University of Reading

# Integrated Fermentative Production and Downstream Processing of 2,3-Butanediol from Sucrose by Non-pathogenic Strains of *Bacillus subtilis* and *Serratia plymuthica*


**By**

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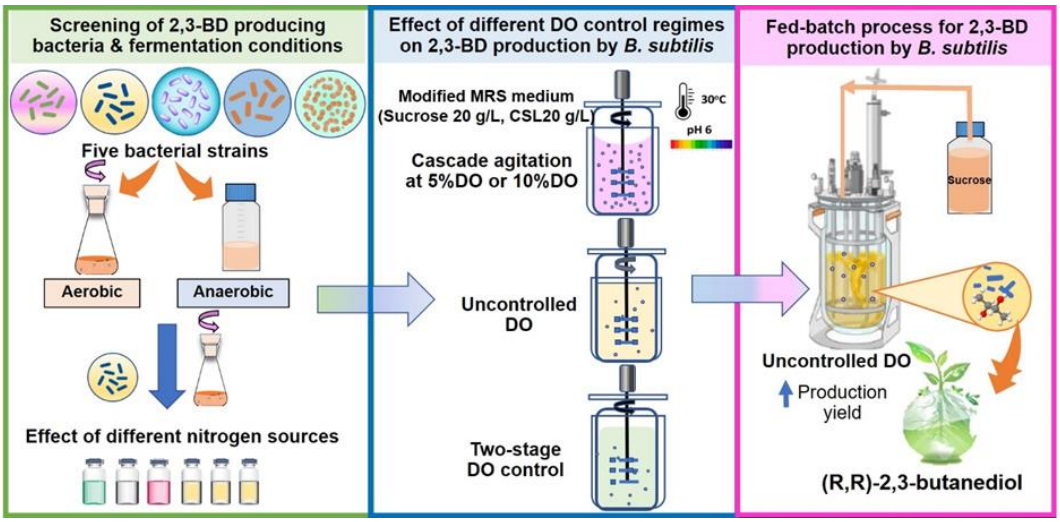
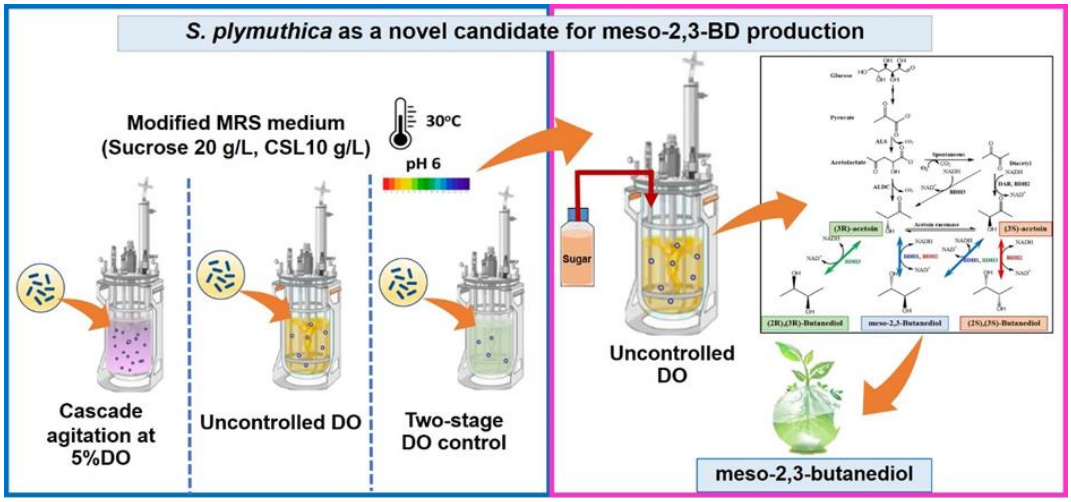
# Graphical Abstract (Chapter 3)




