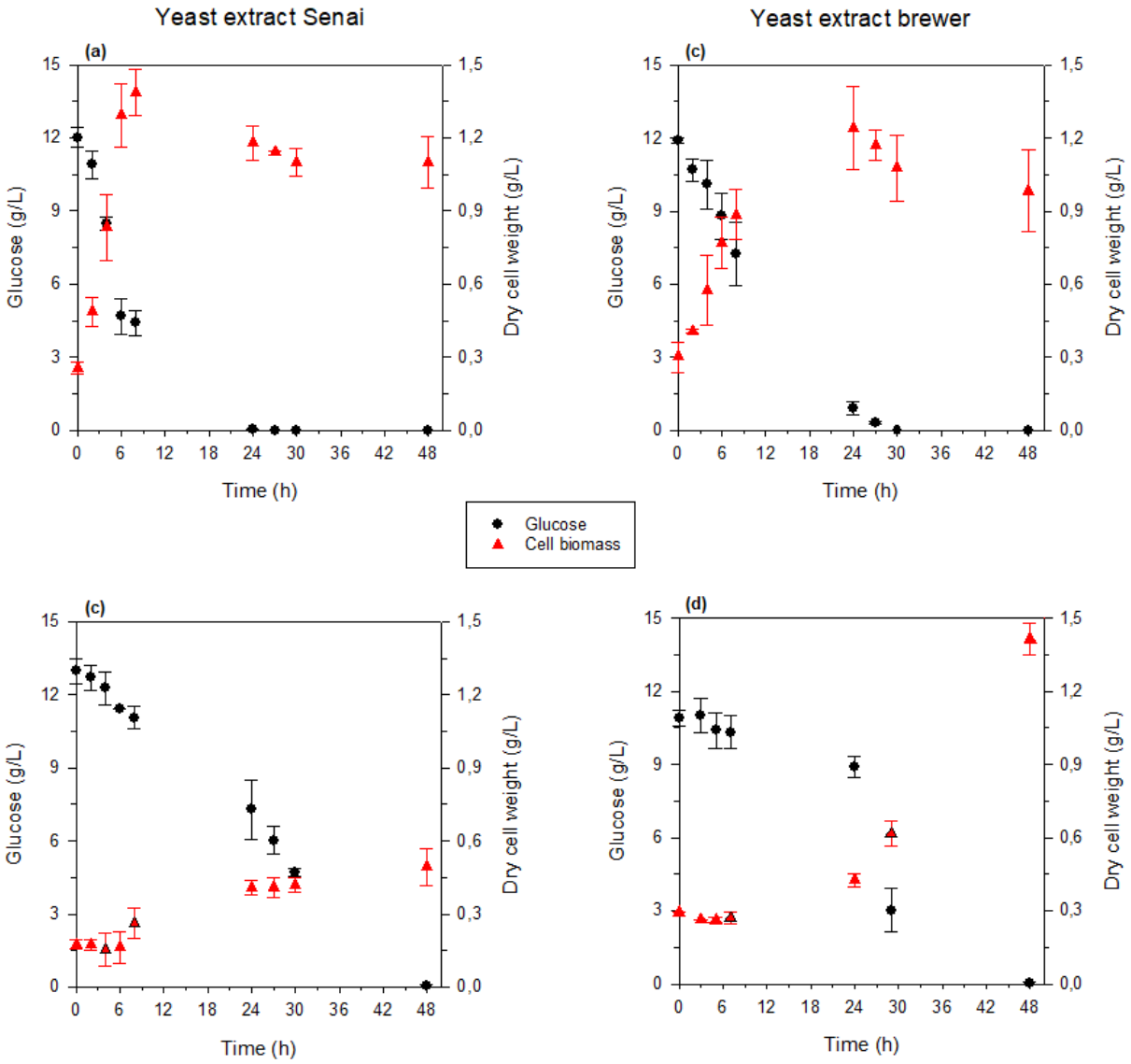

Figure S2: Time courses of glucose consumption and cell biomass formation of *P. peoriae* NRRL BD-62 using different inorganic nitrogen sources in batch fermentation at C/N= 8.5 g/g: (a) $NaNO_3$; (b) NH_4Cl; (c) $(NH_4)_2SO_4$ (d) $(NH_4)_2HPO_4$.

Table S1: Chemical centesimal composition of nitrogen sources used for 2,3-BDO production by *P. peoriae* NRRL BD-62.

