Universidade Federal do Rio Grande Do Sul
Escola de Engenharia
Departamento de Engenharia Química
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2,3-butanediol production by *Pantoea agglomerans* from soybean hull acid hydrolysate in submerged batch bioreactors

Dissertação de Mestrado

Laura Jensen Ourique

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Dissertação submetida ao Programa de Pós-Graduação em Engenharia Química da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Mestra em Engenharia Química

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A Comissão Examinadora, abaixo assinada, aprova a dissertação *"2,3-butanediol production by Pantoea agglomerans from soybean hull acid hydrolysate in submerged batch bioreactors",* elaborada por Laura Jensen Ourique, como requisito parcial para obtenção do título de Mestra em Engenharia Química.

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Resumo

Nesta pesquisa, foi investigada a produção de 2,3-butanodiol (2,3-BD) pela bactéria *Pantoea agglomerans* linhagem BL1 a partir de hidrolisado ácido de casca de soja em reatores submersos operando em modo batelada. O substrato utilizado nos cultivos consistiu em uma mistura de xilose, arabinose e glicose, proveniente da fração hemicelulósica da casca de soja. Foi avaliada a influência do suprimento de oxigênio, controle de pH e adição de agentes exógenos sobre a cinética de crescimento do microrganismo. *P. agglomerans* BL1 foi capaz de metabolizar simultaneamente os três monossacarídeos do meio, sendo a conversão média após 48 h de cultivo correspondente a 74.85 %. As diferentes condições de aeração empregadas demonstraram a via ácida mista de formação de 2,3-BD por enterobactérias. Foram observados melhores resultados nas condições de maior suprimento de oxigênio, contrastando com alguns trabalhos da literatura. Fixando-se a aeração em 2 vvm, obteve-se 14,02 $g \cdot L^{-1}$ de 2,3-BD em 12 h de cultivo, o que corresponde a conversão de 0,53 $g \cdot g^{-1}$ e produtividade de 1,17 $g \cdot L^{-1} \cdot h^{-1}$. Este resultado demonstra o grande potencial de produção de 2,3-BD por *P. agglomerans* BL1, linhagem isolada de um consórcio ambiental, a partir de hidrolisado ácido de casca de soja. Assim, o presente trabalho aponta uma solução para a utilização da fração hemicelulósica da biomassa agroindustrial, carboidratos cuja utilização não é comumente visada em bioprocessos.

Palavras-chave: 2,3-butanodiol, pentoses, hidrolisados de biomassa lignocelulósica, *Pantoea agglomerans*, casca de soja.

Abstract

Production of 2,3-butanediol (2,3-BD) by *Pantoea agglomerans* strain BL1 was investigated using as substrate in batch reactors. The cultivation media consisted of a mixture of xylose, arabinose, and glucose, obtained from the hemicellulosic fraction of this biomass. The influence of oxygen supply, pH control, and media supplementation on the kinetics of growth of the microorganism was evaluated. *P. agglomerans* BL1 was able to simultaneously metabolize all three monosaccharides present in the broth, with average conversions after 48 h of culture corresponding to 74.85 %. The different aeration conditions employed demonstrated the mixed acid pathway of 2,3-BD formation by enterobacteria. Better results of 2,3-BD production were obtained under the highest oxygen supply conditions, contrasting with some reports in the literature. Setting aeration at 2 vvm led to 14.02 $g \cdot L^{-1}$ of 2.3-BD in 12 h of cultivation, which corresponds to yield of 0.53 $g \cdot g^{-1}$ and productivity of 1.17 $g \cdot L^{-1} \cdot h^{-1}$. These results demonstrate the great production potential of 2,3-BD by *P. agglomerans* BL1, strain isolated from an environmental consortium. The present work proposes a solution for the usage of the hemicellulosic fraction of agroindustrial biomasses, carbohydrates whose utilization are not commonly addressed in bioprocess.

Keywords: 2,3-butanediol, pentose sugars, lignocellulosic biomass hydrolysates, *Pantoea agglomerans*, soybean hulls.

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Abbreviations and acronyms

2,3-BD: 2,3-butanediol

D-(-)-2,3-BD: (2R, 3R) -2,3-BD, levorotatory form

L-(+)-2,3-BD:(2S, 3S) -2,3-BD, dextrororatory form

meso-2,3-BD:(R, S) -2,3-BD, optically inactive form

MEK: Methyl Ethyl Ketone

1,3-PD: 1,3-propanediol

1,4-BD:1,4-butanediol

1,2-PD: 1,2-propanediol

α-ALS α-acetolactate synthase

α-LDC: α-acetolactate decarboxylase

BDH: 2,3-BD dehydrogenase (also called acetoin reductase)

DAR: diacetyl reductase

pH: potential of hydrogen

LM: Lignocellulosic material

VC: vitamin C

BiotecLab: Biotechnology & Biochemical Engineering Laboratory

UFRGS: Federal University of Rio Grande do Sul

HPLC: high performance liquid chromatography

LB: Luria-Bertani

C5: pentoses

C6: hexoses

HMF: 5-(hydroxymethyl)furfural

1 Introduction

2,3-Butanediol (2,3-BD; IUPAC, butane-2,3-diol) is a compound whose unique molecular structure allows applications as an intermediate in various branches of the chemical industry. It exists as 3 stereoisomers: (2S, 3S) -BD, (2R, 3R) -BD and meso, the first two having optical activity (CELIŃSKA; GRAJEK, 2009). Some applications of this diol are specific to D-(-)-2,3-BD, as the synthesis of high-price chiral distinct chemicals (PARK; RATHNASINGH; SONG, 2015) and usage as anti-freezing agent (LI et al., 2015). Nevertheless, other relevant uses do not require a specific stereoisomer, as described below (BIAŁKOWSKA, 2016).

For example, 1,3-butadiene, the monomeric unit used in synthetic rubber production, is formed through 2,3-BD dehydration (DE MAS; JANSEN; TSAO, 1988). 1,3-butadiene also has high calorific value - comparable to that of other liquid fuels, such as methanol and ethanol, and high octane number, which permits its utilization as an additive to gasoline and high-grade aviation fuel (GARG; JAIN, 1995). Further 2,3-BD dehydration can also lead to the generation of Methyl Ethyl Ketone (MEK), an industrial solvent that can improve the quality of fuel aviation (VOLOCH et al., 1987). Diacetyl and acetoin, valuable products in the food industry, can be manufactured by 2,3-BD dehydrogenation. Finally, by esterification reactions, 2,3-BD diesters are produced as important precursors of drugs and cosmetics (JI; HUANG; OUYANG, 2011).

2,3-BD production can be carried out via chemical synthesis, based on oil cracking, or employing biotechnological processes (BIAŁKOWSKA, 2016). From this perspective, the use of agroindustrial biomass to obtain 2,3-BD gains appeal, given the current political and social impasses regarding usage of fossil fuels and the growing need to implement more sustainable waste management systems (CELIŃSKA; GRAJEK, 2009). Brazil, in particular, is the second largest soybean producer in the world, just behind the United States (UNITED STATES DEPARTMENT OF AGRICULTURE - USDA, 2019). Soybean hulls are the largest by-product generated in grain processing - up to 8 - 10 % of the whole soybean (LOMAN; JU, 2016). They are currently destined mainly for animal feed production (IPHARRAGUERRE; CLARK, 2010), but many literature studies demonstrate its

potential in bioconversion processes (CHENG et al., 2017; CORTIVO et al., 2018; LOMAN; ISLAM; JU, 2018).

Likewise other lignocellulosic biomasses, the presence of hexoses and pentoses in soybean hulls increases the challenge of efficient bioconversion, as most microorganisms cannot metabolize C5 and C6 sugars simultaneously (LOMAN; JU, 2016). To the best of our knowledge, there are currently no studies in the literature demonstrating the use of soybean hulls for 2,3-BD generation in bioreactors. Several microorganisms are capable of producing this diol (including some yeast and bacteria species), but few are able to so efficiently, which is key to enable industrial-scale production (BIAŁKOWSKA, 2016). In that sense, bacteria who are members of the Enterobacteriaceae family stand out as they can metabolise several substrates and grow fast in simple media (CELIŃSKA; GRAJEK, 2009). The produced stereoisomer varies according to the species and the medium of choice; however a mixture of diols is usually obtained (JI; HUANG; OUYANG, 2011).

2,3-BD production is vital for the microbe physiology, as it prevents the acidification of the broth and also represents a carbon reserve (YANG et al., 2017). The produced stereoisomer varies according to the strain and medium employed. However, a mixture of diols is commonly formed (VOLOCH et al., 1987). Generally, 2,3-BD can be generated from different carbon sources, such as glycerol, some disaccharides, and from most of monosaccharides (ZENG; SABRA, 2011). When using the latter, 2,3-BD is formed based on pyruvate from glycolysis or the pentose phosphate pathway, in a mixed acid pathway, as a result of the formation of various intermediate compounds, such as α-acetolactate, diacetyl, and acetoin, and accompanied by other end-products such as ethanol, lactic acid, and acetic acid (MADDOX, 1996).

In this context, one of the key parameters in the 2,3-BD bioconversion is the oxygen supply in cultures (VOLOCH et al., 1987). Increased oxygen availability enhances the proportion of acetoin:2,3-BD formed (CELIŃSKA; GRAJEK, 2009). However, if aeration supply produces excessive oxygen concentration for the microbe demand, main products shift to cell biomass and carbon dioxide (MADDOX, 1996). Broth pH also affects 2,3-BD bioconversion, as values greater than 6 inhibit the enzymes involved in the bioconversion mechanism (YANG et al., 2017). However,

optimal values of these parameters depend on the microorganism and the culture medium of choice (GUO et al., 2017; GURAGAIN; VADLANI, 2017; LING et al., 2017).

Given these considerations, the objective of this work is to study the metabolism of *Pantoea agglomerans* BL1 in submerged batch bioreactors aiming to the production of 2,3-BD. This strain was isolated from an environmental microbial consortium by Rossi et al. (2011). The formation of this diol in orbital shaker from soybean hull was investigated in a previous work (CORTIVO et al., 2019). However, until now, the cultivation of this microorganism in bioreactors to obtain 2,3-BD has not been reported in the literature.

As substrate, soybean hulls submitted to diluted acid pretreatment were used. This type of operation solubilizes the hemicellulosic fraction, resulting in a broth rich in xylose and arabinose, as well as smaller amounts of glucose. As soybean hulls contain low lignin content compared to other agroindustrial biomasses, efficient hydrolysis can be performed at milder conditions, ensuring greater appeal to the operation (CASSALES et al., 2011). As a consequence, the quantity of inhibitory compounds formed during this treatment, as acetic acid, furfural and 5-(hydroxymethyl)furfural (HMF), is also low (JÖNSSON; MARTÍN, 2016).

1.1 Objectives

The present work aimed to evaluate the biotechnological production of 2,3-BD by *P. agglomerans* BL1 in submerged batch bioreactors, employing as substrate soybean hull acid hydrolysate. Specific objectives included:

- Evaluate the influence of aeration and pH on the production of 2,3-BD by *P. agglomerans* BL1 in soybean hull hydrolysate;
- Evaluate the influence of medium supplementation on this bioprocess in relation to yield and productivity of 2,3-BD.

2 Literature Review

This chapter presents the theoretical background regarding 2,3-butanediol production by different microbes and using different substrates. Key variables and strategies available to improve the investigated bioconversion process are also discussed. The topics covered aim to contextualize the present work and justify the choice of bacterium and substrate studied, as well as their importance in the field of biotechnology.

2.1 2,3 – Butanediol

2,3-BD is an important bulk chemical that exist as three stereoisomers, as described in Figure 1: L-(+)-2,3-BD [(2S, 3S) -2,3-BD, dextrororatory form], D-(-)-2,3-BD [(2R, 3R) -2,3-BD, laevorotary form] and *meso*-2,3-BD [(R, S) -2,3-BD, optically inactive form] (CELIŃSKA; GRAJEK, 2009). Its unique molecular structure allows applications as an intermediate in various branches of the chemical industry (JI; HUANG; OUYANG, 2011). Figure 2 summarizes main 2,3-BD utilization. It should be noted that D-(-)-2,3-BD has distinct uses in the synthesis of high-price chiral distinct chemicals (PARK; RATHNASINGH; SONG, 2015). Furthermore, its low freezing point allows its use as anti-freezing agent (LI et al., 2015). However, other potential applications do not require a specific stereoisomer (BIAŁKOWSKA, 2016).

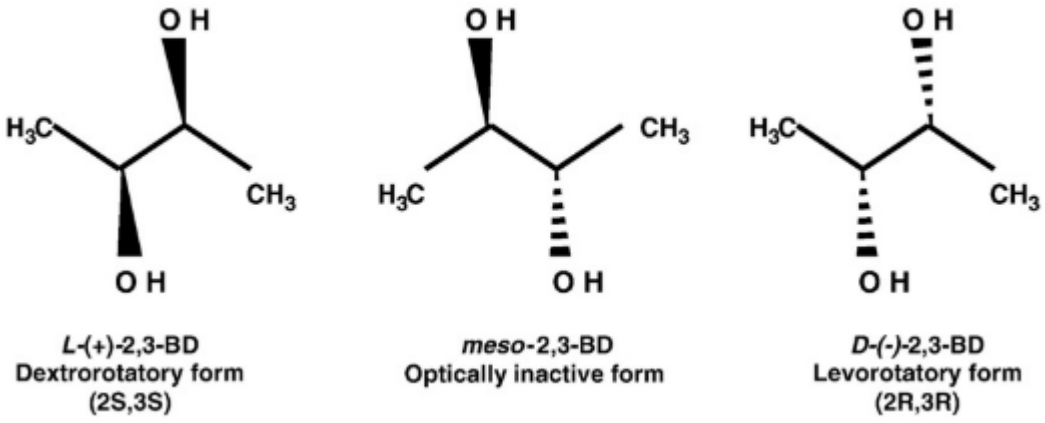

Figure 1: 2,3-butanediol stereoisomers. Extracted from celińska; Grajek (2009).

2,3-BD dehydration, for instance, leads to the formation of 1,3-butadiene, the monomeric unit used in the production of synthetic rubber (JANSEN; TSAO, 2005); Additionally, 1,3-butadiene has high calorific value - comparable to that of other liquid fuels, such as methanol and ethanol – and high octane number, which permits its utilization as an additive to gasoline and high-grade aviation fuel (GARG; JAIN, 1995).

Moreover, 2,3-butanediol dehydration also allows the production of Methyl Ethyl Ketone (MEK), an industrial solvent that can, likewise 1,3-butadiene, boost aviation fuel's quality (VOLOCH et al., 1987).

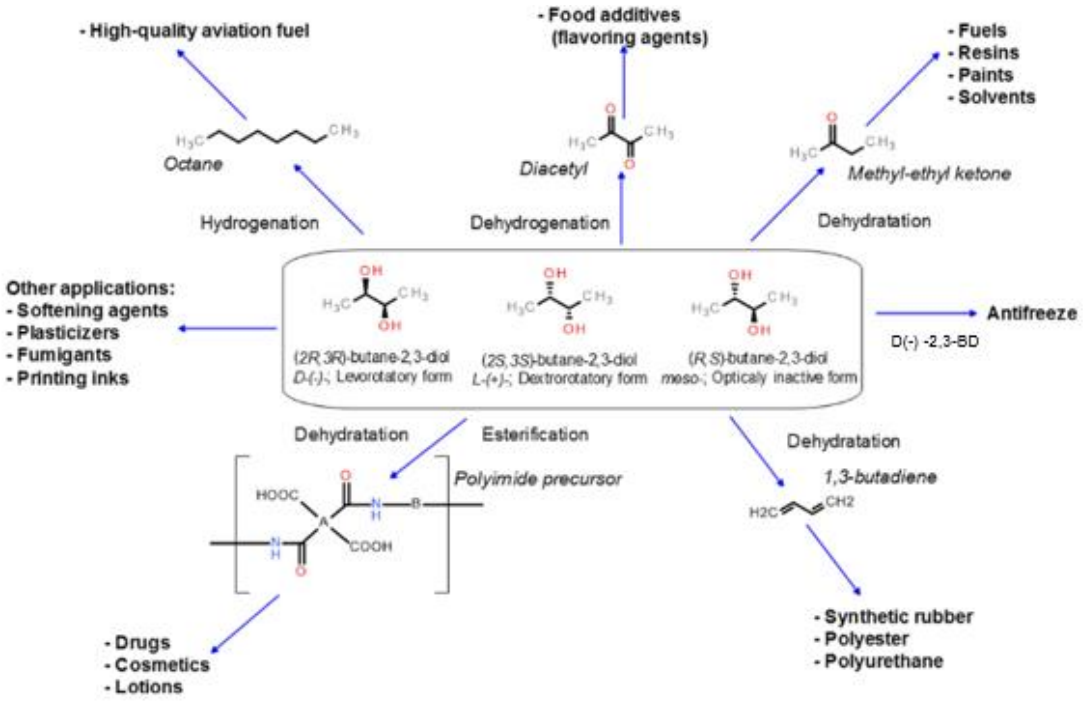

Figure 2: 2,3-butanediol main applications. Extracted from Białkowska (2016).

Other 2,3-BD end-products are diacetyl and acetoin, obtained from 2,3-BD dehydrogenation (JI; HUANG; OUYANG, 2011). They have valuable applications in the food industry. Acetoin is used as an aroma carrier in flavours and essences, diacetyl is a bacteriostatic food additive and can also be a flavouring agent, providing products of a buttery taste (CELIŃSKA; GRAJEK, 2009). Moreover, 2,3-BD diesters, provided by esterification reactions, are important precursors to drugs and cosmetics (JI; HUANG; OUYANG, 2011).

2,3-BD has been described as a bacterial bioconversion product since early 20th century (MADDOX, 1996). However, it was only during World War II that research on this bioproduct had been intensified, as a response to potential shortages of 1,3-butadiene, an strategic chemical (BIAŁKOWSKA, 2016). Nonetheless, efforts in the area were halted, as less expensive routes for chemically producing 1,3-butadiene from petroleum became available. It was only during the 1970s that interest shifted back to its microbiological production, due to expectations of rise in petroleum prices (VOLOCH et al., 1987).

When compared to other well-studied chemicals obtained through biotechnological methods, such as ethanol, acetone/butanol, and 1,3-propanediol (1,3-PD), 2,3-BD production is favoured due to some characteristics of the process. First, 2,3-BD is less toxic to microbial cells, thus permitting higher product concentration. Second, 2,3-BD can be obtained from a variety of substrates, including major sugars (C-6 and C-5 sugars, some disaccharides, glycerol and uronic acids). Finally, 2,3-BD chemical route has higher production costs than the biological approach (ZENG; SABRA, 2011). In that perspective, presently the use of agroindustrial residues to obtain 2,3-BD gains appeal, in view of the current political and economic concerns of fossil fuels and chemical catalysts, in addition to the need to implement more sustainable waste managements (JI; HUANG; OUYANG, 2011).