# Graphical Abstract (Chapter 4)

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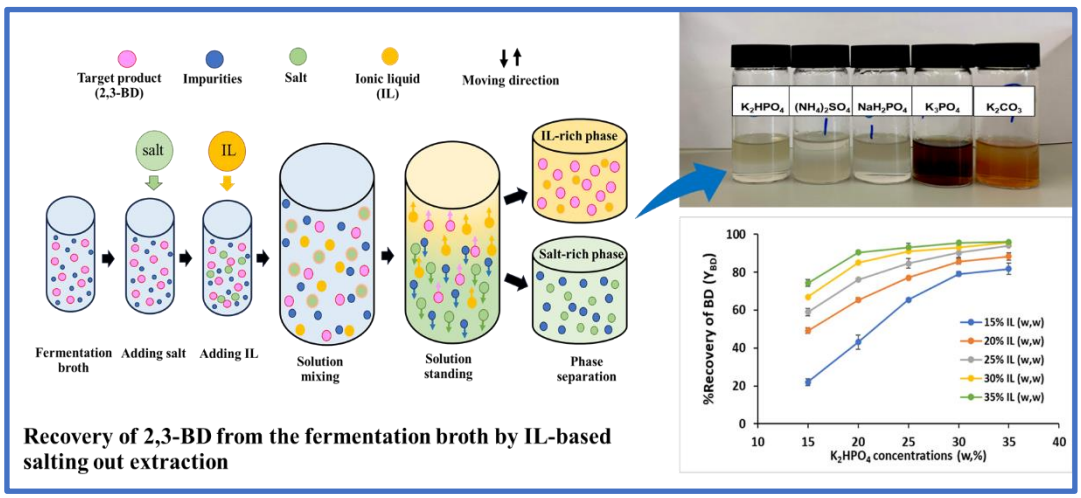

# Declaration of Original Authorship

The work described in this thesis was performed in the Department of Food and Nutritional Sciences, School of Chemistry, Food and Pharmacy, University of Reading, United Kingdom, between September 2019 and September 2023. I confirm that this is my own work and the use of all materials from other sources has been properly and fully acknowledged.

Suthkamol Suttikul

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# Abstract

2,3-Butanediol (2,3-BD) is an important platform chemical of industrial interest due to its extensive applications and high market value. Global warming, environmental impact and limitation of fossil fuel reserves are driving the development of eco-friendly and sustainable bio-based 2,3-BD. A global market of 2,3-BD is anticipated to reach 300 million US dollars in 2030. 2,3-BD stereoisomers are linked to specific industrial applications, with pure chiral 2,3-BD isomers being highly sought after from a commercial point of view. The present study focused on the production of highly pure 2,3-BD stereoisomers by non-pathogenic wild-type bacterial strains and the development of efficient downstream processing. The first task was the screening for non-pathogenic wild-type bacterial strains capable of producing 2,3-BD from sucrose-based media. Among the strains tested, *Bacillus subtilis* and *Serratia plymuthica* showed promising characteristics of producing notable concentrations of 2,3-BD in batch-flask cultures. Corn steep liquor (CSL) was proven as an alternative low-cost nitrogen source to costly nitrogen sources for 2,3-BD production. Furthermore, the impact of dissolved oxygen concentrations on 2,3-BD production was assessed in batch bioreactor process. Limited dissolved oxygen conditions suppressed butanediol dehydrogenase activity and supported maximum (R, R)-2,3-BD production at high purity (100%) in *B. subtilis*, whereas high dissolved oxygen conditions promoted bacterial growth and acetoin formation. *S. plymuthica* was reported for the first time as capable producer of highly pure (95%) meso-2,3-BD. A fed-batch fermentation with optimum DO conditions and a constant residual sucrose feeding regime improved 2,3-BD production in both strains, reaching 42.31 g/L of 2,3-BD with a production yield of 0.52 g/g sucrose in *B. subtilis,*

and 26.23 g/L of 2,3-BD with a production yield of 0.34 g/g sucrose in *S. plymuthica* fermentation. With regards to downstream processing, the yellowish-brown colour of two different fermentation broths (*S. plymuthica* and *B. subtilis*) were successfully reduced through treatment with activated charcoal. Subsequently, the ionic liquid (IL), 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ($[C_2mim][CF_3SO_3]$) was investigated with inorganic salts for 2,3-BD separation. In the case of *S. plymuthica* broth, 95.6% of 2,3-BD was successfully recovered from the fermentation broth with a partition coefficient of 24.6 by using a system composed of 30%$[C_2mim][CF_3SO_3]$ - 35%$K_2HPO_4$ (w/w). In addition, 35%$[C_2mim][CF_3SO_3]$ - 30%$K_2HPO_4$ provided a high yield for recovering 2,3-BD (97.3%) from *B. subtilis* fermentation broth, thus verifying the effectiveness of this separation method. Additionally, the study showed that IL could be effectively recovered (90% to 98%). Hence, non-pathogenic wild-type *B. subtilis* and *S. plymuthica* could be potentially employed as promising strains for high chiral purity of 2,3-BD stereoisomer production. Also, IL-based salting out method enables the development of a novel separation process for efficient recovery of 2,3-BD from diverse fermentation broths.