Nitrogen source	Composition (%)							
	Carbon	**Nitrogen**	**Hydrogen**	**Sulfur**	**Oxygen**	**Phosphorus**	**Sodium**	**Chloride**
Organic								
Yeast extract (commercial)	39.4	10.8	-	-	-	-	-	-
Yeast extract (Senai)	32.4	9.0	6.5	0.5	51.6	0.0	0.0	0.0
Yeast extract (brewer)	35.7	10.	6.5	0.5	47.2	0.0	0.0	0.0
Corn steep liquor	26.5	5.3	4.9	24.4	38.8	0.0	0.0	0.0
Urea	20.0	46.7	6.7	0.0	26.7	0.0	0.0	0.0
Inorganic								
Sodium nitrate – $NaNO_3$	0.0	16.5	0.0	0.0	56.5	0.0	27.1	0.0
Ammonium chloride – NH_4Cl	0.0	26.2	7.5	0.0	0.0	0.0	0.0	66.4
Ammonium sulfate – $(NH_4)_2SO_4$	0.0	21.2	6.1	24.2	48.5	0.0	0.0	0.0
Ammonium phosphate dibasic – $(NH_4)_2HPO_4$	0.0	21.2	6.8	0.0	48.5	23.5	0.0	0.0

*Elemental analysis of yeast extract Senai and brewer, and CSL was performed on a Thermo Scientific™ FlashSmart™ Elemental Analyzer.

Table S2: Free on board (FOB) prices per kg of the culture medium nutrients used for the 2,3-BDO production by *P. peoriae* NRRL BD-62.

Nutrients	NCM/Brand	Description	FOB price per net weight (US$/kg)*	Base year	Reference
Glucose	17023011	Chemically pure glucose	0.84	2021	ComexStat (2022)
K_2HPO_4	28352400	Potassium phosphate	4.55		
KH_2PO_4	28352400	Potassium phosphate	4.55		
KCl	31042010	Potassium chloride, with a potassium oxide (K_2O) content not exceeding 60 % by weight	0.36		
$MnSO_4$	28332990	Other sulfates	2.84		
NaOH	28151100	Sodium hydroxide (caustic soda), solid	0.48		
Yeast Extract (commercial)	Angel Yeast Co., Ltd.	YP108, YP612, YP601, YP602	6.00	2022	Angel Yeast (2022)
Yeast Extract (brewer)	Angel Yeast Co., Ltd.	YP202, YP203	6.00		
Corn steep liquor	23021000	Residues from milling and other corn treatments	0.97	2021	ComexStat (2022)
Urea	31021010	Urea with %N>45	0.54		
$NaNO_3$	31025090	Other sodium nitrates	2.82		
NH_4Cl	28271000	Ammonium chloride	0.88		
$(NH_4)_2SO_4$	31022100	Ammonium sulfate	0.35		
$(NH_4)_2HPO_4$	31053000	Diammonium hydrogen orthophosphate	0.65		
Arginine	29252919	Other arginines and salts	5.65		
Asparagine	29224990	Other amino acids, their esters, and salts	12.90		

*Based on commercial sales prices (Angel Yeast Co., Ltd.) or Brazilian export prices (ComexStat) net weight;
NCM=Common MERCOSUL Nomenclature;
Data for yeast extract Senai not available.

Table S3: Culture medium costs (salts and carbon source) used for the 2,3-BDO production by *P. peoriae* NRRL BD-62.

Nutrients	FOB price per net weight (US$/kg)	Concentration (kg/m³)	Cost/m³ (US$/m³)
Glucose	0.84	10.00	8.40
K_2HPO_4	4.55	2.00	9.11
KH_2PO_4	4.55	0.50	2.28
KCl	0.36	0.30	0.11
$MnSO_4$	2.84	0.0225	0.06
		Total	**19.96**

FOB price= free on board price

Table S1: Preprogrammed values of the fermentation parameters used in the exponential feed rate calculation.

Parameters	Units	Set-point values
μ	h^{-1}	0.11 0.14 0.17
$Y_{X/S}$	g/g	0.134*
S_F	g/L	60.0
S	g/L	0.0
X_0	g/L	0.983*
V_0	L	0.097

*Mean values of the three conditions investigated in batch fermentation
μ= specific cell growth rate; $Y_{X/S}$= dry cell weight/glucose yield; S_F= glucose feed concentration; S= residual glucose concentration; X_0= initial dry cell weight; and V_0= initial culture volume.

Table S2: Glucose exponential feed rate for 2,3-BDO production by *P. peoriae* NRRL BD-62.