The market demand for 2,3-BD is related to the plethora of its possible end-products. According to 360 Research Reports (2019), over the next five years the market of this diol will register a compound annual growth rate of 94.6 % in terms of revenue – which corresponds to a market size of US$ 2 million by 2024. Moreover, synthetic 2,3-BD is currently an unattractive process because of its costly synthesis. Therefore, bio-based 2,3-BD is now capable of replacing the synthetic market segment (TRANSPARENCY MARKET RESEARCH, 2018).

2.2 2,3-BD microbial producers

Several microorganisms are capable of synthesizing and accumulating 2,3-BD, such as bacteria, yeast and microalgae, but few do so in large amounts. In this sense, bacterial species who are members of the Enterobacteriaceae family stand out (CELIŃSKA; GRAJEK, 2009). The produced stereoisomer varies according to the species and the medium employed; but commonly a mixture of diols is formed. Table 1 resumes bacteria commonly used for 2,3-BD production in research, as well as their product yields and conversion efficiencies.

So far, bacteria that belong to the genus *Klebsiella* are the best 2,3-BD producers because of their ability to metabolize several sugars, both hexoses and pentoses, and to grow rapidly in simple media (JANSEN; TSAO, 2005). Nevertheless, they present possible pathogenicity (biosafety level 2), which can make industrial application unattractive (CELIŃSKA; GRAJEK, 2009). On the other hand, if chiral 2,3-BD is the desired product, *P. polymyxa* is a good choice, because it is a non-

pathogenic bacteria that can generate up to 98 % levorotary 2,3-BD (OKONKWO; UJOR; EZEJI, 2017). Yet, *Klebsiella sp.* usually forms at least twice the amount of 2,3-BD attainable using *P. polymyxa* (JI; HUANG; OUYANG, 2011). Therefore, research with new microorganisms is desirable and necessary in order to allow large-scale 2,3-BD biological synthesis.

Table 1: Main bacteria species producers of 2,3-BD. Extracted from Ji; Huang; Ouyang (2011)

Strains	2,3-Butanediol stereoisomer [a]	Microbial 2,3-Butanediol production [b]						References
		Substrates	Methods [c]	Diol concentration (g/L)		Diol productivity [g/(L h)]	Diol yield (g/g)	
				2,3-Butanediol	Acetoin			
Klebsiella pneumoniae	meso-, L-(+)-	Glucose	Fed-batch	150.0	10.0	4.21	0.43	Ma et al. (2009)
Klebsiella pneumoniae	meso-, L-(+)-	Glucose	Fed-batch	92.4	13.1	2.10	0.49	Qin et al. (2006)
Klebsiella pneumoniae	meso-, L-(+)-	Corncob molasses	Fed-batch	78.9	3.6	1.35	0.41	Wang et al. (2010)
Klebsiella pneumoniae	meso-, L-(+)-	Glycerol	Fed-batch	49.2	–	0.18	0.36	Petrov and Petrova (2009)
Klebsiella pneumoniae	meso-, L-(+)-	Glycerol	Fed-batch	70.0	–	0.47	0.39	Petrov and Petrova (2010)
Klebsiella pneumoniae	meso-, L-(+)-	*Jerusalem artichoke* tuber	Fed-batch; SSF	84.0	7.6	2.29	0.32	Sun et al. (2009b)
Klebsiella pneumoniae	meso-, L-(+)-	*Jerusalem artichoke* stalk and tuber	Fed-batch; SSF	67.4	13.1	1.18	1.81 [d]	Li et al. (2010a)
Klebsiella oxytoca	meso-(9), L-(+)-(1)	Corncob hydrolysate	Fed-batch	35.7	–	0.59	0.50	Cheng et al. (2010)
Klebsiella oxytoca	meso-(9), L-(+)-(1)	Glucose	Fed-batch	130.0	1.4	1.64	0.48	Ji et al. (2010)
Klebsiella oxytoca	meso-(9), L-(+)-(1)	Glucose	Batch	95.5	1.9	1.74	0.49	Ji et al. (2009a)
Klebsiella oxytoca	meso-(9), L-(+)-(1)	Molasses	Cell recycle	118.0	2.3	2.40	0.42	Afschar et al. (1991)
Klebsiella oxytoca	meso-(9), L-(+)-(1)	Corn cob cellulose	Batch; SSF	25.0	–	0.36	0.31	Cao et al. (1997)
Klebsiella oxytoca	meso-(9), L-(+)-(1)	Glucose	Fed-batch	85.5	6.4	3.22	0.50	Qureshi and Cheryan (1989b)
Enterobacter aerogenes	meso-(9), L-(+)-(1)	Glucose	Fed-batch	110.0 [e]		5.40	0.49	Zeng et al. (1991)
Serratia marcescens	meso-	Sucrose	Fed-batch	152.0	0.0	2.67	0.41	Zhang et al. (2010a)
Serratia marcescens	meso-	Sucrose	Fed-batch	139.9	6.7	3.49	0.47	Zhang et al. (2010b)
Paenibacillus polymyxa	D-(−)-	*Jerusalem artichoke* tuber	Batch	36.9	–	0.88	0.50	Gao et al. (2010)
Bacillus subtilis	D-(−)-(3), meso-(2)	Glucose	Batch	2.5	0.6	0.33	0.38	Moes et al.(1985)
Bacillus licheniformis	D-(−)-, meso-	Glucose	Flask without shaking	8.7	–	0.47	0.12	Nilegaonkar et al. (1992)
Bacillus amyloliquefaciens	D-(−)-, meso-	Glucose	Batch	33.0	–	–	0.33	Alam et al. (1990)

[a] Based on Celińska and Grajek (2009) and Maddox (1996); in parentheses: relative amount of the isomer is given.

[b] Diol means 2,3-butanediol plus acetoin.

[c] SSF: simultaneous saccharification and fermentation.

[d] Means g diol per (3 g stalks + 4 g tubers).

[e] The total production of 2,3-butanediol and acetoin.

2.3 2,3-BD metabolic pathway

Essentially, 2,3-BD can be generated from different carbon sources as stated above, originated from the hydrolysates of hemicellulosic and cellulosic materials. In bacterial metabolism, the biosynthesis is based on formation of pyruvate (BIAŁKOWSKA, 2016; ZENG; SABRA, 2011). Figure 3 resumes main feedstocks that can be used for 2,3-butanediol production. It is important to note that other diols such as 1,3 – 1,3-PD, 1,4-butanediol (1,4-BD), and 1,2-propanediol (1,2-PD), can also be obtained from these carbon sources (RIPOLL et al., 2016; YANG; ZHANG, 2018)

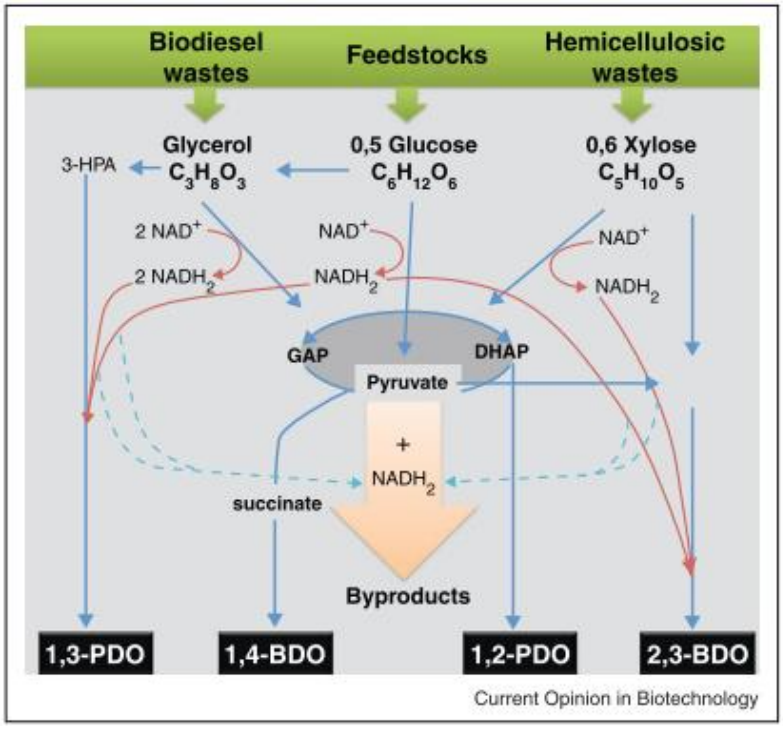

Figure 3: Production of 1,3-propanediol, 1,2-propanediol, 1,4-butnediol and 2,3-butanediol from different feedstocks. Extracted from Zeng; Sabra (2011).

The metabolic route for enterobacteria growing on monosaccharides – the used carbon source in the present work - is extensively described in the literature (CELIŃSKA; GRAJEK, 2009; GARG; JAIN, 1995; MADDOX, 1996; SYU, 2001). From hexoses, pyruvate can be obtained through the glycolysis (Embden–Meyerhof) pathway. Meanwhile, from pentoses, metabolism goes through a combination of pentose phosphate and glycolysis pathway (JI; HUANG; OUYANG, 2011). It involves a mixed acid pathway, as well as the formation of various intermediates, such as α-acetolactate, diacetyl, and acetoin. Other end-products depend on the microorganism employed and culture conditions; i.e., ethanol, acetate, lactate, and formate. Figure 4 illustrates the metabolic pathway for enterobacteria.

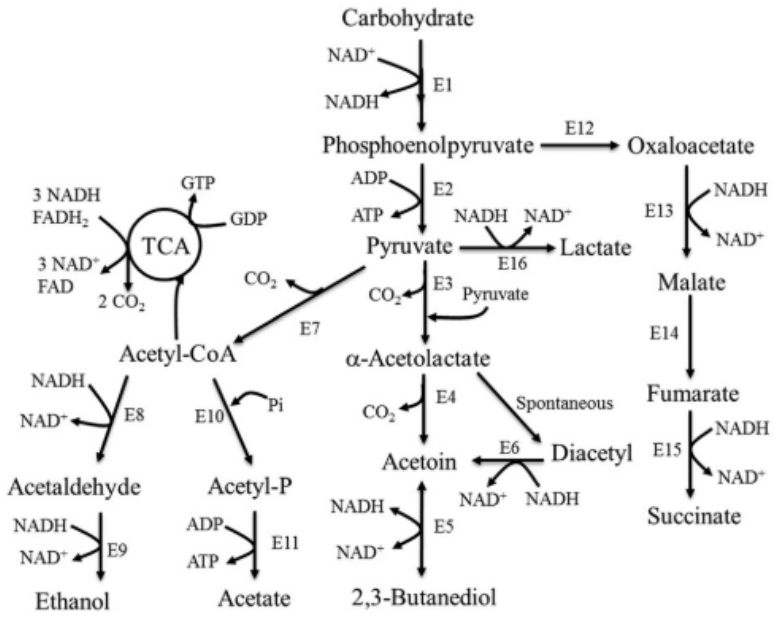

Figure 4: Mixed acid 2,3-BD pathway. E1: Embden–Meyerhof and pentose phosphate pathway enzymes; E2: pyruvate kinase; E3: α -acetolactate synthase; E4: α-acetolactate decarboxylase; E5: acetoin reductase/2,3-butanediol dehydro- genase; E6: diacetyl reductase; E7: pyruvate–formate lyase; E8: acetaldehyde dehydrogenase; E9: ethanol dehydrogenase; E10: phospho-transacetylase; E11: acetate kinase; E12: phosphoenolpyruvate decarboxylase; E13: malate dehydrogenase; E14: fumarase; E15: succinate dehydrogenase; E16: lactate dehydrogenase Extracted from Yang et al. (2017).

There are 3 key enzymes associated to 2,3-BD biosynthesis from pyruvate: α-acetolactate synthase (α-ALS), α-acetolactate decarboxylase (α-LDC), and 2,3-BD dehydrogenase (BDH, also called acetoin reductase). First, α-ALS, whose selectivity is favoured under slightly acidic conditions (pH 6), performs a two-step reaction, generating α-acetolactate as product. Under anaerobic conditions, α-LDC further transforms α-acetolactate into acetoin. Finally, BDH reduces acetoin to 2,3-BD (BIAŁKOWSKA, 2016; JI; HUANG; OUYANG, 2011).

On the other hand, under aerobiosis α-acetolactate can spontaneously be decarboxylated to diacetyl. Afterwards, diacetyl is converted to acetoin by diacetyl reductase (DAR). Yet, under aerobic conditions, pyruvate is mainly turned into acetyl-CoA by the action of the pyruvate dehydrogenase multi-enzyme complex, and then conveyed through the tricarboxylic acid (TCA) cycle (VOLOCH et al., 1987).

Finally, regarding the pool other end-products, besides the bacterial strain, the main determining aspect is oxygen availability. The absence of oxygen benefits formation of succinate and ethanol, formed by succinate dehydrogenase and ethanol dehydrogenase, respectively. When culture conditions are not fully aerobic, lactate dehydrogenase, pyruvate-formate lyase, and α-ALS use pyruvate to generate lactic

acid, formic acid, and 2,3-BD (CELIŃSKA; GRAJEK, 2009; YANG; ZHANG, 2018). Formic acid can be further metabolized to carbon dioxide and hydrogen by the formate-hydrogen lyase complex. Additional increase in oxygen supply benefits acetic acid formation, catalysed by acetate kinase (MADDOX, 1996).

In this manner, formation of 2,3-BD is important to bacterial physiology, as it prevents intracellular acidification by forming a neutral compound instead of organic acids (VOLOCH et al., 1987). Moreover, acid supplementation promotes 2,3-BD biosynthesis, which implies that 2,3-BD counterbalances excessive acidification of the medium (NAKASHIMADA et al., 2000). The reversible conversion of acetoin to 2,3-BD relies on a NADH/NAD^+ transformation, so this pathway might also take part in the appropriate regulation of these co-factors ratio (CELIŃSKA; GRAJEK, 2009). Microorganisms can also utilize acetoin and 2,3-BD as carbon sources when other supplies have been depleted. Thus, microbial acetoin and 2,3-BD synthesis represents a carbon and energy storing strategy (BIAŁKOWSKA, 2016).

Concerning optical purity of 2,3-BD, recent research focuses on characterization of enzymes involved in the biosynthesis pathway. Because BDH catalyses the reversible conversion between acetoin and 2,3-BD, the stereoisomer of 2,3-BD is determined by the specificity of BDH (LI et al., 2015; ZHANG et al., 2016, 2018). Therefore, hosts for efficient production of optically pure 2,3-BD can be constructed (YANG; ZHANG, 2018).

Increasing titer and reducing process costs drive most research about engineering natural bacterial 2,3-BD producers. Mutants were developed showing enhanced substrate utilization, as well as increased efficiency for 2,3-BD production and showing minimal by-product formation, (ZENG; SABRA, 2011). According to Yang and collaborators (2017), recent strategies include overexpressing enzymes involved in glycolysis pathway (YANG et al., 2013), manipulating the NADH level (BAO et al., 2015), blocking by-product formation (JI et al., 2010) and engineering 2,3-BD pathway by blocking acetoin dissimilation (ZHANG; ZHANG; BAO, 2013) or overproducing α-ALS and/or α-LDC (XIAO; LU, 2014).

When using sugars obtained from biomass as carbon source, such as those from soybean hull hydrolysate, the biggest challenge for the bacterial culture is the cell physiology towards the mixture of different monosaccharides liberated in the

medium, which might cause catabolite repression due to preferential use of glucose by cells (ZENG; SABRA, 2011). Therefore, there have been efforts to regulate expression of genes responsible for sucrose, xylose, and glucose assimilations in order to overcome this problem (YANG et al., 2017; YANG; ZHANG, 2018).

2.4 *Pantoea agglomerans*

The bacterium *Pantoea agglomerans* was designated in 1989 to differentiate among three enterobacteria previously classified in other genera, which were recognized to be synonymous - *Enterobacter agglomerans*, *Erwinia herbicola*, and *Erwinia milletiae* (BEIJERINCK, 1888; GAVINI et al., 2019; TINDALL, 2014). The genus *Pantoea* is derived from the Greek word *'pantoios*', meaning *of all sorts and sources,* whereas the specific name '*agglomerans*' means *forming into a ball*, which illustrates the peculiar property of this organism to form aggregates, which play a role in the colonization bacterial process (DUTKIEWICZ et al., 2015).

Pantoea spp. belong to the family Enterobacteriaceae, gammaproteobacteria class that contains facultatively anaerobic and fermentative Gram-negative bacteria (DUTKIEWICZ et al., 2015). The genus is close to *Tatumella* and *Erwinia*, wherein the three form a monophyliletic group related to other enterobacterial genera: *Escherichia*, *Salmonella*, *Citrobacter, Enterobacter*, *Klebsiella,* and *Cronobacter* (WALTERSON; STAVRINIDES, 2015). *Pantoea spp*. form straight rods that are motile by peritrichous flagella and are non-capsulated, non-sporing (MORIN, 2014).

Pantoea genera is composed of 20 different phenotypically similar species. They are probably one of the most widespread organisms in the world, as they can be found in a variety of materials and environments, such as plants, seeds, fruits, soil, water, human and animal gastrointestinal tracts, dairy products, blood and urine (DUTKIEWICZ et al., 2015; WALTERSON; STAVRINIDES, 2015). Some species of *Pantoea* are plant pathogens and some can be infectious agents in immunocompromised humans, causing wound, blood, and urinary-tract infections. Because there are clinical reports of *Pantoea spp.* as opportunistic human pathogen, they are listed as a biosafety level 2 organisms, along with other enterobacteria (MORIN, 2014).

Pantoea strains isolated from different environments have useful characteristics, which have been explored for commercial and industrial applications:

resistance and/or degradation of arsenic (SULTANA et al., 2011), degradation of herbicides (PILEGGI et al., 2012), biosorption of heavy metals as chromium, cadmium, and copper from water (OZDEMIR et al., 2004) and the production of antibiotics in order to manage plant diseases (SMITS et al., 2011; VANNESTE; YU; CORNISH, 2008). Some strains are able to fix nitrogen (MACCOLLOM et al., 2009) and promote plant growth (KIM et al., 2012), which can be useful for agricultural applications.

Regarding biofuels production, research report the ability of *Pantoea spp.* to produce 1,3-propanediol (CASALI et al., 2012; ROSSI et al., 2012), and hydrogen (LIU et al., 2012; LIU; WANG, 2015; MA et al., 2015). However, to the best of our knowledge, the first work reporting the ability of *P. agglomerans* to form 2,3-BD was from our research group (CORTIVO et al., 2019). In the present work, this production process is being scaled up to bioreactors, in which it is possible to have a better control of the cultivation parameters of this robust microorganism.