# List of Publications

1. Suthkamol Suttikul, Dimitris Charalampopoulos and Afroditi Chatzifragkou, Optically Pure 2,3-Butanediol by *Bacillus subtilis* Based on Dissolved Oxygen Control Strategy, Fermentation 2023, 9(1), 15. **Published. (Chapter 3)**
2. Suthkamol Suttikul, Dimitris Charalampopoulos and Afroditi Chatzifragkou, *Serratia plymuthica* as a novel candidate for meso-2,3-butanediol production. **In preparation for submission. (Chapter 4)**
3. Suthkamol Suttikul, Dimitris Charalampopoulos and Afroditi Chatzifragkou, Integration of activated charcoal treatment and ionic liquids for 2,3-butanediol extraction from fermentation broth. **In preparation for submission. (Chapter 5)**
4. Suthkamol Suttikul, Dimitris Charalampopoulos and Afroditi Chatzifragkou, Biotechnological fermentation and downstream processing for bio-based 2,3-butanediol production: Current state and advances (a review). **In preparation for submission. (Chapter 2)**

# List of Conferences

1. Oral presentation (3 minutes) at the 2nd Food and Nutritional Sciences Research Symposium on 5th November 2019, in University of Reading, UK.
2. Poster presentation at the 3rd Food and Nutritional Sciences Research Symposium on 3rd - 4th November 2020, in University of Reading, UK.
3. Oral presentation at the 4th Food and Nutritional Sciences Research Symposium on 2nd - 3rd November 2021, in University of Reading, UK.
4. Oral Presentation at Department of Food and Nutritional Sciences Seminar on 2nd March 2022, in University of Reading, UK.
5. Oral presentation at the Total Food Conference 2022, University of Nottingham, 13th -14th July 2022, in University of Reading, UK.
6. Oral presentation at the 5th Food and Nutritional Sciences Research Symposium, 31st October - 1st November 2022, in University of Reading, UK.

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# Abbreviations

| | |
|---|---|
| AR | Acetoin reductase |
| α-ALS | α-acetolactate synthase |
| ALDC | Acetolactate decarboxylase |
| AC | Activated charcoal |
| adhE | Alcohol dehydrogenase gene |
| AMS | Ammonium sulphate |
| AMP | Diammonium hydrogen phosphate |
| ANOVA | Analysis of variance |
| ASTM | American Society for Testing and Materials |
| ATP | Adenosine triphosphate |
| ATPS | Aqueous-two phase system |
| 2,3-BD | 2,3-Butanediol |
| BDH | Butanedioldehydrogenase |
| budA | Alpha-acetolactate decarboxylase gene |
| budB | Alpha-acetolactate synthase gene |
| budC | Butanediol dehydrogenase gene |
| CA | Casamino acids |
| C/N ratio | Carbon to nitrogen molar ratio |
| CSL | Corn steep liquor |
| CSLP | Corn steep liquor powder |
| $^{o}C$ | Degree Celsius |
| $[C_2mim][CF_3SO_3]$ | 1-ethyl-3-methylimidazolium trifluoromethanesulfonate |

| | |
|---|---|
| [$C_4$mim][Cl] | 1-n-butyl-3-methylimidazolium chloride |
| DAR | Diacetyl reductase |
| DCW | Dry Cell weight |
| DHA | Dihydroxyacetone |
| DAD | Diode Array detector |
| DO | Dissolved oxygen |
| FAN | Free amino nitrogen |
| FDH | Formate-hydrogen lyase |
| FID | Flame ionization detector |
| gapA | Glyceraldehyde-3-phosphate dehydrogenase gene |
| GC | Gas Chromatography |
| GHG | Greenhouse gas |
| g/L | Gram (s) per liter |
| g/L/h | Gram (s) per liter per hour |
| h | Hour (s) |
| HPLC | High performance liquid chromatography |
| IL | Ionic liquid |
| IPTG | Isopropyl-β-D-thiogalactoside |
| $K_{BD}$ | Partition coefficient of 2,3-BD |
| LDH | Lactate dehydrogenase |
| ldhA | Lactate dehydrogenase-A gene |
| MEK | Methyl ethyl ketone |
| mL | Mililitre |
| min | Minute (s) |