Inoculation time (h)	Feed rate (10^{-3} L/h)*		
	μ_{set} (h^{-1})		
	0.11	**0.14**	**0.17**
8	1.30	1.66	2.02
9	1.46	1.91	2.39
10	1.63	2.20	2.83
11	1.81	2.53	3.36
12	2.03	2.91	3.98
13	2.26	3.34	4.72
14	2.52	3.85	5.59
15	2.82	4.42	6.63
16	3.15	5.09	7.86
17	3.51	5.85	9.31
18	3.92	6.73	11.04
19	4.37	7.74	13.08
20	4.88	8.91	15.51
21	5.45	10.25	18.38
22	6.09	11.79	21.78
23	6.79	13.56	25.82
24	7.58	15.60	30.61

μ= pre-programmed specific cell growth rate.
*Equations: $F = 1.30 \exp(0.11t)$ (1); $F = 1.66 \exp(0.14t)$ (2); $F = 2.02 \exp(0.17t)$ (3)

Table S1: Dissolved oxygen (DO) correlation to dry cell weight (DCW) for each carbon and nitrogen source used for the 2,3-BDO production by *P. peoriae* NRRL BD-62.

Carbon source	Nitrogen source	1 $DO_{600\ nm}$ is equal to DCW (g/L)
Glucose		0.466 ± 0.012
Fructose		0.596 ± 0.004
Sucrose	Yeast extract	0.486 ± 0.083
Xylose		0.488 ± 0.036
Glycerol		0.942 ± 0.174
	Yeast extract	0.640 ± 0.077
Sugarcane molasses	Corn steep liquor	1.582 ± 0.169
	Ammonium chloride	0.790 ± 0.060

Table S2: Free on board (FOB) prices per kg of the culture medium nutrients used for the 2,3-BDO production by *P. peoriae* NRRL BD-62.

Nutrients	NCM/Brand	Description	FOB price per net weight (US$/kg)*	Base year	Reference
Salts					
K_2HPO_4	28352400	Potassium phosphate	4.55	2021	COMEXSTAT (2022)
KH_2PO_4	28352400	Potassium phosphate	4.55		
KCl	31042010	Potassium chloride, with a potassium oxide (K_2O) content not exceeding 60 % by weight	0.36		
$MnSO_4$	28332990	Other sulfates	2.84		
Nitrogen source					
Yeast Extract	Angel Yeast Co., Ltd.	YP108, YP612, YP601, YP602	6.00	2022	ANGEL YEAST (2022)
Corn steep liquor	23021000	Residues from milling and other corn treatments	0.97	2021	COMEXSTAT (2022)
Ammonium chloride	28271000	Ammonium chloride	0.88		
Carbon source					
Glucose	17023011	Chemically pure glucose	0.84		
Sucrose	17019900	Other cane sugars, beet, chemically pure sucrose, sol.	0.38		
Molasses	17031000	Sugarcane molasses	0.44		

*Based on commercial sales prices (Angel Yeast Co., Ltd.) or Brazilian export prices (ComexStat) net weight;
NCM=Common MERCOSUL Nomenclature.

Table S3: Culture medium nutrients costs used for the 2,3-BDO production by *P. peoriae* NRRL BD-62 in batch fermentation at $C/N_{initial}$= 8.5 g/g.

Nutrients	FOB price per net weight (US$/kg)	Concentration (kg/m³)[a]	Cost/m³ (US$/m³)
Salts			
K_2HPO_4	4.55	2.0	9.11
KH_2PO_4	4.55	0.5	2.28
KCl	0.36	0.3	0.11
$MnSO_4$	2.84	0.0225	0.06
Nitrogen source			
Yeast Extract	6.00	22.5	135.00
Yeast Extract (C/N= 11 g/g)		15	90.00
Corn steep liquor	0.97	63	61.21
Ammonium chloride	0.88	5.37	4.74
Carbon source			
Glucose	0.84	30	25.31
Sucrose	0.38	30	11.32
Molasses*	0.44	60	26.39

[a]Based on an initial 30 g/L substrate to maintain C/N= 8.5 g/g.
*Molasses composition: 50% sugar.
C/N ratio= carbon/nitrogen ratio; FOB price= free on board price.

Table S1: Bioreactor designs used for 2,3-BDO scaling-up production by *P. peoriae* NRRL BD-62.

System	Model (Brand)	Total volume (L)	Vessel dimensions		Agitation and aeration accessories			Power
			Internal diameter (m)	Height (m)	Impeller type	Impeller diameter (m)	Air supply (21% O_2/79% N_2)	Np
Mini bioreactor	DASbox® Mini Bioreactor System (Eppendorf)	0.35	0.064	0.120	Rushton-type impeller with 6-blade (stainless steel)	0.030	Submerged (via L-sparger)	4.5-6.5
Benchtop bioreactor	New Brunswick™ BioFlo®/CelliGen® 310 (Eppendorf)	3.00	0.120	0.240	Rushton-type impeller with 6-blade (stainless steel)	0.060	Submerged (via ring-sparger)	4.5-6.5
Pilot bioreactor	Pilot Plant of Bioprocesses (LaBiM-UFRJ)	200	0.650	0.900	Rushton-type impeller with 4-blade (stainless steel)	0.150	Overlay	3.2-4.5

Np= power number.