2.5 Alternative substrates

There are many economic, environmental, and political issues regarding the use of fossil fuels and chemical syntheses. In this perspective, as there is the need to utilize substrates other than food resources, second-generation biorefineries emerge as an alternative to achieve a sustainable economy (HASSAN; WILLIAMS; JAISWAL, 2018). Wild-type bacterial strains such as *P. agglomerans* can synthesize 2,3-BD from various carbon sources (JI; HUANG; OUYANG, 2011). Hence, the use of lignocellulosic material (LM) as substrate to produce 2,3-BD has gained attention over the years, as it is the most abundant source of carbohydrates on earth available for microbial conversion (GEDDES; NIEVES; INGRAM, 2011). Other alternative substrates suitable for second 2,3-BD generation are non-lignocellulosic biomass, which includes crude glycerol, a biodiesel industry by-product, and food industry residues such as molasses, whey, starch hydrolysates from cereals or potatoes (CELIŃSKA; GRAJEK, 2009).

In particular, Brazil is the largest producer of sugarcane in the world, the second largest of soybean and is among the top ten largest suppliers of rice (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, 2017). In the

light of these considerations, in this section, peculiarities of exploring LM in bioprocess will be further scrutinized.

2.5.1 Lignocellulosic material

LM suitable for second-generation biofuel and other chemicals can be generated from agricultural crops and their by-produtcs, such as wheat, barley, maize and rice straws, rice hulls, corn stalks, sugarcane bagasse, among others, or forests management residues, urban wood, and garden residues (KUMARI; SINGH, 2018). Generally, LM consists of three main polymers: cellulose (35–50 %), hemi-cellulose (20–35 %) and lignin (15–20 %), as well as ash and other compounds (15–20 %) (MENON; RAO, 2012). LM structure forms a highly complex arrangement, illustrated in figure 5. Each component has a role in the main structure, which is described below.

- **Cellulose:** it is the structural base of the plant cells and main component of LM. It is a linear biopolymer formed by D-glucose monomers connected by β-(1-4) glycosidic bonds, forming the cellobiose dimer. It forms long chains, joined by hydrogen bonds and Van der Waals forces (JÖNSSON; MARTÍN, 2016). Usually, the largest fraction of cellulose has a crystalline structure. Yet, the amorphous conformation is more susceptible to acid or enzymatic degradation (SÁNCHEZ, 2009).

- **Hemicellulose:** it is a reserve carbohydrate of plants. It has a branched biopolymer with short lateral chains consisting of different sugars (SÁNCHEZ, 2009). It is composed of pentoses (ß-D-xylose, α-L-arabinose), hexoses (ß-D-mannose, β-D-glucose and/or α-galactose), and/or uronic acids (α-D-glucuronic, α-D-4-O-methyl-galacturonicanda-D-galacturonicacids) (SHIRKAVAND et al., 2016). Hemicellulose shows a random and amorphous structure, linked by ß-(1-4) and ß-(1-3) glycosidic bonds, which confers resistance to hydrolysis, unlike cellulose (SINGH et al., 2011).

- **Lignin:** this fraction is a complex aromatic, amorphous, non-water soluble and optically inactive biopolymer. Its structure is formed from fenil propanoid precursors, such as syringyl, guaiac, and p-hydroxyphenol. Covalently attached to hemicellulose and cellulose. Its function is to protect the biomass from degradation, to transport the water inside the stem of the

plant, and to strengthen its mechanical resistance (SÁNCHEZ, 2009; SHIRKAVAND et al., 2016).

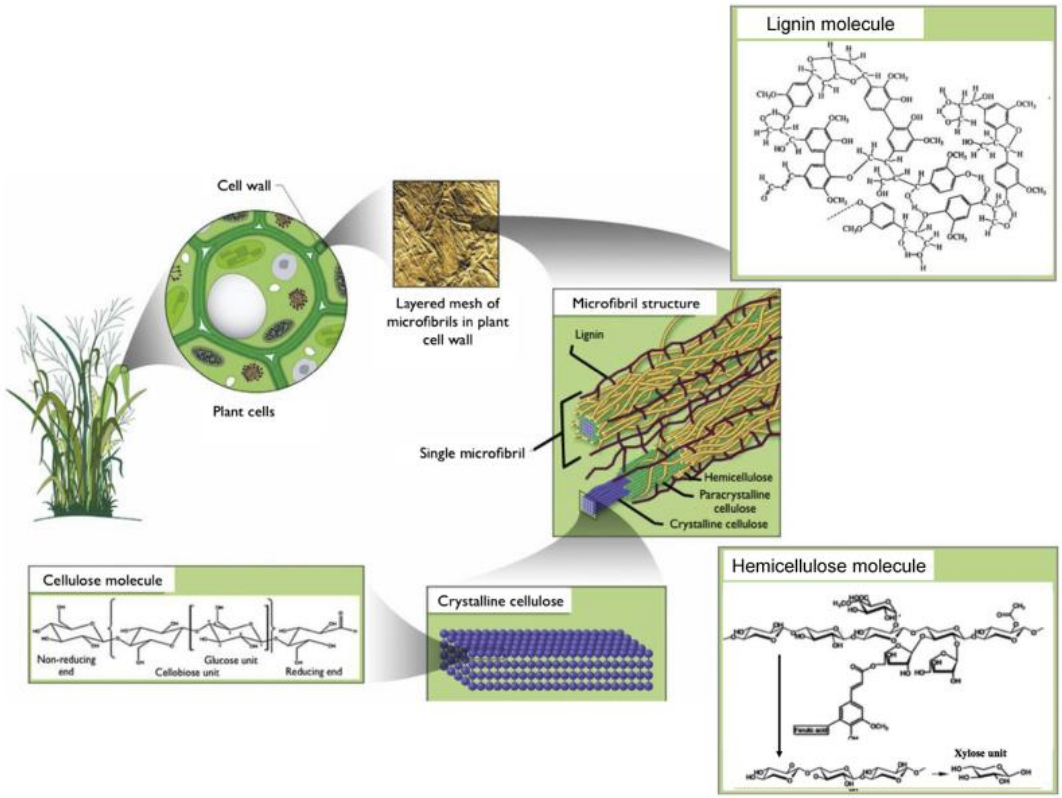

Figure 5: Lignocellulosic biomass structure. Extracted from Seidl; Goulart (2016).

The percentage of lignocellulosic structure components varies greatly according to the nature of the biomass, in addition to climatic and soil characteristics where the vegetal is cultured (JÖNSSON; MARTÍN, 2016). Table 2 illustrates the composition of main LM generated in Brazil. Soybean hulls and stalks, as well as sugarcane bagasse, stand out for their low lignin content and high cellulose and hemicellulose content. Comparatively, rice straws and husks possess higher proportions of lignin and ash compared to the other biomasses. This characteristic may represent additional difficulties for the efficient hydrolysis of these materials, requiring severe physicochemical conditions and higher amounts of toxic compounds being formed (LIM et al., 2012).

Table 2: percentage composition of dry lignocellulosic biomass generated from different sources

Lignocellulosic Biomass	Cellulose	Hemicelullose	Lignin	Reference
Sugarcane bagasse	43	31	11	(SHIRKAVAND et al., 2016)
Barley husk	34	38	19	
Corn straw	38	26	17	
Wheat straw	30	22	17	
Rice straw	32	36	22	(LIM et al., 2012)
Rice husk	29	29	24	
Soybean stalks	35	25	10	
Soybean hull	39.7	25.5	9.1	(CASSALES et al., 2011)

Moreover, soybean hulls are the largest by-product generated in the soybean grain processing, representing 8 to 10 % of the grain mass (LOMAN; JU, 2016) and have marginal commercial value compared to protein and oil. Thus, they are mainly used for animal feed production (IPHARRAGUERRE; CLARK, 2010). Nevertheless, studies in literature demonstrate the potential of using soybean hull in solid-state fermentation in order to produce enzymes such as cellulases (BRIJWANI; OBEROI; VADLANI, 2010), and xylanases (COFFMAN; LI, 2014; MENEZES; MISTURINI; AYUB, 2017). Soybean hulls can also be employed in submerged reactors, as hydrolysates, to obtain ethanol and xylitol (HICKERT et al., 2014), as well as other organic molecules of higher commercial value (CHENG et al., 2017; LOMAN; ISLAM; JU, 2018; YU et al., 2015). Nonetheless, the first study to report production of 2,3-BD from soybean hull hydrolysate in orbital shaker was from our research group (CORTIVO et al., 2019), whose process was scaled up in the present work.

2.5.2 Lignocellulosic material hydrolysis

As described above, in order to be used as medium in submerged cultures to produce 2,3-BD, lignocellulosic biomass needs first to be converted to monosaccharides because it is resistant to depolymerization (GEDDES; NIEVES; INGRAM, 2011). Therefore, bioconversion process in this case can be summarized in three major steps: (i) pre-treatment (also known as delignification); (ii) depolymerization and (iii) cultivation. Pre-treatment allows altering the structure of lignocellulose by increasing the surface area and porosity of the biomass; modification and removal of lignin, partial depolymerization and extraction of hemi-

celluloses and reduction of cellulose crystallinity (SINGH et al., 2014). Depolymerization involves the use of enzymes, such as xynalases and cellulases, to hydrolyse reactive intermediates to bioconvertible sugars.

In this perspective, although lignocellulosic structure represents the main natural resource of polysaccharides for bioprocess, it also presents a major challenge when implementing such operations. According to Galbe; Zacchi (2012) and Jönsson; Martín (2016), this is mainly because:

- The complex chemical conformation of biomass makes high yields of hydrolysis difficult to achieve;
- The resulting mixture of sugars from hydrolysis, in the form of C-5 and C-6, can cause problems during fermentation as pentoses are not as easily assimilated as hexoses by most microorganisms;
- Because of side reactions, there can occur the formation of undesirable toxic compounds during pretreatment, such as acetic acid, formic acid, phenolic compounds, 2-furaldehyde (furfural), and 5-(Hydroxymethyl)furfural (HMF). Most of them are formed during solubilization and degradation of lignin and/or hemicellulose. However, products obtained during fermentation can also originate them. Toxicity may vary according to the microbe, but it can inhibit or even block bioconversion process. The choice of pretreatment technology directly influences the nature and quantity of toxic products.

Strategies to address these setbacks include microbial genetic engineering to turn microorganisms more resistant to culture media under harsh conditions; selection of lignocellulosic raw materials such as to generate lower amounts of inhibitory compounds; detoxification steps following pretreatment for removal of inhibitory compounds, if necessary (JÖNSSON; MARTÍN, 2016). Nonetheless, many authors in literature claim pretreatment is the most crucial step for lignocellulosic biomass microbial utilization, each technology having particular characteristics and being generally designed to a specific type of carbohydrate and/or lignin (HASSAN; WILLIAMS; JAISWAL, 2018). Thus, choosing the appropriate pretreatment process relies only on the desired application (KUMAR; SHARMA, 2017).

Driven by these facts, there are several researches in the literature aiming to optimize and compare different pretreatment technologies suitable for lignocellulosic biomass. According to Galbe and Zacchi (2012), a good pretreatment should be cost effective, environment friendly, result in high recovery of all carbohydrates and present low concentrations of toxic compounds. Recent reviews on the subject categorize conventional types of pretreatment as physical, chemical, physicochemical and biological approaches (CHEN et al., 2017).

The latest efforts to improve efficiency of the pretreatment step include integration of conventional pretreatment techniques, as reducing the number of process steps can be beneficial and minimize production of undesirable inhibitors (KUMAR; SHARMA, 2017). As reviewed by Hassan, Williams and Jaiswal (2018), other studies have focused on the use of emerging technologies as pretreatment for lignocellulosic biomass, such as exposition to radiation and pulsed-electric field. Finally, some review articles highlight application of green solutions, like steam-explosion and biological pretreatment, because most common options are not environmentally friendly (CAPOLUPO; FARACO, 2016; KUMARI; SINGH, 2018).

So far, acid treatment remains the most commonly used method for lignocellulosic biomass hydrolysis on an industrial scale (KUMARI; SINGH, 2018). Sulphuric acid is the main acid used because it produces higher yields of hydrolysis and is cheaper. There are two types of acid operations: high temperature (above 180 °C), for short time (1 to 5 min) and low temperature (<120 °C), for long time (30 to 90 min), respectively. The amount of acid used also varies: being concentrated when above 30 % (volume fraction) and diluted when used in concentrations lower than 10 % (volume fraction). The latter configuration is more attractive, because it does not requires the recycling of the acid stream and requires less robust equipments (CHEN et al., 2017; KUMAR; SHARMA, 2017)

In the present work, soybean hulls were submitted to diluted acid pretreatment. This type of operation solubilizes polysaccharides (mainly the hemicellulosic fraction), resulting in a broth that is rich in arabinose and xylose, as well as smaller amounts of glucose (KUMARI; SINGH, 2018). Nonetheless, depending on operating conditions, some toxic degradation compounds of sugars and lignin can also be generated, such as aliphatic carboxylic acids and phenylic compounds (JÖNSSON; MARTÍN, 2016).

2.6 2,3-BD bioconversion process

The production of 2,3-BD is strongly affected by the media and microorganisms employed. Moreover, other variables that affect the physiology of the microbe, such as oxygen supply, pH, temperature, substrate initial concentration, can also influence 2,3-BD formation. Like other extracellular metabolites, optimizing 2,3-BD production process involves scaling up cultivations to bioreactors, in order to have a better control of the cultivation parameters. In that sense, there has been several different strategies described in the literature (CELIŃSKA; GRAJEK, 2009)

2.6.1 Oxygen supply

Many studies in literature claim that oxygen supply is the most important parameter in 2,3-BD production (VOLOCH et al., 1987). Although a product of anaerobic metabolism, aeration rate influences 2,3-BD production, productivity, by-product formation, and conversion by two factors, as shown in Figure 6 (JI; HUANG; OUYANG, 2011). First, most of 2,3-BD producing microbes are facultative anaerobes, enabling them to obtain energy through respiration and fermentation. If aeration is too high, metabolism is shifted for cell growth. Then, NADH from formation of pyruvate is regenerated via respiration. On the other hand, if there is no oxygen available in the culture, respiration cannot occur. Hence, balance of co-factors is maintained through 2,3-BD and other metabolites pathways (MADDOX, 1996; VOLOCH et al., 1987).

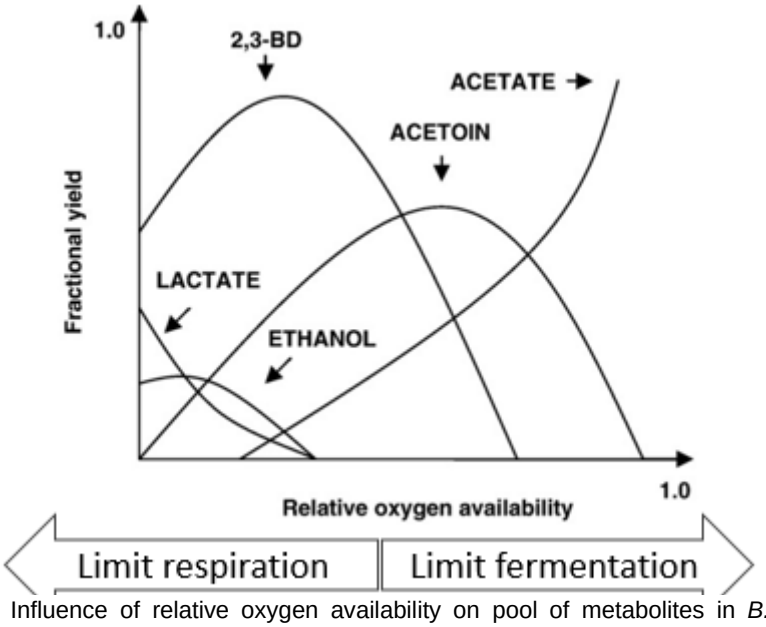

Figure 6 Influence of relative oxygen availability on pool of metabolites in *B. polymyxa* (figure modified, based on de Mas; Jansen; Tsao, 1988).

Therefore, when availability of oxygen is too low, cell mass growth is also compromised, thus 2,3-BD generation is decreased. In other words, maintaining an internal redox balance during glycolysis benefits formation of 2,3-BD, being maximized when both energy producing pathways - respiration and fermentation - are operating simultaneously (CELIŃSKA; GRAJEK, 2009; VOLOCH et al., 1987).

The second factor is that oxygen supply also influences the pool of metabolites obtained in the mixed acid pathway. When aeration rate is high, formation of acetate, biomass and acetoin are favoured. When it is absent, there is formation of ethanol and lactic acid in addition to 2,3-BD. Therefore, boosting aeration rate toward the microbial oxygen demand results in an increment of acetoin:2,3-BD proportion (MADDOX, 1996).

2.6.2 Potential of hydrogen

In bacterial metabolism, potential of hydrogen (pH) is a key variable, especially in processes that include multiple end-product formation. Typically, alkaline conditions benefit organic acids formation, decreasing 2,3-BD yields. Acidic pH, on the other hand, can reduce organic acid synthesis, more than 10-fold, while enhancing diol production up to 3 to 7 times (GARG; JAIN, 1995). Because 2,3-BD is produced through a mixed acid pathway, the pH of media naturally decreases during its bioconversion process. Eventually, cell growth and substrate consumption can be ceased in the culture because of its own toxic products effects (BIEBL et al., 1998). In that sense, as explored in section 2.3, 2,3-BD pathway is triggered in acid conditions: it is strategic to the microorganism because it is less toxic and prevents intracellular acidification (CELIŃSKA; GRAJEK, 2009).

Notwithstanding, the best strategy of pH control for 2,3-BD production is directly related to the microorganism and substrate employed (CELIŃSKA; GRAJEK, 2009). Recent work in which pH-controlled strategies were evaluated for different strains and media reported that pH can influence microbial growth (GUO et al., 2017), the nature of end-products formed from pyruvate (WONG et al., 2014), as well as 2,3-BD productivity and yields (MAINA et al., 2019). Therefore, in the present study, influence of controlled pH was investigated on *P. agglomerans* 2,3-BD production.

2.6.3 Media supplementation and substrate initial concentration

Some authors suggest that it is not acid pH in the media that triggers 2,3-BD production but rather the accumulation of acidic compounds. Størmer (1977) demonstrated that ionized acetate can induce α-ALS formation, which catalyses pyruvate transformation into 2,3-BD. Nakashimada et al. (2000) reported that supplementation with acetate, propionate, pyruvate, and succinate at appropriate amounts, improved 2,3-BD conversion by *B. polymyxa* ATCC 12321; whereas the addition of butyrate, valerate, formate, lactate, and malate did not influence 2,3-BD formation.

In this perspective, the addition of acetic acid was tested in some researches, with different strains and media for the production of 2,3-BD. This is particularly relevant when it comes to utilizing lignocellulosic biomass as substrate, as acetic acid is naturally generated during hydrolysis step (JÖNSSON; MARTÍN, 2016) (section 2.5.2). Joo et al. (2016) reported that in cultures of *Enterobacter aerogenes* KCTC 2190, although cell growth was slightly lowered, formation of 2,3-BD in media utilizing as sole carbon source glucose or xylose was improved with the addition of acetic acid (12 $g \cdot L^{-1}$ and 9 $g \cdot L^{-1}$ respectively). Cheng and collaborators (2010) reported that controlled pH at 6.3 during fed-batch experiments could alleviate inhibition by acetic acid formation and improve 2,3-BD production by *Klebsiella* oxytoca ACCC 10370 using corncob acid hydrolysate as substrate, with low acetic acid concentration. Lee and co-authors (2017) enhanced 2,3-BD production by *Enterobacter aerogenes* SUMI014 ldhA mutant employing a pulsed fed-batch strategy of acetate (50 %) and glucose (stock solution was 800 $g \cdot L^{-1}$) addition.