| | |
|---|---|
| mM | Milli-molar |
| MRS | De Man, Rogosa and Sharpe |
| NA | Nutrient agar |
| NAD | Nicotinamide adenine dinucleotide (Oxidised form) |
| NADH | Nicotinamide adenine dinucleotide (Reduced form) |
| NOX | NADH oxidase |
| OD | Optical cell density |
| PEG | Polyethylene glycol |
| PT | Peptone |
| PFLB | Pyruvate-formate lyase |
| pflB | Pyruvate-formate lyase gene |
| PTS | Phosphotransferase system |
| PVA | Poly vinyl alcohol |
| R | Phase volume ratio |
| RI | Refractive index |
| rpm | Revolutions per minutes |
| RQ | Respiratory quotient |
| s | Second (s) |
| SBM | Soybean meal |
| sp. | Species (singular) |
| SSF | Simultaneous saccharification and fermentation |
| $S_{SU}$ | Selectivity of BD over sucrose |
| $S_{AC}$ | Selectivity of BD over acetoin |
| $Ss_{A}$ | Selectivity of BD over succinic acid |

| | |
|---|---|
| $S_{AA}$ | Selectivity of BD over acetic acid |
| TCD | Total colour difference |
| TP | Tryptone |
| U/mg | Unit per miligram |
| vvm | Gas volume flow per unit of liquid volume per minute |
| w/v | Weight per volume |
| (w/w) | Weight per weight |
| YE | Yeast extract |
| $Y_{BD}$ | Recovery of 2,3-BD |
| $Y_{IL}$ | Recovery of IL |

# Chapter 1

# General Introduction

Globally, more than 80 million tonnes of industrial chemicals are produced every year [9]. The development of bio-based chemicals has attracted great attention due to increasing concerns on depletion of fossil resources, climate change and other environmental problems and has opened a door for innovation in the chemical industry [10]. Many chemicals that are conventionally produced from fossil fuel via chemical synthesis, can also be biologically produced from renewable resources such as simple sugars, starch, glycerol, as well as lignocellulosic biomass, through microbial fermentation processes [11]. 2,3-Butanediol (2,3-BD), an emerging platform chemical, has gained much attention due to its potential applications in chemicals, food, agriculture, pharmaceuticals and cosmetics. The global market for 2,3-BD is anticipated to reach USD 300 million by 2030 [12]. The biological production of 2,3-BD presents economic and environmental advantages over the chemical synthesis route, especially through the use of low-cost renewable resources, the reduction of greenhouse gas emissions, and the selective production of 2,3-BD stereoisomers [13]; however, its commercial-scale implementation has not been full established yet. The main challenges for its industrial implementation are due to safety risks of 2,3-BD producing strains, stereoisomer purity, as well as substrate and downstream processing costs [14].

Examples of efficient 2,3-BD producers are strains belonging to *Klebsiella* species, *Serratia marcescens*, *Enterobacter aerogenes*, and *Paenibacillus polymyxa* [1]. However, most of high-performing native 2,3-BD producing strains (*Klebsiella* species,

*Serratia marcescens*) are regarded as risk group 2 pathogens, which hinder their applicability for industrial scale production [1, 15]. Many studies have demonstrated the ability of genetic engineered or mutant strains to reach high yields of 2,3-BD production [16-18]. However, these microorganisms usually require nutrient-rich fermentation media as well as chemical inducers and antibiotics, to ensure plasmid stability in the host microorganisms; both factors increase the production cost [19]. In general, native 2,3-BD producing strains can produce a mixture of two or three stereoisomers of 2,3-BD, depending on the presence of different stereospecific 2,3-BD dehydrogenases. 2,3-BD stereoisomers can have a variety of applications. Specifically, the (R,R)-2,3-BD can be used as an anti-freezing agent due to its low freezing point at (-60 $^{o}$C) [15]. Moreover, it can be applied in the asymmetric synthesis of valuable chiral compounds, such as chloro-boronic esters [20]. Besides, (R,R) 2,3-BD exhibits multiple applications in agriculture as plant growth-promoting compound [21], while (S,S)-2,3-BD could also be employed in the production of printing inks, solvents and resins [22]. Meso-2,3-BD can be applied as a humectant and antiseptic in cosmetic products [5]. Various bioprocessing factors have been reported for influencing the formation of 2,3-BD and other by-products. Oxygen supply is considered as the most important factor impacting the production of 2,3-BD. It has been demonstrated that relatively low dissolved oxygen (DO) concentrations favour 2,3-BD production, whereas relatively high DO saturation promotes microbial growth and acetoin production [23, 24]. Therefore, an effective DO control approach is required to minimise or even suppress acetoin formation and other by-products while improve 2,3-BD production.