Other chemicals can also be supplemented to the substrate in order to obtain higher 2,3-BD yields. Commonly used substances are yeast extract, urea, peptone and, ammonium salts, which can be used as nitrogen sources for microbial growth (GAO et al., 2010; RIPOLL et al., 2016; YANG et al., 2015). Other alternatives are addition of compounds such as acid disodium salt (Na_2EDTA) (WANG et al., 2016), mineral-trace elements (JI et al., 2009), and vitamin C (VC) (DAI et al., 2014).

Finally, another important aspect regarding substrate in 2,3-BD bioprocess is the carbon source initial concentration. In general, lignocellulosic biomass studies employ starting concentrations of carbohydrates in the range of 5-10 % (mass

fraction). This relatively low amount is partly because as sugar concentration in a raw material increases, the amount of toxic substances (weak acids, furan derivatives, and phenolics, section 2.5.2) also increase (GARG; JAIN, 1995). For this reason, some works suggest a detoxification step prior to cultivation is necessary to improve efficiency when utilizing such substrates (JOO et al., 2016; LEE et al., 2015). On the other hand, studies that use synthetic media with pure sugars, like glucose, can present initial sugar concentrations of up to 200 $g \cdot L^{-1}$, as no inhibitory compounds are present in the substrate (CELIŃSKA; GRAJEK, 2009).

2.6.4 Temperature

Bioprocesses efficiencies are strictly correlated to temperature, as enzymatic activity and cellular maintenance varies greatly upon temperature changes (GARG; JAIN, 1995). Because distinct microbes may have different performances according to temperature variations, optimal values should be attained for each strain and used substrate independently (CELIŃSKA; GRAJEK, 2009). In previous study of our group, the influence of temperature on the production of 2,3-BD by *P. agglomerans*, cultivated in acid soybean hull hydrolysate was investigated and better results were obtained at 37 °C (CORTIVO et al., 2019). Thus, all cultivations in the present work were carried out at 37°C.

3 Methodology

In this chapter, the methodology employed to carry out this work is detailed. All experiments were performed at the Biotechnology & Biochemical Engineering Laboratory (BiotecLab), located at Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul, Brazil.

3.1 Chemicals, microorganism, and cell maintenance

P. agglomerans BL1 was isolated from a sludge of an upflow anaerobic bacterial blanket reactor (UASB) at a local soybean treatment plant (Esteio, Brazil). The strain was isolated and chemically characterized in a previous work (ROSSI et al., 2011). Its viable stocks belong to BiotecLab Microbiology Culture Collection (UFRGS, Brazil) and are kept frozen in 50 % glycerol-Luria-Bertani (LB) medium in an ultrafreezer at – 80 °C. For the pre-inocula of all cultures, 1 mL of the microorganism stock was inoculated into 500 mL Erlenmeyers flasks containing 250 mL of LB liquid medium. Pre-inocula were cultured for 14 h on orbital shaker (30 °C and 120 rpm), until the cell concentration corresponded to an optical density (600 nm) of 1. All chemicals used in this research were purchased from Sigma-Aldrich (MO, St. Louis) and were of analytical grade.

3.2 Soybean hull acid hydrolysate and substrate initial conditions

Soybean hulls used in this study were obtained from local mills (Rio Grande do Sul State, geo-coordinated: 30° 51'04”S and 51° 48'44”O) as dry material. No further treatment was imposed on them before hydrolysis. The solubilization of the hemicellulosic fraction was performed in an autoclave (1 % (volume fraction) H_2SO_4 solution, solid:liquid ratio of 1:10, at 121 °C and 40 min) as optimized by Cassales et al. (2011). The resulting liquid fraction was recovered by filtration followed by centrifugation (3500 g, 4 °C, 15 min) and its sugar loading was analysed by HPLC (section 3.4). Hydrolysis yields calculation was performed according to a previous work (CORTIVO et al., 2019) and corresponded to 62 % of liberation of sugars of the total present in the hemicellulose fraction.

No subsequent enzymatic hydrolysis was performed with the remaining solid fraction of soybean hull. In order to decrease the water content of the liquid fraction and obtain a fixed concentration to be used in cultivation experiments, soybean acid hydrolysate was vacuum concentrated at 60 °C. Additional detoxification or

supplementation of culture medium was not carried out. Prior to the experiments, the initial pH of the medium was adjusted to 6.0 using NaOH (solid, micropearls). This procedure causes precipitation of several compounds contained in the medium. Therefore, to minimize the quantity of undissolved particles, soybean acid hydrolysate was centrifuged again (3500 g, 4 °C, 15 min). Finally, to ensure sterility before inoculation, it was autoclaved (30 min, 111 °C).

To evaluate the potential of acid soybean hull hydrolysate as substrate, after all the described preparation steps the media was chemically characterized (NIELSEN, 2017). Mineral profile was established by inductively coupled plasma atomic emission spectrometry (ICP-AES). Total nitrogen content was measured through the Kjeldahl method, using the N x 6.25 conversion factor. Dry ashes were determined by weight difference before and after incineration of samples in a muffle furnace at 600 °C for 4 h. Nitrogen and ashes analyses were conducted in triplicate. Furthermore, initial culture conditions (after inoculation) were analysed. Osmotic pressure was measured by osmometry (30 µL samples) in an osmometer (VAPRO 5520, USA). Concentration of acetic acid, furfural, HMF, and monosaccharides were obtained by HPLC (section 3.4).

3.3 Culture conditions

All cultivations (orbital shaker and bioreactor batch experiments) were run at 37 °C and performed in duplicate. Bioreactor batch cultivations were carried out in fully equipped 2 L bioreactors (Biostat B model, Braun Biotech International, Germany) and in all experiments stirring speed was set at 300 rpm. In each culture, 150 mL of pre-inoculum was added to soybean hull hydrolysate medium, resulting in 1500 mL of working volume. Cultivation kinetics were monitored by collecting 2 mL of samples at regular intervals up to 48 h. Antifoam was added to the broth when needed.

The influence of oxygen supply was evaluated by either controlling it at an anaerobic condition (no aeration, but stirring speed set at 300 rpm), or setting aeration rate at 0.5; 1, or 2 vvm through a needle valve and a rotameter. By addition of NaOH or H_3PO_4 (1 M) solutions, pH was either set at 6.0 on the start of cultivation (uncontrolled pH experiments) or maintained at 6.0 (controlled pH experiment). To evaluate influence of yeast extract supplementation, properly concentrated soybean

hull hydrolysate and yeast extract solutions were autoclaved separately, in order to obtain a final broth containing 5 $g \cdot L^{-1}$ of yeast extract and 30 $g \cdot L^{-1}$ of xylose (main sugar of soybean hull hydrolysate). To investigate the effect of addition of Vitamin C (VC) on *P. agglomerans* metabolism, VC stock solution (89.28 $g \cdot L^{-1}$) was continuously added into the bioreactor by a peristaltic pump at a constant flow rate of 2.24 $mL \cdot h^{-1}$ after 7 h of cultivation, resulting in a concentration in the broth of 2.22 $mg \cdot L^{-1}$ of VC. The VC stock solution was sterilized by filtration through a 0.22 µm sterile filter before adding into the culture.

For the orbital shaker experiments, cultivation kinetics were monitored by collecting 2 mL of samples at regular intervals up to 72 h. 125 mL Erlenmeyers flasks filled with 62.5 mL of working volume (inoculum corresponded to 10 % volume fraction), and flasks were incubated at 37 ˚C and 120 rpm. Stock solutions of yeast extract, peptone and sodium acetate were autoclaved separately. Supply solutions of trace elements, $MgCl_2$, K_2HPO_4 and Na_2EDTA were individually sterilized by filtration utilizing a 0.22 µm sterile filter. Trace element solution consisted of (in $g \cdot L^{-1}$): 0.1 $MnCl_2 \cdot 4\ H_2O$, 0.06 H_3BO_3, 0.0037 $CuSO_4 \cdot 5H_2O$, 0.2 $CoCl_2 \cdot 6H_2O$, 0.025 $NiCl_2 \cdot 6H_2O$, 0.035 $Na_2MoO_4 \cdot 2H_2O$, 0.14 $ZnSO_4 \cdot 7H_2O$, and 0.9 mL of HCl (37 % volume fraction).

3.4 Analytical methods

Sugars (xylose, arabinose and glucose), metabolites (2,3-BD, acetoin, acetic acid, lactic acid, succinic acid, formic acid, and ethanol), and inhibitory compounds (furfural and HMF) concentrations were determined by HPLC (Shimadzu, Japan). Culture samples were centrifuged (3500 g, 4 °C, 15 min) to pellet the cells, in order to prepare them for analysis. Samples were diluted (1:10) in distilled water, and filtered using cellulose acetate membrane (pore size 0.22 µm, Sartorius, Germany).

Sugar and metabolites were estimated using a RID-10A refractive index detector (Shimadzu) and an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad, USA). Mobile phase was kept in isocatric mode and consisted on 5 mM H_2SO_4 solution; sample injection volume was 20 µL. For the quantification of xylose, arabinose, glucose, ethanol, succinic acid, acetic acid, lactic acid and formic acid, analytical temperature of 45 °C and flow rate of 0.6 $mL \cdot min^{-1}$ was used, whereas for 2,3-BD and acetoin, 65 °C and 0.5 $mL \cdot min^{-1}$ were the conditions. Aminex HPX-78H allows for a good distinction of meso and optically active forms of 2,3-BD, but does

not differ D-(-) and L-(+)-2,3-BD. For this reason, in this work 2,3-BD concentration was considered as the sum of all diols stereoisomers produced by *P. agglomerans*, without further differentiation.

Inhibitory compounds were separated using a C_{18} column (4.6 mm x 250 mm, 5 mm particle size, Shimadzu) and quantified using a DAD detector (Shimadzu). Analysis parameters were modified from conditions previously described in detail by Rodrigues; Mariutti and Mercadante (2013). Flow rate was kept at 0.9 $mL \cdot min^{-1}$, column temperature at 29 °C, sample injection volume corresponding to 20 µL. Mobile phase consisted of a water:formic acid mixture [99.5:0.5 ($v \cdot v^{-1}$)] (solvent A) and an acetonitrile:formic acid mixture [99.5:0.5 ($v \cdot v^{-1}$)] (solvent B) in a linear gradient, as follows: from 99:1 ($v \cdot v^{-1}$) $A \cdot B^{-1}$ to 69.6:30.4 ($v \cdot v^{-1}$) $A \cdot B^{-1}$ over 30 min; from 69.6:30.4 ($v \cdot v^{-1}$) $A \cdot B^{-1}$ to 50:50 ($v \cdot v^{-1}$) $A \cdot B^{-1}$ over 3 min; from 50:50 ($v \cdot v^{-1}$) $A \cdot B^{-1}$ to 1:99 ($v \cdot v^{-1}$) $A \cdot B^{-1}$ for 7 min, from 1:99 ($v \cdot v^{-1}$) $A \cdot B^{-1}$ to 99:1 ($v \cdot v^{-1}$) $A \cdot B^{-1}$ for 5 min. Then, the former ratio [99:1 ($v \cdot v^{-1}$) $A \cdot B^{-1}$] was maintained for an additional 3 min, totalizing 53 min of running time. The UV−vis spectra were evaluated (200 nm to 600 nm), and chromatograms were quantified at 276 and 283 nm for furfural and HMF, respectively.

3.5 Kinetic parameters calculation and statistical analysis

In this study, kinetic parameters were calculated at the cultivation time when 2,3-BD production curve showed maximum productivity. 2,3-BD yields ($Y_{P/S}$, $g \cdot g^{-1}$) was defined as the ratio between the amount of 2,3-BD produced and the total sugars consumed at the chosen interval. Volumetric productivity (Q_P, $g \cdot L^{-1} \cdot h^{-1}$) was determined by the ratio between the concentration of 2,3-BD and the cultivation time to reach it. Experiments were conducted in duplicate and the obtained results were submitted to analysis of variance and Tukey's test at 5 % significance level. All statistical analyses were performed using Statistica 12.0 software (StatSoft Inc., São Paulo, Brazil).

4 Results and discussion

In this chapter, results obtained in the cultivations of *P. agglomerans* strain BL1 in soybean acid hydrolysate will be presented and discussed.

4.1 Soybean hull acid hydrolysate characterization

Prior to experiments, the hydrolysate was diluted to obtain an initial xylose (main monosaccharide) concentration of 30 $g \cdot L^{-1}$. However, soybean hull composition naturally varies in different lots. Moreover, exposure to heat can lead to degradation sugar reactions (JÖNSSON; MARTÍN, 2016). In all experiments, it was noted that, although substrate was centrifuged, after autoclavation there was precipitation of several particles within the broth. In view of these facts, initial composition of the culture media was detailed individually for every batch reactor experiment (Table 3).

Initial proportion of each sugar in the broth corresponded to ($w \cdot v^{-1}$, %): 55.12, xylose; 33.44, arabinose; 11.42, glucose. Results compare to previous research reports (CORTIVO et al., 2018; HICKERT et al., 2014), in which soybean acid hydrolysate was characterized as having high pentose and low hexose content. As a consequence of its composition, it was observed a high medium osmotic pressure, which can affect oxygen transfer and bacteria metabolism (HICKERT et al., 2013). Even so, *P. agglomerans* BL1 was able to consume all sugars in the broth and produce 2,3-BD efficiently, demonstrating an adaptable metabolism.

Characterization of the culture media also revealed a broth rich in terms of protein (23.61 ± 0.76 $g \cdot L^{-1}$ of total nitrogen) and ashes (89.03 ± 1.91$g \cdot L^{-1}$). Mineral profile examination of the substrate (Table 4) suggests a balanced composition for microbial growth. Relatively higher amounts of sulphur and sodium are related to pretreatment steps performed to solubilize hemicellulose fraction.

Acetic acid is expected to form during pre-treatment because of lignocellulosic acetyl group hydrolysis (GALBE; ZACCHI, 2012). As explored in section 2.6.3, its initial concentration is also an important parameter, as different studies demonstrated that it can benefit 2,3-BD production, even though it is detrimental to microbial growth (CHENG et al., 2010). Hence, in the present work, relatively low amounts of acetic acid possibly enhanced 2,3-BD generation by *P. agglomerans* BL1. As comparison, Joo et al. (2016) reported that yellow poplar (hardwood), larix (softwood), and rice hull acid hydrolysates had (in $g \cdot L^{-1}$) 3.53, 1.83, and 0.87 of acetic acid, respectively.

Table 3: initial culture conditions of batch experiments.

Tested condition	Xylose ($g \cdot L^{-1}$)	Arabinose ($g \cdot L^{-1}$)	Glucose ($g \cdot L^{-1}$)	Total sugars ($g \cdot L^{-1}$)	Acetic Acid ($g \cdot L^{-1}$)	Osm. Pressure[1] ($mmol \cdot kg^{-1}$)	HMF ($mg \cdot L^{-1}$)
Anaerobiose [2]	29.17 ± 1.24 ^{ab}	18.43 ± 0.16 ^{a}	4.45 ± 0.80 ^{c}	52.04 ± 1.88 ^{abc}	1.20 ± 0.19 ^{ab}	2038.50 ± 30.41 ^{bc}	2.96 ± 2.39 ^{bc}
0.5 vvm [2]	30.54 ± 0.4 ^{a}	18.79 ± 4.12 ^{a}	5.40 ± 1.15 ^{abc}	54.74 ± 3.46 ^{abc}	1.05 ± 0.18 ^{b}	2085.50 ±103.94 ^{abc}	4.37 ± 1.11 ^{abc}
1 vvm [2]	28.60 ± 0.11 ^{ab}	16.47 ± 1.69 ^{a}	4.76 ± 1.15 ^{bc}	49.82 ± 0.64 ^{bc}	0.98 ± 0.13 ^{b}	2102.00 ± 91.92 ^{abc}	2.25 ± 0.33 ^{c}
2 vvm [2]	27.11 ± 0.71 ^{b}	14.69 ± 0.18 ^{a}	4.75 ± 0.10 ^{bc}	46.81 ± 0.99 ^{c}	1.08 ± 0.05 ^{b}	1832.0 ± 2.83 ^{c}	12.01 ± 6.01 ^{abc}
2 vvm + ye [2]	30.32 ± 0.45 ^{ab}	18.64 ± 0.06 ^{a}	8.17 ± 0.18 ^{a}	57.12 ± 0.56 ^{ab}	1.41 ± 0.26 ^{ab}	2186.50 ± 24.75 ^{ab}	5.27 ± 0.97 ^{abc}
2 vvm + pH 6	31.82 ± 1.40 ^{a}	18.98 ± 1.45 ^{a}	7.43 ± 0.72 ^{ab}	58.23 ± 3.57 ^{a}	1.30 ± 0.13 ^{ab}	2191.00 ± 39.60 ^{ab}	13.86 ± 3.40 ^{ab}
2 vvm + VC [2]	28.67 ± 0.83 ^{ab}	18.89 ± 0.37 ^{a}	7.81 ± 0.02 ^{a}	55.36 ± 1.22 ^{ab}	1.97 ± 0.32 ^{b}	2339.00 ± 120.21 ^{a}	15.58 ± 1.80 ^{a}

[1] Osmotic pressure

[2] Initial pH of 6.0 (uncontrolled pH experiments)

Mean values ± standard deviation (*n* = 2)

Different letters overlapped in the same column indicate significant statistical difference ($p < 0.05$).

2 vvm + Ye: cultivation with oxygen supply at 2 vvm supplemented with 5 $g.L^{-1}$ of yeast extract; 2 vvm +pH6: cultivation with oxygen supply at 2 vvm and controlled pH at 6,0; 2 vvm + VC: cultivation with oxygen supply at 2 vvm and addition of vitamin C.

Table 4: Mineral profile of soybean acid hydrolysate prior to inoculation.

Conc. ($mg \cdot L^{-1}$)	K	Ca	Mg	Cu	Zn	Fe	Mn	Na	Al	Co	S	P
Mineral	5905.4	364.2	794.5	0.8	22.7	90.6	3.1	32027.4	99.9	<0.4	14002.8	329.5

Soybean acid hull hydrolysate did not seem to present toxicity. Formic acid was not detected in any media, furfural concentration was below 0.5 $mg \cdot L^{-1}$ and HMF corresponded to only 8.04 ± 5.77 $mg \cdot L^{-1}$. Furfural is formed due to pentose deterioration, whereas HMF, hexose degradation (GALBE; ZACCHI, 2012). Aliphatic carboxylic acids, such as formic acid, are the final degradation state of monossacharides degradation (JÖNSSON; MARTÍN, 2016). Thus, results suggest mainly glucose was degraded during pretreatment steps. As comparison, Lee and co-authors (2015) reported that *Miscanthus* biomass submitted to acid pretreatment presented concentrations of furfural below 0.1 $g \cdot L^{-1}$ and HMF of 0.06 $g \cdot L^{-1}$, but high amounts of formic acid (2.01 $g \cdot L^{-1}$), indicating that most furfural was conveyed to the acid form.

Other studies that utilized soybean hull hydrolysates found comparable results (QING et al., 2017). As this type of LM present lower lignin content than other lignocellulosic biomass (section 2.5.1), milder pre-treatment conditions are enough to hydrolyse this material efficiently (CASSALES et al., 2011). Thus, inhibitory compounds released are also present in small amounts (LOMAN; JU, 2016).

4.2 Kinetics of substrate uptake

The sugar consumption pattern of the microorganism was similar among different oxygen conditions tested (Figure 7). All monosaccharides present in the broth were simultaneously metabolized, indicating low glucose initial concentration (average of 11.42 % based on total monossacharides) did not induce catabolic repression (JI et al., 2011). In contrast, in a previous research, consumption of xylose by *P. agglomerans* BL1 was completely repressed when glucose was the main sugar in the medium (81.00 % of total initial sugars) (CORTIVO et al., 2019).

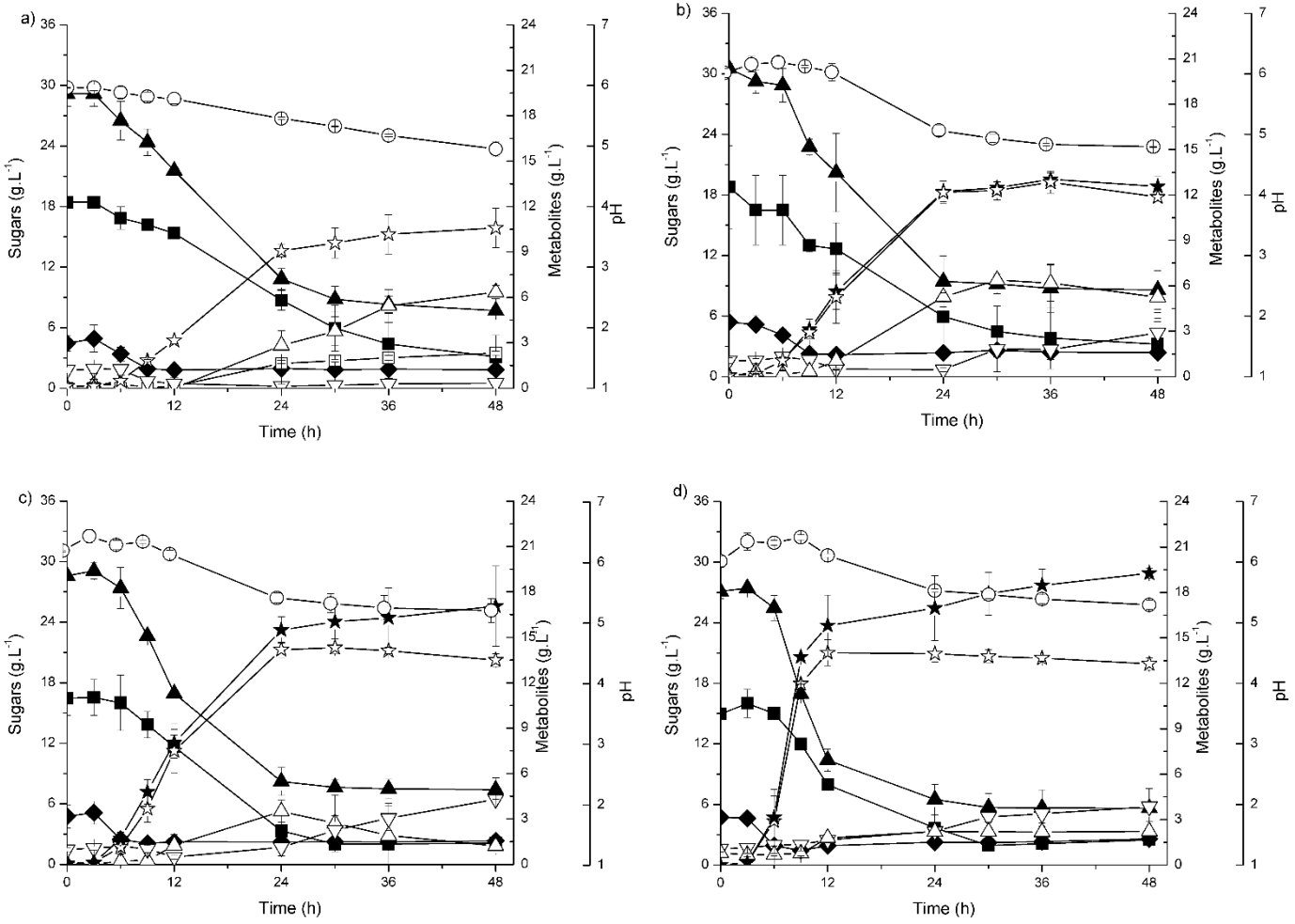

Figure 7: Kinetics profile of P. aglommerans in soybean acid hydrolysate under a) anaerobiosis, b) 0.5 vvm, c) 1 vvm and d) 2 vvm. Cultivations were carried at Initial pH of 6.00; 37 ° C; 300 rpm. (▲) xylose, (■) arabinose, (♦) glucose, (☆) 2,3-BD, (★) 2,3-BD + acetoin, (∆) lactic acid, (∇) acetic acid, (□) ethanol and (○) pH. Data represent the mean of two independent samples and error bars, standard deviation.

Glucose catabolite repression (GCR) has been reported in the literature for several enteric bacteria. It is related to the ability of glucose to block the induction of genes responsible for the use of substrates such as xylose (JI et al., 2011) and is one of the major drawbacks of employing lignocellulosic biomass as substrates, as usually a mixture of sugars is present (GALBE; ZACCHI, 2012). Guragain et al. (2017) suggested lignocellulosic biomass hydrolysates are not suited for efficient 2,3-BD production by *B*. *licheniformis* DSM 8785. In their study, even though xylose could be metabolized as a sole carbon source in synthetic media, the mixture of glucose: xylose (2:1 and 3:1, respectfully) in wood and sorghum hydrolysates resulted in diauxic growth and inefficient xylose uptake.

Another interesting phenomenon regarding the sugar utilization profile by *P. agglomerans* BL1 is the concomitant and similar consumption of xylose and arabinose. Wang and co-authors (2010) reported *K. pneumoniae* SDM metabolizes sugars from corncob molasses in bioreactor batch fermentations at the preferential order: glucose> arabinose> xylose (initial concentration in cultivations: 9 % glucose, 14.6 % arabinose and 45 % xylose, initial sugars: 70.4 $g \cdot L^{-1}$).

In retrospective, after 48 h of cultivation, *P. agglomerans* BL1, consumed (%): 76.31, 85.36, and 52.77 of xylose, arabinose and glucose, respectively. Within the concept of biorefineries, these results are highly interesting, showing the potential of using this bacterium, and at least this biomass hydrolysate, for the commercial production of bioproducts with industrial importance (KUMARI; SINGH, 2018; MENON; RAO, 2012).

4.3 Influence of oxygen and pH on *P. agglomerans* BL1 metabolism

2,3-BD was the main bioconversion product in all conditions tested. It is commonly stated in literature that microaerophilic conditions are best to obtain higher efficiency in 2,3-BD production process, as high aeration supply in the broth shifts the microbe's metabolism for cell growth (BARRETT et al., 1983; CELIŃSKA; GRAJEK, 2009; CONVERTI; PEREGO; DEL BORGHI, 2003) (section 2.6.1). In the present work, 2,3-BD titer after 24 h was lower under anaerobiosis (Table 5). However, increasing aeration rate did not result in statistically higher diol titer. Sugar consumption rate increased when more air was provided to the broth, probably because of more cell formation (Table 5 and Figure 7). Consequently, 2,3-BD

productivity (Q_P) was higher at 2 vvm. Under anaerobiosis, 0.5 vvm and 1 vvm, maximum Qp corresponded to 24 h of cultivation, whereas when aeration rate was kept at 2 vvm maximum productivity was obtained within 12 h.

Table 5: Yield, productivity and sugar total consumption percentage obtained by *P. agglomerans* after 24 h in soybean acid hydrolyate under different conditions. Cultures were performed at 37 ° C, 300 rpm and initial pH = 6.00. Data represent the average of two independent samples.

Tested condition	Total sugar consumption (%)	C 2.3-BD ($g \cdot L^{-1}$)	$Y_{P/S}$ ($g \cdot g^{-1}$)	Q_P ($g \cdot L^{-1} \cdot h^{-1}$)
An	58.60 ± 4.23 ^{bcd}	9.08 ± 0.40 ^{c}	0.30 ± 0.02 ^{b}	0.38 ± 0.02 ^{d}
0.5 vvm	67.85 ± 8.08 ^{abc}	12.17 ± 0.74 ^{bc}	0.33 ± 0.00 ^{b}	0.51 ± 0.03 ^{c}
1 vvm	76.20 ± 3.33 ^{ab}	14.18 ± 0.51 ^{ab}	0.39 ± 0.01 ^{ab}	0.60 ± 0.02 ^{bc}
2 vvm	73.78 ± 6.60 ^{ab} (56.78 ± 2.05 $^{cd\ 1}$)	14.02 ± 0.86 ^{ab} (13.96 ± 0.38 $^{1\ ab}$)	0.40 ± 0.01 ^{ab} (0.53 ± 0.04 $^{1\ a}$)	0.58 ± 0.02 ^{bc} (1.17 ± 0.07 $^{1\ a}$)
2 + ye	78.40 ± 4.51 ^{a}	15.93 ± 1.07 ^{a}	0.36 ± 0.00 ^{ab}	0.66 ± 0.04 ^{b}
2 + pH 6	43.76 ± 1.01 ^{d}	11.14 ± 2.55 ^{bc}	0.44 ± 0.12 ^{ab}	0.46 ± 0.11 ^{d}
2 + VC	43.95 ± 2.31 ^{d}	10.61 ± 0.76 ^{bc}	0.44 ± 0.00 ^{ab}	0.44 ± 0.03 ^{d}

^{1} after 12 h of cultivation

Mean values ± standard deviation (n = 2)

Different letters overlapped in the same column indicate significant statistical difference ($p < 0.05$).

An: anaerobioses;2 vvm + Ye: cultivation with oxygen supply at 2 vvm supplemented with 5 g.L-1 of yeast extract; 2 vvm +pH6: cultivation with oxygen supply at 2 vvm and controlled pH at 6.00; 2 vvm + VC: cultivation with oxygen supply at 2 vvm and continuous Vc addition (2.22 mg·L-1).

As comparison, Dai et al. (2014) reported decrease in 2,3-BD titer (19.02 $g \cdot L^{-1}$ to 16.83 $g \cdot L^{-1}$ after 18 h of cultivation) when utilizing aeration rate of 0.2 and 0.4 vvm, respectively, in synthetic glucose cultures of *P. polymyxa* CJX518. Moreover, Yang et al. (2016) found similar results in synthetic glucose cultures of a three-gene 2,3-BDO pathway construct *Z. mobilis* 9C-BC1. After 20 h, the recombinant strain produced 15 $g \cdot L^{-1}$ and 12 $g \cdot L^{-1}$ of acetoin and 2,3-BD when dissolved oxygen was kept at 1 % by sparging N_2 in the bioreactor, whereas titer of acetoin and 2,3-BD were of 10 $g \cdot L^{-1}$ and 3 $g \cdot L^{-1}$, respectively, when dissolved oxygen corresponded to 10 %.

Other than the diol, under low oxygen conditions (anaerobiosis and 0.5 vvm) the main products were lactic acid, succinic acid and ethanol (Table 6). As described in sections 2.3 and 2.6.1, because *P. agglomerans* is a facultative anaerobe, balance of co-factors is maintained through 2,3-BD and other metabolites pathways when there is no oxygen available in the broth (VOLOCH et al., 1987). Formation of lactic acid, ethanol and 2,3-BD from pyruvate require NADH, whereas acetoin generation occurs mainly under high NAD^+ availability (YANG et al., 2017).

Table 6 Pool of metabolites obtained at the moment of maximum productivity of the 2,3-BD production curve for different oxygen supply conditions by *P. agglomerans* in soybean acid hydrolysate. Cultures were performed at 37 ° C, 300 rpm and initial pH = 6.00. Data represent the average of two independent samples.

Oxygen supply	Acetic Acid ($g \cdot L^{-1}$)	Lactic Acid ($g \cdot L^{-1}$)	Succinic Acid ($g \cdot L^{-1}$)	Ethanol ($g \cdot L^{-1}$)	Acetoin ($g \cdot L^{-1}$)	2.3-BD ($g \cdot L^{-1}$)
An	0.14 ± 0.14 ^{a}	2.83 ± 0.98 ^{ab}	3.14 ± 0.34 ^{a}	1.64 ± 0.01 ^{a}	0.03 ± 0.04 ^{b}	9.08 ± 0.04 ^{b}
0.5 vvm	0.47 ± 0.14 ^{a}	5.26 ± 0.26 ^{a}	2.86 ± 0.74 ^{a}	0.30 ± 0.42 ^{b}	0.31 ± 0.28 ^{b}	12.17 ± 0.73 ^{a}
1 vvm	1.12 ± 0.55 ^{ab}	3.50 ± 0.73 ^{ab}	2.71 ± 0.76 ^{a}	0.32 ± 0.45 ^{b}	1.30 ± 0.36 ^{b}	14.18 ± 0.51 ^{a}
2 vvm	2.23 ± 0.61 ^{b}	2.21 ± 0.64 ^{b}	2.25 ± 0.71 ^{a}	0.06 ± 0.08 ^{b}	3.02 ± 0.42 ^{a}	13.96 ± 0.56 ^{a}

Mean values ± standard deviation (n = 2)

Different letters overlapped in the same column indicate significant statistical difference ($p < 0.05$).

At higher aeration rates, it was noted gradual decrease in ethanol formation, whereas acetoin and acetic acid increased (Table 6). Because in these case there was more oxygen in the broth, regeneration of NADH could also be performed by conveying acetyl-CoA (formed from pyruvate by dehydrogenase multi-enzyme complex) to the tricarboxylic acid (TCA) cycle (CELIŃSKA; GRAJEK, 2009). Hence, this metabolism shift should be accompanied by enhancement in biomass formation (MADDOX, 1996).

Unfortunately, in this work, due to the physicochemical characteristics of the medium, with its high osmotic pressure and composition, dissolved oxygen concentration was not possible to measure. Thus, we could not establish the k_La under different oxygen environments. Moreover, because soybean hull hydrolysate has a dark colour, spectrophotometric quantification of cell growth was not possible. In addition, the substrate contained undissolved particles (formed during sterilization in autoclave) that precluded determination of dry cells. Estimation of biomass growth was attempted by cell counting (CFU, colony forming units) plated on LB medium. This technique did not result in representative results (data not shown) and dilution rates necessary to enable counting were too high (up to 10^{-30}). Therefore, biomass growth was not quantified.

In the 2,3-BD mixed-acid pathway, end-products formation vary according to the strain and culture conditions employed (section 2.3) (YANG; ZHANG, 2018). For instance, Li; Dai; Xiu (2010) reported that *K. pneumoniae* CICC 10011 growing on Jerusalem artichoke stalk and tuber under low aeration rates (0.2 vvm) produces lactic acid in similar quantities to that of 2,3-BD (39.75 $g \cdot L^{-1}$ and 49.55 $g \cdot L^{-1}$, respectively; initial reducing sugar corresponding to 160.08 $g \cdot L^{-1}$). However, when oxygen supply increased (0.5 vvm) in this broth, formation of lactic acid was not

detected. Moreover, formation of 2,3-BD (55.13 $g \cdot L^{-1}$) was enhanced, accompanied by formation of acetoin (14.03 $g \cdot L^{-1}$) and acetic acid (less than 1.5 $g \cdot L^{-1}$).

In light of the previous results, in all forthcoming experiments, aeration rate was kept 2 vvm. On average, in uncontrolled pH experiments the culture pH stabilized at 5 within 24 h because of organic acids formation. To test whether this fact could explain smaller sugar consumption rates after 24 h, experiments in which the culture pH was kept at 6.0 were carried out (Figure 8), but this strategy did not improve sugar consumption. Moreover, maintaining pH at 6 probably resulted in a longer lag phase, as 2,3-BD started to be produced only after 12 h. Therefore, results observed in this work suggest that controlling the pH at 6 as a strategy does not improve process efficiency.

The proper approach of pH control for 2,3-BD production is directly related to the microorganism and substrate of choice (CELIŃSKA; GRAJEK, 2009). Guo et al. (2017) reported complete inhibition of *Klebsiella pneumoniae* CICC 10781 growth in cheese whey medium when keeping pH at 5.5. However, the authors obtained best results when implementing a two-stage pH control (6.5 for the first 8 h to promote cell development, and then pH at 6.0 till the end of cultivation to enhance 2,3-BD generation). Guragain; Vadlani, (2017) investigated *K. oxytoca* ATCC 8724 growth in synthetic glucose media and found that keeping pH at 7.0 leads to lactic acid formation as the main product (73.6 $g \cdot L^{-1}$ of lactic acid and 29.1 $g \cdot L^{-1}$ of 2,3-BD). In their study, better results were obtained when pH was initially set at 7, but not controlled (final pH was 5.3).