Additionally, to achieve an economically viable microbial 2,3-BD production, less expensive carbon sources have been extensively investigated, such as food and agricultural industry wastes. Nevertheless, less work has sought the use of low-cost

nitrogen sources, which possess the second largest proportion of substrate, to replace costly nitrogen components in commercial culture media. The utilisation of agri-food wastes and residues as substrate for 2,3-BD production is a promising trend, aiming to increase the value of the waste as well as reduce waste disposal in agri-food industries.

On the other hand, the cost of downstream separation process of 2,3-BD exceeds 50% of the total production cost [25]. Hence, it is important to continually develop a cost effective, easy to operate as well as "clean" method to facilitate the commercialization of 2,3-BD. The fermentation broth generally contains a large amount of water, small number of polysaccharides, monosaccharides, proteins, and several impurities such as short chain fatty acids, ethanol, and inorganic salts. Moreover, 2,3-BD high boiling point, high viscosity, and high hydrophilicity renders traditional distillation energy intensive for 2,3-BD separation [3]. Therefore, downstream processing is considered a critical bottleneck hindering the industrial production of 2,3-BD. Solvent extraction and reactive extraction methods have been effectively developed for 2,3-BD separation. However, the toxicity and properties (i.e. highly volatile and flammable) of most organic solvents are not desirable for downstream separation of 2,3-BD from the fermentation broth [3]. Thus, separation methods that deliver high efficiency, are less toxic, more practical and cost effective should be developed for promoting cleaner production of bio-based 2,3-BD.

### 1.1 Research Objectives

The overall aim of this research was to develop a biotechnological process for the production and separation of 2,3-BD in sucrose-based media with alternative low-

cost nitrogen source. The specific objectives that were set out in order to achieve individual research goals were:

1. Evaluation of dissolved oxygen control strategies for the biotechnological production of optically pure (R,R)-2,3-BD, by employing a non-pathogenic wild-type *Bacillus subtilis* strain (Chapter 3)
2. Investigation of the effect of dissolved oxygen and alternative low-cost nitrogen sources on meso-2,3-BD production utilising *Serratia plymuthica* as a novel 2,3-BD producing candidate (Chapter 4)
3. Development of green separation processes for the recovery of 2,3-BD from fermentation broths (Chapter 5)

## 1.2 Research Hypotheses

The following hypotheses were stated for the purpose of the thesis:

A suitable oxygen control strategy could enhance 2,3-BD production while reduce by-products formation in non-pathogenic wild-type *B. subtilis* and *S. plymuthica* strains. Alternative low-cost nitrogen source can be potentially employed to replace expensive nitrogen sources in commercial MRS medium for 2,3-BD production by both strains. 2,3-BD could be effectively recovered from the fermentation broths with high yield by integrated techniques, specifically activated charcoal pretreatment and ionic liquid based- salting out extraction.