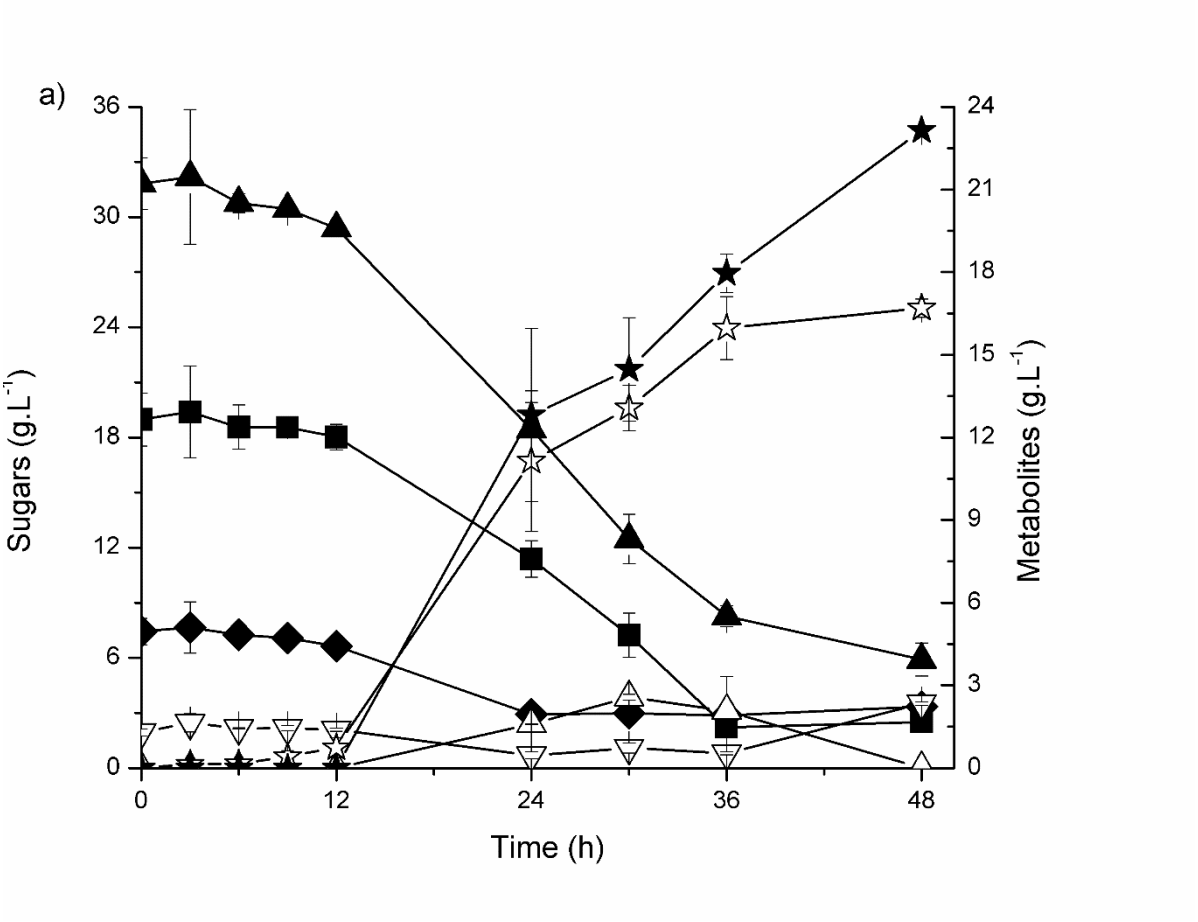

Figure 8: Kinetics profile of *P. aglommerans* in soybean acid hydrolysate under 2 vvm and controlled pH at 6.00; 37 ° C; 300 rpm. (▲) xylose (■) arabinose; (◆) glucose, (☆) 2,3-BD, (★) 2,3-BD + acetoin, (Δ) lactic acid, (∇) acetic acid, (○) pH. Data represent the mean of two independent samples and error bars, standard deviation.

4.4 Impact of medium supplementation

Besides incomplete sugar consumption within 48 h in all tested conditions, it was noted the accumulation of acetoin in the broth at the expense of 2,3-BD consumption after 24 h when aeration was provided to the broth, as oxygen availability enhances acetoin generation (section 2.6.1) (MADDOX, 1996). Reversible reaction of acetoin ↔ 2,3-BD is also affected by intracellular NADH/ NAD^+ ratio (section 2.3) (JI; HUANG; OUYANG, 2011). In this manner, Guragain; Vadlani (2017) reported a boost in acetoin titer as the aeration rate increased from 0.33 to 1 vvm in cultivations of *K. oxytoca* ATCC 8724 in glucose medium. In their study, to promote biomass growth and funnel sugar consumption to 2,3-BD production at later stage, a two-step aeration strategy was proposed:1 vvm during the first 8 h, followed by 0.33 vvm till the end. Meanwhile, Zhang et al. (2013) proposed a two-stage speed agitation control strategy by stirring at 350 rpm during 24 h increased to 500 rpm up to 50 h of

cultivation in order to increase acetoin production by *B. amyloliquefaciens* FMME044 in synthetic glucose medium.

In this work, we regarded such strategies as not reproducible on an industrial context, as aeration rate and stirring are parameters not suitable for bioprocess scaling up. Whenever scaling up of aerated bioprocess is required, the volumetric oxygen uptake rate of culture, the k_La, is the main parameter that should be used (ATKINSON; MAVITUNA, 1991). As previously stated, we could not measure the k_La in the broth (section 5.3), so we opted to investigate whether accumulation of acetoin and incomplete sugar consumption could be addressed by supplementation strategies that enhanced 2,3-BD production, as described in other researches. Addition of peptone (GAO et al., 2010), mineral trace elements (JI et al., 2009), and yeast extract (RIPOLL et al., 2016) have been reported to promote microbial growth. Inclusion of Na_2EDTA enhances mass transfer of nutrients during bioconversion process by improving cell permeability (JIANG et al., 2012; LORETTA, 1965). Finally, acetic acid can induce enzymes involved in 2,3BD pathway (section 2.6.3) (JOO et al., 2016).

In light of this considerations, seven sets of experimental cultivations in orbital shaker were carried out, in which inclusion of different supplementations were tested:

1. **Trace elements + peptone (TEP):** trace element solution [2 % ($v \cdot v^{-1}$)] + Mg^{+2} (0.2 $g \cdot L^{-1}$) + P^{-3} (0.52 $g \cdot L^{-1}$) + peptone (10 $g \cdot L^{-1}$);
2. **Trace elements (TE):** trace element solution [2 %($v \cdot v^{-1}$)] + Mg^{+2} (0.2 $g \cdot L^{-1}$) + P^{-3} (0.52 $g \cdot L^{-1}$);
3. **Yeast extract + peptone (YESP):** 5 $g \cdot L^{-1}$ and 10 $g \cdot L^{-1}$, respectfully;
4. **Yeast extract (YES):** 5 $g \cdot L^{-1}$;
5. **Acetic acid (AC):** sodium acetate (4.8 $g \cdot L^{-1}$);
6. **Na_2EDTA (ED):** 1.2 ($g \cdot L^{-1}$);
7. **Control (C):** soybean hull hydrolysate without supplementation. Distilled sterile water was added to the culture medium in the same proportion added to flasks from 1 to 6.

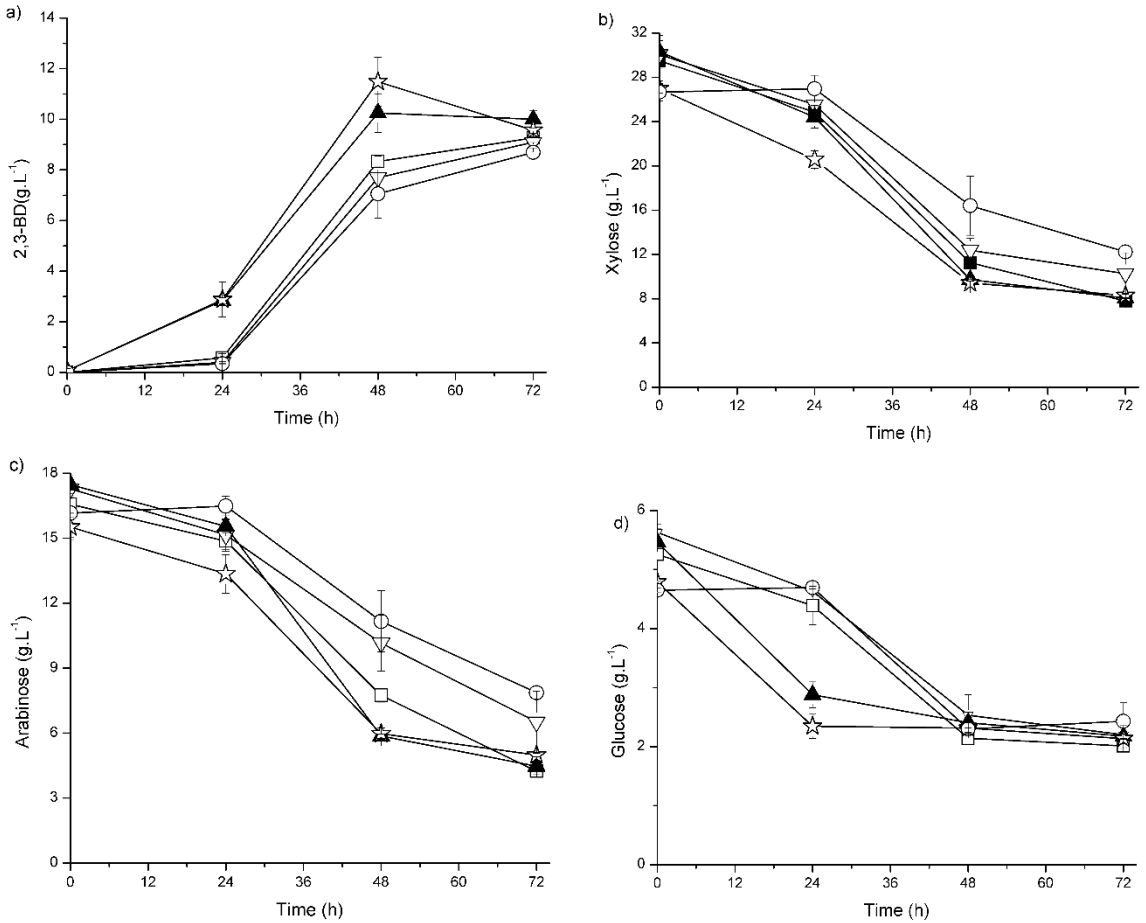

Figure 9: effect of different supplements on a) 2,3-BD production, b) xylose, c) arabinose and d) glucose consumption. (□) TEP, (∇) TE, (▲) YESP, (☆) YES (○) C. The results are the mean of duplicates and error bars represent standard deviations.

TEP: Trace elements + peptone; TE: trace elements; YESP: yeast extract + peptone; YES: yeast extract; C: control.

Figure 9 illustrates the obtained results. pH tendency was not affected by any supplementation (average initial 6.0, decreasing to average final 5.0 after 72 h, not shown in graphs). The combinations ED or AC hindered substrate uptake and 2,3-BD production by *P. agglomerans* BL1 (not shown in graphs). This could be attributed to low tolerance of these compounds by the strain, in contrast to other studies from literature that employed different bacteria species. Wang et al. (2016) reported simultaneous addition of yeast extract, acetic acid, and Na_2EDTA (35.2, 1.2, and 4.5 $g \cdot L^{-1}$, respectively) and enhanced production of 2,3-BD by K. *pneumoniae* HR521 LDH.

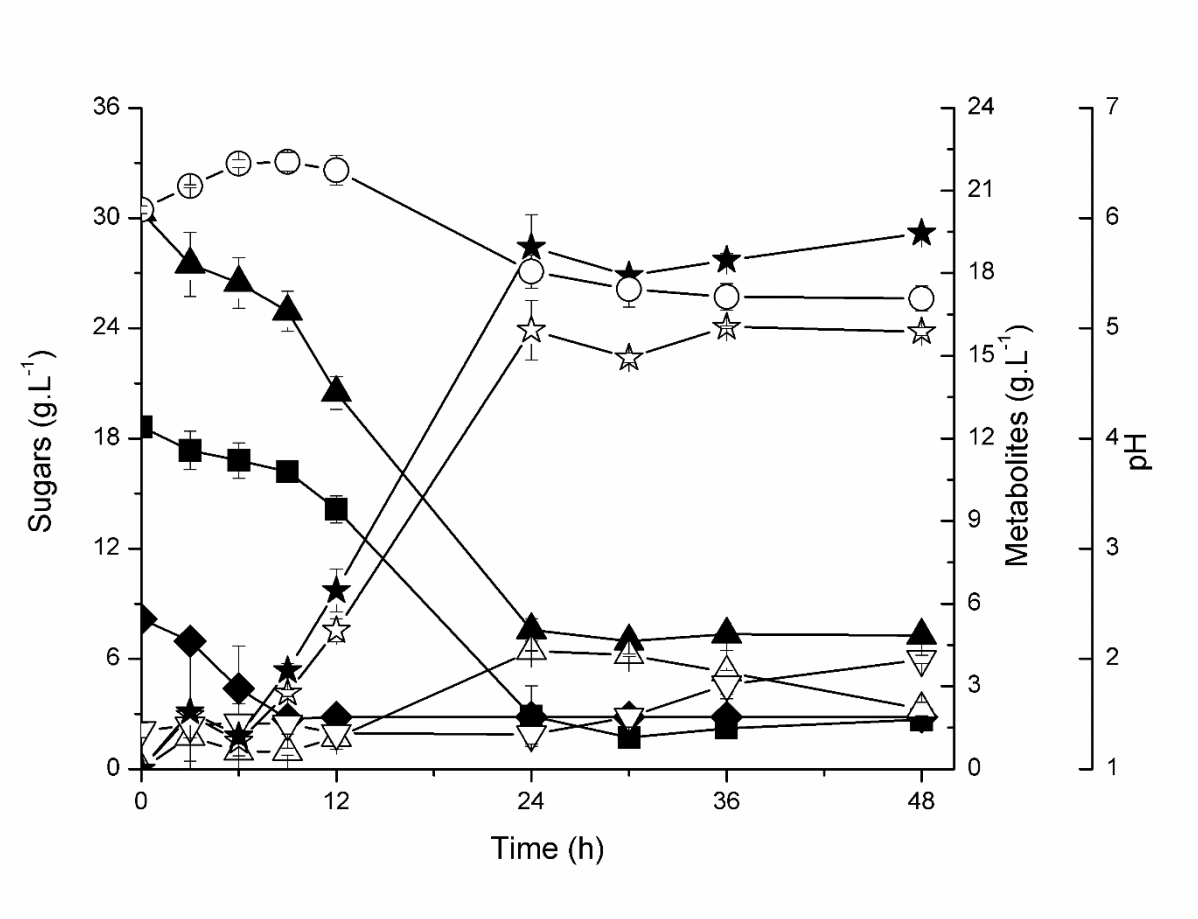

Figure 10: Kinetics profile of P. aglommerans in soybean acid hydrolysate under 2 vvm and addition of yeast extract (5 g·L-1), initial pH 6.00; 37 ° C; 300 rpm. (▲) xylose (■) arabinose; (♦) glucose, (☆) 2,3-BD, (★) 2,3-BD + acetoin, (Δ) lactic acid, (∇) acetic acid, (○) pH. Data represent the mean of two independent samples and error bars, standard deviation.

Nitrogen supplementation seemed to boost substrate consumption rate and diols production when compared to VC, TEP and TE in orbital shaker experiments. Because YES represents lower production costs and its effect was similar to YESP,

YES combination was further scaled up to bioreactors (Figure 10) Yeast extract addition in bioreactor cultures probably favoured biomass generation (it was noted the media turned thicker than usual while the reaction occurred), but did not benefit 2,3-BD productivity and yields (Table 6). Interestingly, under this condition although 2,3-BD production ceased after 24 h, formation of acetoin in this period was also not observed. Bao et al. (2015) demonstrated that the formation of 2,3-BD in the early stages of cultivation of *B. subtilis* 168 is directly related to high NADH availability, whereas consumption of the diol to form acetoin is correlated to low availability of this co-factor. Hence, addition of yeast extract should have helped to maintain NADH/ NAD^+ ratio appropriate for microbial growth, in which regeneration of NADH is possible through carrying acetyl-CoA to the tricarboxylic acid (TCA) cycle (VOLOCH et al., 1987).

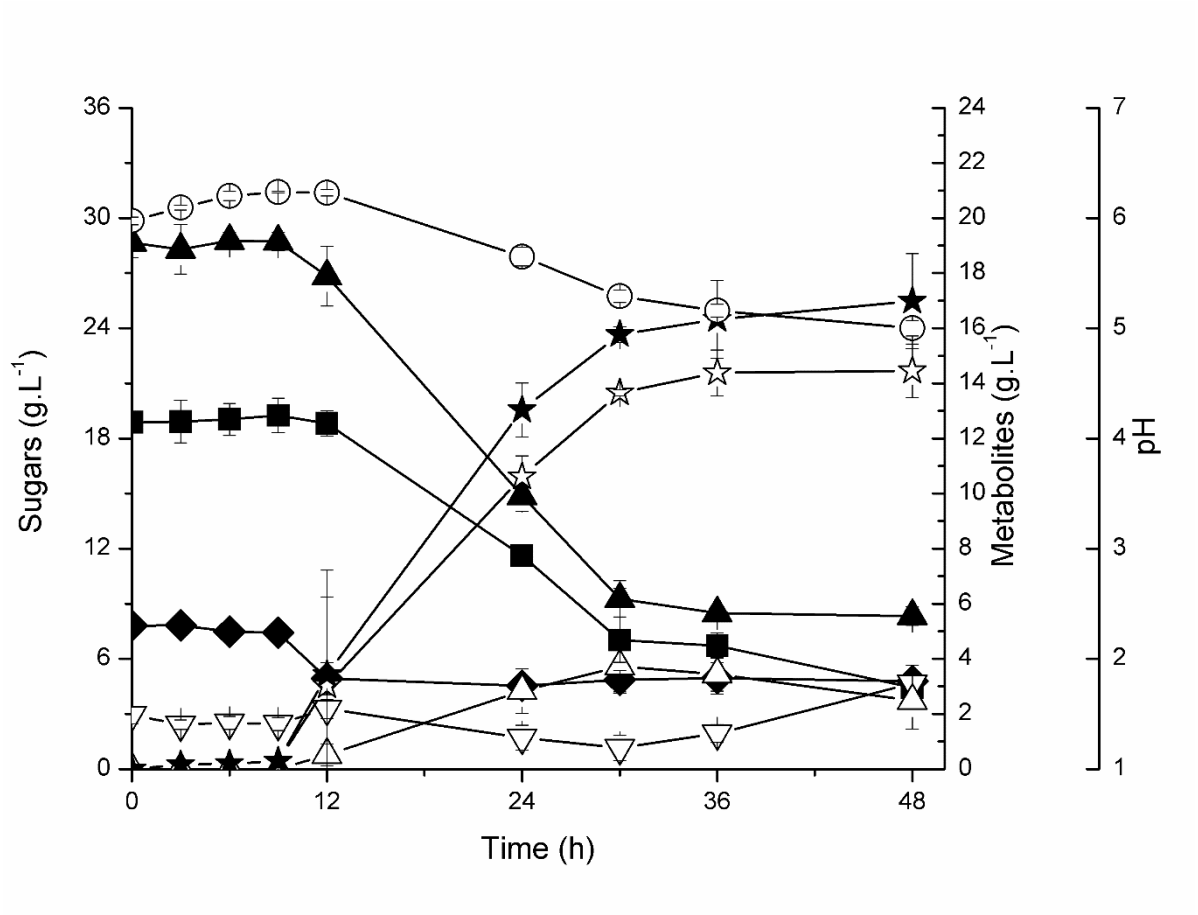

Figure 11: Kinetics profile of *P. aglommerans* BL1 in soybean acid hydrolysate under 2 vvm and continuous VC addition (2.22 mg·L^{-1}), initial pH 6.00; 37 ° C; 300 rpm. (▲) xylose; (■) arabinose; (◆) glucose, (☆) 2,3-BD, (★) 2,3-BD + acetoin, (Δ) lactic acid, (∇) acetic acid and (○) pH. Data represent the mean of two independent samples and error bars, standard deviation.

Accumulation of acetoin in the latter stage of cultivation under uncontrolled pH, 2 vvm condition suggests co-factors deficiency in *P. agglomerans* BL1 cells. Dai et al. (2014) showed that increasing NADH/NAD+ ratio is possible by adjusting extracellular oxidoreduction potential through addition of VC in cultures of *P. polymyxa* CJX518 growing in synthetic glucose media. VC was periodically added to the broth every 3 h after 6 h cultivation and improved 2,3-BD productivity (from 0.80 $g \cdot L^{-1} \cdot h^{-1}$ to 1.07 $g \cdot L^{-1} \cdot h^{-1}$ within 24 h), as well as glucose consumption rate. In the present study, this strategy was adapted to enable VC feeding continuously after 7 h of cultivation (concentration in the broth of 2.22 $mg \cdot L^{-1}$).

In our experiments, VC did not improve *P. agglomerans* metabolism (Figure 11 and Table 6), but it was clear that the addition of it affected extracellular oxidoreduction potential over the course of the bioconversion process. Formation of acetoin at the expense of 2,3-BD consumption was not observed, contrasting with the uncontrolled pH experiments without supplementation (Figure 12). Bespalov; Zhulin; Taylor (1996) reported that electron transport between oxidation and reduction states can be benefited by extracellular change in redox potential. However, addition of an exogenous reducing agent, such as VC, within the early step of cultivation can be toxic to cells (DAI et al., 2014). Therefore, further studies should be carried out to investigate influence of VC addition to medium at a later stage. Moreover, NADH and NAD^+ titer can also be evaluated during the bioconversion process, in order to clarify the relationship of the ratio of these co-factors and 2,3-BD productivity for *P. agglomerans* BL1 in soybean hull hydrolysate.

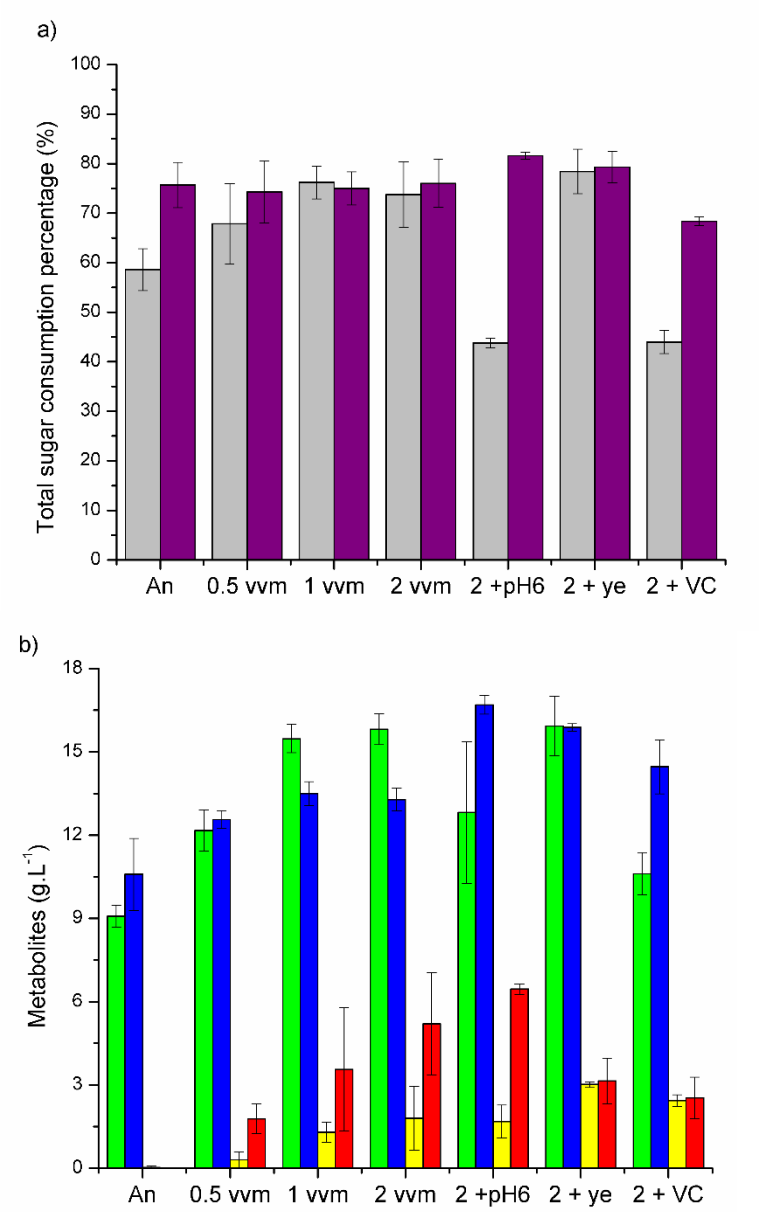

Figure 12: Effect of the 7 tested conditions on P. agglomerans BL1 conversion of substrate to 2,3-BD and acetoin. a) total monosaccharides (sugar) consumption percentage: (gray) in 24 h of cultivation and (purple) in 48 h of cultivation. b) metabolites titer: (green) 2,3-BD in 24 h of cultivation and (blue) 2,3-BD in 48 h of cultivation; (yellow) acetoin titer in 24 h of cultivation and (red) acetoin titer in 48 h of cultivation. Data represent the mean of two independent samples and error bars, standard deviation.

An: anaerobiose

2 vvm + Ye: cultivation with oxygen supply at 2 vvm supplemented with 5 g.L-1 of yeast extract.

2 vvm + pH6: cultivation with oxygen supply at 2 vvm and controlled pH at 6.0.

2 vvm + VC: cultivation with oxygen supply at 2 vvm and continuous VC addition (2.22 mg·L-1).

4.5 Soybean hull hydrolysate as a potential substrate for 2,3-BD production

In the present work, in contrast to studies in literature, higher yields and productivities of 2,3-BD were paralleled with higher oxygen supply conditions, as explored in section 4.1. *P. agglomerans* BL1 was able to simultaneously consume all

monosaccharides present in soybean hull hydrolysate and, after 48 h of cultivation, 74.85 % of initial sugars were depleted. Kinetics of substrate uptake under supplementation of yeast extract or VC was similar to that obtained in experiments carried out without supplementation. Incomplete sugar utilization can be attributed to catabolite repression (LING et al., 2017), high initial osmotic pressure of the broth (HICKERT et al., 2014) and/or NADH deficiency in the late stage of cultivation of *P. agglomerans* (BAO et al., 2015). It is also possible that 2,3-BD might present some toxicity to microbial cell at higher levels, as demonstrated for *P. polymyxa* DSM 365 cultivations (OKONKWO; UJOR; EZEJI, 2017).

Initial pH at 6.0, aeration rate at 2 vvm and no media supplementation resulted in 14.02 $g \cdot L^{-1}$ of 2,3-BD in 12 h of cultivation, which corresponds to yields and productivities of 0.53 $g \cdot g^{-1}$ and 1.17 $g \cdot L^{-1} \cdot h^{-1}$, respectively. These results demonstrate great production potential of 2,3-BD by *P. agglomerans* from soybean hull acid hydrolysate, when compared to other studies that investigated the use of lignocellulosic biomass and wild-type strains of bacteria in bioreactors. Moreover, it seems that there is a compromise between high 2,3-BD titer, productivity, and initial media composition when using these types of substrates. For example, Ling et al. (2017) observed preferential glucose consumption over xylose in *E. cloacae* CICC 10011 batch cultivations from corncob subjected to alkaline treatment followed by enzymatic hydrolysis (total initial sugars: 100 $g \cdot L^{-1}$, 60 % glucose and 40 % xylose). In their work, 2,3-BD titer, maximum yield and productivity corresponded to 52.5 $g \cdot L^{-1}$, 0.42 $g \cdot g^{-1}$ and 0.81 $g \cdot L^{-1} \cdot h^{-1}$, respectively.

Cheng et al. (2010) also utilized corncob as substrate, which was submitted to acid hydrolysis for the cultivation of *K. oxytoca* ACCC 10370 (initial substrate composition ($g \cdot L^{-1}$): 120.1, xylose; 20.2, glucose; 12.8, arabinose; 0.8, cellobiose; 1.2, galactose; 0.7, mannose; 7.4, acetate). They obtained 35.7 $g \cdot L^{-1}$, 0.49 $g \cdot g^{-1}$, 0.54 $g \cdot L^{-1} \cdot h^{-1}$ of 2,3-BD titer, yield, and productivity, respectively. Longer lag phase in this case was attributed to high initial acetate concentration. Finally, Hazeena et al. (2019) using the approach of simultaneous saccharification and fermentation and employing as substrate oil palm front submitted to alkali pretreatment (60 $g \cdot L^{-1}$ of initial total sugars) in *E. cloacae* sp.SG1 cultures obtained 12.53 $g \cdot L^{-1}$, 0.32 $g \cdot g^{-1}$ and 0.13 $g \cdot L^{-1} \cdot h^{-1}$ of 2,3-BD titer, yield, and productivity, respectively. The authors in this work attributed less attractive results in terms of productivity to high initial inhibitors

concentration, when comparing it to cultures of the same strain in synthetic glucose media.

Higher titer were obtained in researches in which other non-lignocellulosic biomass were used as substrates, because these by-products do not require any pre-treatment operations to convert carbohydrates into monosaccharides avoiding the formation of toxic compounds (BIAŁKOWSKA, 2016). For instance, Guo et al. (2017) achieved 57.63 $g \cdot L^{-1}$, 0.413 $g \cdot g^{-1}$ and 1.23 $g \cdot L^{-1} \cdot h^{-1}$ of 2,3-BD titer, yield and productivity on *K. pneumoniae* CICC 10781 pulsed fed-batch fermentation using cheese whey (60 $g \cdot L^{-1}$ initial lactose). Moon et al. (2018) developed a semi-continuous, two-stage simultaneous saccharification and fermentation (STSSF) process using cassava (initial concentration of 308 $g \cdot L^{-1}$) as carbon source. The recombinant *K. oxytoca ΔldhA ΔpflB* strain used by the authors was able to produce 108 $g \cdot L^{-1}$ of 2,3-BD, which corresponded to yields of 0.45 $g \cdot g^{-1}$ and productivities of 3.0 $g \cdot L^{-1} \cdot h^{-1}$.

5 Conclusions

In this research, it was studied the influence of oxygen supply, pH control and the impact of media supplementation on production of 2,3-BD by *P. agglomerans* BL1 using soybean acid hydrolysate as substrate in batch reactors. Higher aeration rates enhanced 2,3-BD yield and productivity, in contrast to some reports in literature. Maintaining pH at 6 did not benefit kinetics growth of the microbe in the broth. Addition of acetic acid and Na_2EDTA hindered substrate uptake, whereas inclusion of mineral trace elements did not affect bioprocess efficiency. Finally, supplementation with yeast extract increased biomass growth, but did not improve 2,3-BD yield and productivity. VC addition impacted extracellular oxidoreduction potential over the course of the bioconversion process, however the addition strategy in the early stage of cultivation was toxic to the microbe.

Best results were obtained in cultivations carried out at 2 vvm, initial pH at 6 and without supplementation. Within 12 h, 14.02 $g \cdot L^{-1}$ of 2.3-BD were obtained, which corresponds to yields of 0.53 $g \cdot g^{-1}$ and productivity of 1.17 $g \cdot L^{-1} \cdot h^{-1}$. Thus, this research points out a promising approach to produce 2,3-BD utilizing as substrate soybean hull acid hydrolysate. In addition, *P. agglomerans* BL1, strain isolated from an environmental consortium, proved to be a robust bacterium, as it was capable of efficiently convert sugars from this substrate into 2,3-BD and other value-added products. Thus, results represent an alternative to ensure proper utilization of the hemicellulose fraction of agroindustrial biomass within the biorefinery concept.

For future work aiming further optimization of this bioprocess, initially it is recommended investigation of an effective method for biomass growth quantification. Therefore, mathematical modelling of experimental data is enabled and the impact of fed-batch strategies on the microbe physiology can be studied, in order to increase 2,3-BD titer and productivity (MORCELLI et al., 2018). In this perspective, the relationship between 2,3-BD production and $NADH/NAD^+$ ratio should also be elucidated aiming to provide alternatives to enhance substrate uptake and minimize acetoin conversion in latter cultivation cells.

Moreover, the impact of VC addition on cultivations can be explored, as results demonstrate it positively influenced co-factors equilibrium, but the tested feeding strategy did not benefit conversion efficiency. It is also proposed performance of

enzymatic studies to investigate the metabolic pathway utilized by *P. agglomerans* BL1 in soybean hull acid hydrolysate. This effort leads to the suggestion of genetic modifications on the strain and different process control strategies that potentially benefit 2,3-BD production.

Finally, experiments addressing 2,3-BD possible toxicity during kinetics growth can be carried out. This approach enables studies involving the adaptation of the bioprocess to cell-immobilized bioreactors, as this design can provide microbe protection against inhibitory metabolites, as well as higher productivity, when compared with free-cell bioreactors (GABARDO; RECH; AYUB, 2012).

References

360 RESEARCH REPORTS. **GLOBAL 2,3 BUTANEDIOL MARKET GROWTH 2019-2024**. Disponível em: <https://www.360researchreports.com/global-2-3-butanediol-market-growth-2019-2024-13902505>. Acesso em: 7 may. 2019.

ATKINSON, B.; MAVITUNA, F. **Biochemical engineering and biotechnology handbook**. 2nd. ed. New York, N.Y: Stockton Press, 1991.

BAO, T. et al. Regulation of the NADH pool and NADH / NADPH ratio redistributes acetoin and 2 , 3-butanediol proportion in Bacillus subtilis. **Biotechnology Journal**, p. 1298–1306, 2015.

BARRETT, E. L. et al. Production of 2,3-Butylene Glycol from Whey by Klebsiella pneurnoniae and Enterobacter aerogenes. **Journal of Dairy Science**, v. 66, n. 12, p. 2507–2514, 1983.

BEIJERINCK, M. W. Die bakterien der papilionaceenknoïllchen. **Botanische Zeitung**, v. 46, n. 1, p. 740–750, 1888.

BESPALOV; ZHULIN, I. B.; TAYLOR, B. L. Behavioral responses of Escherichia coli to changes in redox potential. **Proc Natl Acad Sci U S A**, v. 93, n. 19, p. 10084–10089, 1996.

BIAŁKOWSKA, A. M. Strategies for efficient and economical 2,3-butanediol production: new trends in this field. **World Journal of Microbiology and Biotechnology**, v. 32, n. 12, p. 1–14, 2016.

BIEBL, H. et al. Fermentation of glycerol to 1,3-propanediol and 2,3-butanediol by Klebsiella pneumoniae. **Applied Microbiology and Biotechnology**, v. 50, n. 1, p. 24–29, 1998.

BRIJWANI, K.; OBEROI, H. S.; VADLANI, P. V. Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. **Process Biochemistry**, v. 45, n. 1, p. 120–128, 2010.

CAPOLUPO, L.; FARACO, V. Green methods of lignocellulose pretreatment for biorefinery development. **Applied Microbiology and Biotechnology**, v. 100, n. 22, p. 9451–9467, 2016.

CASALI, S. et al. Development of a biofilm technology for the production of 1,3-propanediol (1,3-PDO) from crude glycerol. **Biochemical Engineering Journal**, v. 64, p. 84–90, 2012.

CASSALES, A. et al. Optimization of soybean hull acid hydrolysis and its

characterization as a potential substrate for bioprocessing. **Biomass and Bioenergy**, v. 35, n. 11, p. 4675–4683, 2011.

CELIŃSKA, E.; GRAJEK, W. Biotechnological production of 2,3-butanediol - Current state and prospects. **Biotechnology Advances**, v. 27, n. 6, p. 715–725, 2009.

CHEN, H. et al. A review on the pretreatment of lignocellulose for high-value chemicals. **Fuel Processing Technology**, v. 160, n. 1, p. 196–206, 2017.

CHENG, C. et al. Polymalic acid fermentation by Aureobasidium pullulans for malic acid production from soybean hull and soy molasses : Fermentation kinetics and economic analysis. **Bioresource Technology**, v. 223, p. 166–174, 2017.

CHENG, K. K. et al. Improved 2,3-butanediol production from corncob acid hydrolysate by fed-batch fermentation using Klebsiella oxytoca. **Process Biochemistry**, v. 45, n. 4, p. 613–616, 2010.

COFFMAN, A. M.; LI, Q. Effect of Natural and Pretreated Soybean Hulls on Enzyme Production by Trichoderma reesei. **Journal of the American Oil Chemists' Society**, v. 91, n. 8, p. 1331–1338, 2014.

CONVERTI, A.; PEREGO, P.; DEL BORGHI, M. Effect of specific oxygen uptake rate on Enterobacter aerogenes energetics: Carbon and reduction degree balances in batch cultivations. **Biotechnology and Bioengineering**, v. 82, n. 3, p. 370–377, 2003.

CORTIVO, P. et al. Production of 2,3-butanediol by Klebsiella pneumoniae BLh-1 and Pantoea agglomerans BL1 cultivated in acid and enzymatic hydrolysates of soybean hull. **Biotechnology Progress**, n. September 2018, p. 1–8, 2019.

CORTIVO, P. R. D. et al. Fermentation of oat and soybean hull hydrolysates into ethanol and xylitol by recombinant industrial strains of Saccharomyces cerevisiae under diverse oxygen environments. **Industrial Crops & Products**, v. 113, n. December 2017, p. 10–18, 2018.

DAI, J. J. et al. Regulation of extracellular oxidoreduction potential enhanced (R,R)-2,3-butanediol production by Paenibacillus polymyxa CJX518. **Bioresource Technology**, v. 167, p. 433–440, 2014.

DE MAS, C.; JANSEN, N. B.; TSAO, G. T. Production of optically active 2,3-butanediol by Bacillus polymyxa. **Biotechnology and Bioengineering**, v. 31, n. 4, p. 366–377, 1988.

DUTKIEWICZ, J. et al. Pantoea agglomerans: A mysterious bacterium of evil and

good. Part I. Deleterious effects: Dust-borne endotoxins and allergens - Focus on cotton dust. **Annals of Agricultural and Environmental Medicine**, v. 22, n. 4, p. 576–588, 2015.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. **FAOSTAT: Crops**. Disponível em: <http://www.fao.org/faostat/en/#data/QC/visualize>. Acesso em: 22 jul. 2019.

GABARDO, S.; RECH, R.; AYUB, M. A. Performance of different immobilized-cell systems to efficiently produce ethanol from whey : fluidized batch , packed-bed and fluidized continuous bioreactors ˆ. **Chemical Technology and Biotechnology**, v. 87, p. 1194–1201, 2012.

GALBE, M.; ZACCHI, G. Pretreatment : The key to efficient utilization of lignocellulosic materials. **Biomass and Bioenergy**, v. 6, p. 0–8, 2012.

GAO, J. et al. Optimization of medium for one-step fermentation of inulin extract from Jerusalem artichoke tubers using Paenibacillus polymyxa ZJ-9 to produce R,R-2,3-butanediol. **Bioresource Technology**, v. 101, n. 18, p. 7076–7082, 2010.