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# Chapter 2

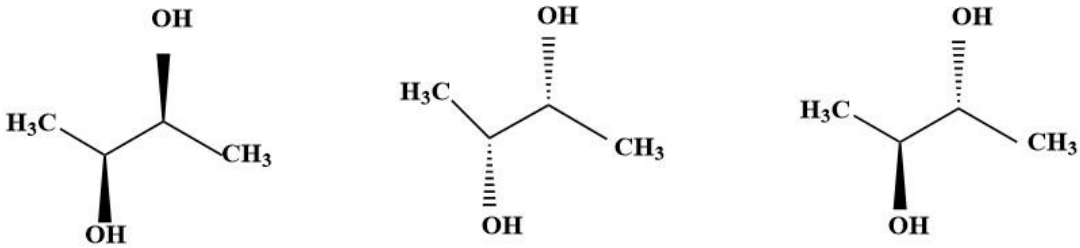
# Literature review

## 2.1 Introduction

Global warming and limitation of fossil fuel reserves is driving the development of ecologically friendly and sustainable bio-based chemicals. A number of chemicals can be produced biologically from renewable resources by microorganisms [1, 2]. 2,3-butanediol (2,3-BD) is considered as one of the most valuable chemical products, and has attracted considerable attention recently due to its diverse range of applications including fuel additives, synthetic rubber, printing ink, perfumes, fumigants, moistening and softening agents as well as food products [3-8]. Production of microbial 2,3-BD from renewable resources could diminish the reliance on petroleum supply for the manufacturing of platform chemicals [9, 10]. Furthermore, biotechnological processes are considered as environmentally friendlier through the reduction of greenhouse gas (GHG) emissions as the process can be developed with less water, and energy utilisation compared to conventional chemical processes. Bioprocess development strategies using efficient microbes and relatively cheap substrates can improve yield and fermentation efficiency of microbial 2,3-BD production as well as promote effective product recovery processes. This could lead to alternative biotechnological routes for economically viable 2,3-BD production compared to the existing chemical synthesis route.

The 2,3-BD ($C_4H_{10}O_2$) is also known as 2,3-butylene glycol, dimethylethylene glycol, dimethylene glycol, and 2,3-dihydroxybutane. The molecular weight is

90.12 g/mol. 2,3-BD exists in three stereoisomeric forms: (2S, 3S)-BD (Dextro- form), (2R, 3R)-BD (levo-form), and (2R, 3S)-BD (meso-form) (Figure 2.1).




L-(+)-2,3-BD D-(-)-2,3-BD *meso*-2,3-BD

(2S,3S); Dextrorotatory form (2R,3R); Levorotatory form (2R,3S); Optically inactive-form

**Figure 2.1** Stereoisomeric forms of 2,3-BD (modified from Ji et al., 2011 [11]).

The enantiomers (S,S)-2,3-BD and (R,R)-2,3-BD are optically active while meso-2,3-BD is optically inactive. The optically active form of isomer is more stable than the optically inactive form because the methyl ($CH_3$) groups present in the optically active form are anti, whilst those in the optically inactive (meso) form are gauche [12]. 2,3-BD is a colourless, odourless, soluble in water, and transparent liquid at room temperature. The boiling points of different stereoisomers of 2,3-BD are slightly different, ranging from 177 to 182$^{o}$C. The low freezing point of 2,3-BD (-60$^{o}$C) is the basis of the commercial interest in its application as an anti-freezing agent [9].

This review focuses on the recent advances of 2,3-BD production. Factors that influence 2,3-BD productivity such as the source and type of substrate, cultivation conditions, and the type of microorganism employed are critically discussed. Moreover, recent advances in 2,3-BD downstream processing are also covered in this review.

## 2.2 Applications of 2,3-BD

Butanediol has different isomers (1,2-, 1,3-, 1,4-, 2,3-BD), which have common applications as plasticizer, solvent, and resin. Specifically, 2,3-BD is only native occurring 2,3-BD isomer that can be produced by natural microorganisms [13]. 2,3-BD has unique applications; it is typically employed as a crosslinking component for rubber materials, blending additives, solvent for dyes and paints as well as can potentially be used in various industries [14]. As shown in Figure 2.2, 2,3-BD could serve as an anti-freeze agent because it has a low freezing point (-60$^{o}$C) [9]. Furthermore, 2,3-BD has a potential as fuel additive with a high heating value of 27198 J $g^{-1}$ [15]. Moreover, 2,3-BD has high octane number, thus, it could also be employed as an octane booster in gasoline or as high quality of aviation fuel [9]. The global market of 2,3-BD is expected to reach USD 300 million by 2030, growing at a compound annual growth rate (CAGR) of 3.5% from 2020 to 2030 [16]. 2,3-BD and its derivatives had a global market of over 32 million tonnes (USD 43 billion) in 2018 [12]. The most important derivative of 2,3-BD is the intermediate chemical 1,3-butadiene, which can be exploited as monomer for the production of synthetic rubber, polyester, as well as other polymers. The global market volume of 1,3-butadiene was estimated to reach 14 million tonnes, accounting for USD 33.01 billion by 2020. Interestingly, other 2,3-BD derivatives including acetoin and diacetyl also have new trends of applications in the food, pharmaceutical, and cosmetic industry [11]. Another important application of 2,3-BD involves the production methyl ethyl ketone (MEK) as efficient fuel additive and solvent for paints and resins [9].