GARG, S. K. K.; JAIN, A. Fermentative production of 2, 3-butanediol: a review. **Bioresource Technology**, v. 51, n. 2, p. 103–109, 1995.

GAVINI, F. et al. Transfer of Enterobacter agglomerans (Beijerinck 1888) Ewing and Fife 1972 to Pantoea gen . nov . as Pantoea agglornerans comb . nov . and Description of Pantoea dispersa sp . nov . n. July 1989, p. 337–345, 2019.

GEDDES, C. C.; NIEVES, I. U.; INGRAM, L. O. Advances in ethanol production. **Current Opinion in Biotechnology**, v. 22, n. 3, p. 312–319, 2011.

GUO, X. et al. Efficient production of 2,3-butanediol from cheese whey powder (CWP) solution by Klebsiella pneumoniae through integrating pulsed fed-batch fermentation with a two-stage pH control strategy. **Fuel**, v. 203, p. 469–477, 2017.

GURAGAIN, Y. N. et al. Appropriate lignocellulosic biomass processing strategies for efficient 2,3-butanediol production from biomass-derived sugars using Bacillus licheniformis DSM 8785. **Food and Bioproducts Processing**, v. 4, p. 147–158, 2017.

GURAGAIN, Y. N.; VADLANI, P. V. 2,3-Butanediol production using Klebsiella oxytoca ATCC 8724: Evaluation of biomass derived sugars and fed-batch fermentation process. **Process Biochemistry**, v. 58, n. May, p. 25–34, 2017.

HASSAN, S. S.; WILLIAMS, G. A.; JAISWAL, A. K. Emerging technologies for the pretreatment of lignocellulosic biomass. **Bioresource Technology**, v. 262, n. March, p. 310–

318, 2018.

HAZEENA, S. H. et al. Simultaneous saccharification and fermentation of oil palm front for the production of 2,3-butanediol. **Bioresource Technology**, v. 278, n. October 2018, p. 145–149, 2019.

HICKERT, L. R. et al. Ethanogenic fermentation of co-cultures of Candida shehatae HM 52 . 2 and Saccharomyces cerevisiae ICV D254 in synthetic medium and rice hull hydrolysate. **Bioresource Technology**, v. 131, p. 508–514, 2013.

HICKERT, L. R. et al. Fermentation kinetics of acid-enzymatic soybean hull hydrolysate in immobilized-cell bioreactors of Saccharomyces cerevisiae, Candida shehatae, Spathaspora arborariae, and their co-cultivations. **Biochemical Engineering Journal**, v. 88, p. 61–67, 2014.

IPHARRAGUERRE, I. R.; CLARK, J. H. Soyhulls as an Alternative Feed for Lactating Dairy Cows: A Review. **Journal of Dairy Science**, v. 86, n. 4, p. 1052–1073, 2010.

JANSEN, N. B.; TSAO, G. T. Bioconversion of pentoses to 2,3-butanediol by Klebsiella pneumoniae. **Pentoses and Lignin**, p. 85–99, 2005.

JI, X. et al. Engineering Klebsiella oxytoca for efficient 2 ,3-butanediol production through insertional inactivation of acetaldehyde dehydrogenase gene. **Applied Microbiology and Biotechnology**, v. 85, n. 6, p. 1751–1758, 2010.

JI, X. J. et al. Development of an industrial medium for economical 2,3-butanediol production through co-fermentation of glucose and xylose by Klebsiella oxytoca. **Bioresource Technology**, v. 100, n. 21, p. 5214–5218, 2009.

JI, X. J. et al. Elimination of carbon catabolite repression in Klebsiella oxytoca for efficient 2,3-butanediol production from glucose-xylose mixtures. **Applied Microbiology and Biotechnology**, v. 89, n. 4, p. 1119–1125, 2011.

JI, X. J.; HUANG, H.; OUYANG, P. K. Microbial 2,3-butanediol production: A state-of-the-art review. **Biotechnology Advances**, v. 29, n. 3, p. 351–364, 2011.

JIANG, L. Q. et al. Production of 2,3-butanediol from acid hydrolysates of Jatropha hulls with Klebsiella oxytoca. **Bioresource Technology**, v. 107, p. 405–410, 2012.

JÖNSSON, L. J.; MARTÍN, C. Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. **Bioresource Technology**, v. 199, p. 103–112, 2016.

JOO, J. et al. Improved fermentation of lignocellulosic hydrolysates to 2,3-butanediol

through investigation of effects of inhibitory compounds by Enterobacter aerogenes. **Chemical Engineering Journal**, v. 306, p. 916–924, 2016.

KIM, H. J. et al. Draft genome sequence of Pantoea ananatis B1-9, a nonpathogenic plant growth-promoting bacterium. **Journal of Bacteriology**, v. 194, n. 3, p. 729, 2012.

KUMAR, A. K.; SHARMA, S. Recent updates on different methods of pretreatment of lignocellulosic feedstocks : a review. **Bioresources and Bioprocessing**, 2017.

KUMARI, D.; SINGH, R. Pretreatment of lignocellulosic wastes for biofuel production: A critical review. **Renewable and Sustainable Energy Reviews**, v. 90, n. May 2017, p. 877–891, 2018.

LEE, S. J. et al. Phenolic compounds: Strong inhibitors derived from lignocellulosic hydrolysate for 2,3-butanediol production by Enterobacter aerogenes. **Biotechnology Journal**, v. 10, n. 12, p. 1920–1928, 2015.

LEE, S. J. et al. Process strategy for 2,3-butanediol production in fed-batch culture by acetate addition. **Journal of Industrial and Engineering Chemistry**, v. 56, p. 157–162, 2017.

LI, D.; DAI, J. Y.; XIU, Z. L. A novel strategy for integrated utilization of Jerusalem artichoke stalk and tuber for production of 2,3-butanediol by Klebsiella pneumoniae. **Bioresource Technology**, v. 101, n. 21, p. 8342–8347, Nov. 2010.

LI, L. et al. Metabolic engineering of Enterobacter cloacae for high-yield production of enantiopure (2R,3R)-2,3-butanediol from lignocellulose-derived sugars. **Metabolic Engineering**, v. 28, p. 19–27, 2015.

LIM, J. S. et al. A review on utilisation of biomass from rice industry as a source of renewable energy. **Renewable and Sustainable Energy Reviews**, v. 16, p. 3084–3094, 2012.

LING, H. Z. et al. Corncob Mild Alkaline Pretreatment for High 2,3-Butanediol Production by Spent Liquor Recycle Process. **Bioenergy Research**, v. 10, n. 2, p. 566–574, 1 Jun. 2017.

LIU, H. et al. Improvement of hydrogen production by transposon- mutagenized strain of Pantoea agglomerans BH18. **International Journal of Hydrogen Energy**, v. 37, n. 10, p. 8282–8287, 2012.

LIU, H.; WANG, G. Microbial Fe (III) reduction and hydrogen production by a transposon-mutagenized strain of Pantoea agglomerans BH18. **Energy**, v. 81, p. 691–695,

2015.

LOMAN, A. A.; ISLAM, S. M. M.; JU, L. Production of arabitol from enzymatic hydrolysate of soybean flour by Debaryomyces hansenii fermentation. p. 641–653, 2018.

LOMAN, A. AL; JU, L. K. Soybean carbohydrate as fermentation feedstock for production of biofuels and value-added chemicals. **Process Biochemistry**, v. 51, n. 8, p. 1046–1057, 2016.

LORETTA, L. Release of lipopolysaccharide by EDTA treatment of E coli. **Biochemical and Biophysical Research Communications**, v. 21, n. 4, p. 290–296, 1965.

MA, Y. et al. Biohydrogen production via the interaction of nitrogenase and anaerobic mixed-acid fermentation in marine bacteria. **International Journal of Hydrogen Energy**, v. 40, n. 1, p. 176–183, 2015.

MACCOLLOM, G. B. et al. Association and Attraction of Blueberry Maggot Fly Curran (Diptera: Tephritidae) to Pantoea (Enterobacter) agglomerans. **Environmental Entomology**, v. 38, n. 1, p. 116–120, 2009.

MADDOX, I. S. Microbial Production of 2,3-Butanediol. In: REHM, H. J.; REED, G. (Eds.). . **Biotechnology**. 2. ed. Weinheim: VCH Verlagsgesellschaft, 1996. p. 270–288.

MAINA, S. et al. Improvement on bioprocess economics for 2,3-butanediol production from very high polarity cane sugar via optimisation of bioreactor operation. **Bioresource Technology**, v. 274, n. September 2018, p. 343–352, 2019.

MENEZES, B. DA S.; MISTURINI, D. M.; AYUB, M. A. Z. Screening of filamentous fungi to produce xylanase and xylooligosaccharides in submerged and solid-state cultivations on rice husk , soybean hull , and spent malt as substrates. **World Journal of Microbiology and Biotechnology**, v. 33, n. 58, 2017.

MENON, V.; RAO, M. Trends in bioconversion of lignocellulose : Biofuels , platform chemicals & biorefinery concept. **Progress in Energy and Combustion Science**, v. 38, n. 4, p. 522–550, 2012.

MOON, S. et al. Development of a semi-continuous two-stage simultaneous saccharification and fermentation process for enhanced 2 , 3-butanediol production by Klebsiella oxytoca. 2018.

MORCELLI, A. et al. Exponential Fed-Batch Cultures of Klebsiella pneumoniae under Anaerobiosis Using Raw Glycerol as a Substrate to Obtain Value-Added Bioproducts. **Journal of the Brazilian chemical socienty**, v. 29, n. 11, p. 2278–2286, 2018.

MORIN, A. Pantoea. **Encyclopedia of Food Microbiology: Second Edition**, v. 3, p. 1028–1032, 2014.

NAKASHIMADA, Y. et al. Enhanced 2,3-Butanediol Production by Addition of Acetic Acid in Paenibacillus polymyxa. **Journal of Bioscience and Bioengineering**, v. 90, n. 6, p. 661–664, 2000.

NIELSEN, S. S. (ED.). **Food Analysis**. 4. ed. New York: Springer International Publishing, 2017.

OKONKWO, C. C.; UJOR, V.; EZEJI, T. C. Investigation of relationship between 2,3-butanediol toxicity and production during growth of Paenibacillus polymyxa. **New Biotechnology**, v. 34, p. 23–31, 2017.

OZDEMIR, G. et al. Biosorption of chromium(VI), cadmium(II) and copper(II) by Pantoea sp. TEM18. **Chemical Engineering Journal**, v. 102, n. 3, p. 249–253, 2004.

PARK, J. M.; RATHNASINGH, C.; SONG, H. Enhanced production of (R,R)-2,3-butanediol by metabolically engineered Klebsiella oxytoca. **Journal of Industrial Microbiology and Biotechnology**, v. 42, n. 10, p. 1419–1425, 2015.

PILEGGI, M. et al. Isolation of mesotrione-degrading bacteria from aquatic environments in Brazil. **Chemosphere**, v. 86, n. 11, p. 1127–1132, 2012.

QING, Q. et al. Comparison of alkaline and acid pretreatments for enzymatic hydrolysis of soybean hull and soybean straw to produce fermentable sugars. **Industrial Crops & Products**, v. 109, n. September, p. 391–397, 2017.

RIPOLL, V. et al. Novel biocatalysts for glycerol conversion into 2,3-butanediol. **Process Biochemistry**, v. 51, n. 6, p. 740–748, 2016.

RODRIGUES, E.; MARIUTTI, L. R. B.; MERCADANTE, A. Z. Carotenoids and phenolic compounds from Solanum sessiliflorum, an unexploited amazonian fruit, and their scavenging capacities against reactive oxygen and nitrogen species. **Journal of Agricultural and Food Chemistry**, v. 61, n. 12, p. 3022–3029, 2013.

ROSSI, D. M. et al. Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel. **International Journal of Hydrogen Energy**, v. 36, n. 8, p. 4814–4819, 2011.

ROSSI, D. M. et al. Bioconversion of residual glycerol from biodiesel synthesis into 1,3-propanediol and ethanol by isolated bacteria from environmental consortia. **Renewable Energy**, v. 39, n. 1, p. 223–227, 2012.

SÁNCHEZ, C. Lignocellulosic residues : Biodegradation and bioconversion by fungi. **Biotechnology Advances**, v. 27, p. 185–194, 2009.

SEIDL, P. R.; GOULART, A. K. Pretreatment processes for lignocellulosic biomass conversion to biofuels and bioproducts. **Current Opinion in Green and Sustainable Chemistry**, v. 2, p. 48–53, 2016.

SHIRKAVAND, E. et al. Combination of fungal and physicochemical processes for lignocellulosic biomass pretreatment – A review. **Renewable and Sustainable Energy Reviews**, v. 54, p. 217–234, 2016.

SINGH, A. et al. Enhanced saccharification of rice straw and hull by microwave-alkali pretreatment and lignocellulolytic enzyme production. **Bioresource Technology**, v. 102, n. 2, p. 1773–1782, 2011.

SINGH, R. et al. A review on delignification of lignocellulosic biomass for enhancement of ethanol production potential. **Renewable and Sustainable Energy Reviews**, v. 32, p. 713–728, 2014.

SMITS, T. H. M. et al. Metabolic versatility and antibacterial metabolite biosynthesis are distinguishing genomic features of the fire blight antagonist pantoea vagans C9-1. **PLoS ONE**, v. 6, n. 7, 2011.

STØRMER, F. C. Evidence for regulation of pH 6 acetolactate-forming enzyme by acetate ion. **Biochemical and Biophysical Research Communications**, v. 74, n. 3, p. 898–902, 1977.

SULTANA, M. et al. Bacterial communities in Bangladesh aquifers differing in aqueous arsenic concentration. **Geomicrobiology Journal**, v. 28, n. 3, p. 198–211, 2011.

SYU, M. J. Biological production of 2,3-butanediol. **Applied Microbiology and Biotechnology**, v. 55, n. 1, p. 10–18, 2001.

TINDALL, B. J. The combination Enterobacter agglomerans is to be cited as Enterobacter agglomerans (Beijerinck 1888) Ewing and Fife 1972 and the combination Pantoea agglomerans is to be cited as Pantoea agglomerans (Beijerinck 1888) Gavini et al. 1989. Opinion 90. **International Journal of Systematic and Evolutionary Microbiology**, v. 64, p. 3582–3583, 2014.

TRANSPARENCY MARKET RESEARCH. **Butanediol (1,4 BDO & 2,3 BDO), 1,3 Butadiene And Methyl Ethyl Ketone (MEK) Market: Applications (THF, PU, PBT, SBR, ABS, NBR Etc.), Bio-Based Alternatives, Downstream Potential, Market Size And Forecast, 2010 - 2018**. Disponível em:

<https://www.transparencymarketresearch.com/butanediol-butadiene-and-mek-market.html>. Acesso em: 5 jul. 2019.

UNITED STATES DEPARTMENT OF AGRICULTURE - USDA. **Oilseeds: World Markets and Trade**. Disponível em: <https://apps.fas.usda.gov/psdonline/app/index.html#/app/downloads>. Acesso em: 7 jun. 2019.

VANNESTE, J. L.; YU, J.; CORNISH, D. A. Presence of genes homologous to those necessary for synthesis of microcin MccEh252 in strains of Pantoea agglomerans. **Acta Horticulturae**, v. 793, p. 391–396, 2008.

VOLOCH, M. et al. 2,3-Butanediol. In: **Comprehensive biotechnology: the principles, applications and regulations of biotechnology in industry, agriculture and medicine.** 26. ed. UK: Pergamon/Elsevier, 1987. p. 933–944.

WALTERSON, A. M.; STAVRINIDES, J. Pantoea: Insights into a highly versatile and diverse genus within the Enterobacteriaceae. **FEMS Microbiology Reviews**, v. 39, n. 6, p. 968–984, 2015.

WANG, A. et al. Production of 2,3-butanediol from corncob molasses, a waste by-product in xylitol production. **Applied Microbiology and Biotechnology**, v. 87, n. 3, p. 965–970, 2010.

WANG, X. X. et al. The implementation of high fermentative 2,3-butanediol production from xylose by simultaneous additions of yeast extract, Na2EDTA, and acetic acid. **New Biotechnology**, v. 33, n. 1, p. 16–22, 2016.

WONG, C. L. et al. Effects of pH and fermentation strategies on 2,3-butanediol production with an isolated Klebsiella sp. Zmd30 strain. **Bioresource Technology**, v. 152, p. 169–176, 2014.

XIAO, Z.; LU, J. R. Strategies for enhancing fermentative production of acetoin : A review. **Biotechnology Advances**, v. 32, n. 2, p. 492–503, 2014.

YANG, S. et al. Metabolic engineering of Zymomonas mobilis for 2,3-butanediol production from lignocellulosic biomass sugars. **Biotechnology for Biofuels**, v. 9, n. 1, p. 1–15, 2016.

YANG, T. et al. Improved Production of 2,3-Butanediol in Bacillus amyloliquefaciens by Over-Expression of butanediol Dehydrogenase. **PLoS ONE**, v. 8, n. 10, p. 1–9, 2013.

YANG, T. et al. Economic conversion of spirit-based distillers' grain to 2,3-butanediol

by Bacillus amyloliquefaciens. **Process Biochemistry**, v. 50, n. 1, p. 20–23, 2015.

YANG, T. et al. Metabolic engineering strategies for acetoin and 2,3-butanediol production: advances and prospects. **Critical Reviews in Biotechnology**, v. 37, n. 8, p. 990–1005, 2017.

YANG, Z.; ZHANG, Z. Recent advances on production of 2,3-butanediol using engineered microbes. **Biotechnology Advances**, n. March, p. 0–1, 2018.

YU, L. et al. Metabolic Engineering of Clostridium tyrobutyricum for n-Butanol Production Through Co-Utilization of Glucose and Xylose. v. 112, n. 10, p. 2134–2141, 2015.

ZENG, A. P.; SABRA, W. Microbial production of diols as platform chemicals: Recent progresses. **Current Opinion in Biotechnology**, v. 22, n. 6, p. 749–757, 2011.

ZHANG, L. et al. Mechanism of 2 ,3-butanediol stereoisomers formation in a newly isolated Serratia sp . T241. **Nature Publishing Group**, n. September 2015, p. 1–12, 2016.

ZHANG, L. et al. Production of R,R -2 ,3-butanediol of ultra-high optical purity from Paenibacillus polymyxa ZJ-9 using homologous recombination. **Bioresource Technology**, v. 261, n. April, p. 272–278, 2018.

ZHANG, X.; ZHANG, R.; BAO, T. Moderate expression of the transcriptional regulator ALsR enhances acetoin production by Bacillus subtilis. **Journal of Industrial Microbiology & Biotechnology**, v. 40, n. 9, p. 1067–1076, 2013.

ZHANG, Y. et al. Acetoin production enhanced by manipulating carbon flux in a newly isolated Bacillus amyloliquefaciens. **Bioresource Technology**, v. 130, p. 256–260, 2013.