A particular stereoisomeric form of 2,3-BD has a specific industrial application. The (R,R)-2,3-BD presents multiple applications in agriculture such as a plant growth-

promoting compound [17], and can be used in the asymmetric synthesis of valuable chiral compounds, such as chloro-boronic esters [18]. Moreover, the (S,S)-2,3-BD is a building block for the asymmetric synthesis of valuable chiral compounds and it can also be used in the production of printing inks, resins and solvents [19]. In addition, the meso-2,3-BD can be employed as an antiseptic and humectant for cosmetic products [20].

2,3-BD can be typically manufactured via chemical process from petroleum materials. Recently, the production of 2,3-BD has regarded as an alternative perspective in biomass conversion to biorefinery products. This diol can be biologically produced from renewable materials, namely wood hydrolysates and food-waste products, mainly by bacterial strains of *Klebsiella*, *Serratia*, *Bacillus* as well as *Enterobacter* species.

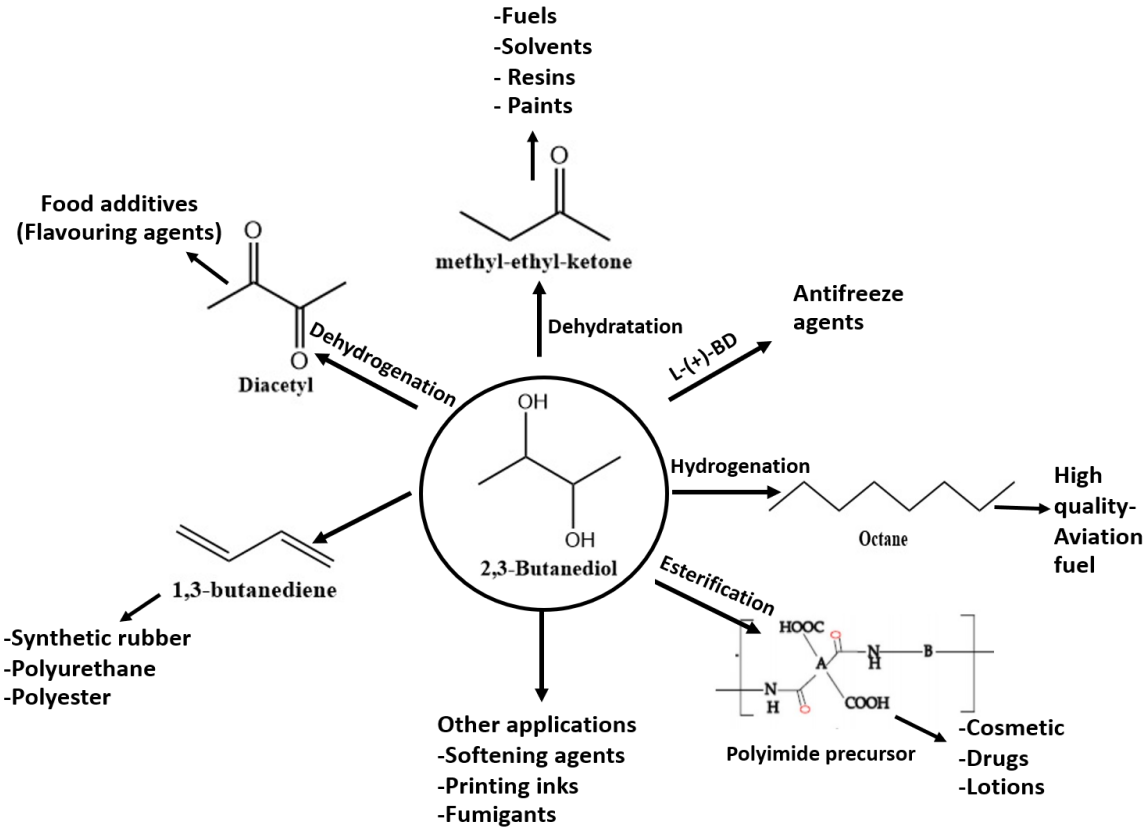



**Figure 2.2** Derivatives of 2,3-butanediol and their potential applications (adapted from Biakowska, 2016; Ji et al., 2011; Hakizimana et al., 2020 [4, 11, 21]).

## 2.3 Chemical Synthesis of 2,3-BD

Butenes derived from crack gases are used as raw material for chemical synthesis of 2,3-BD. Briefly, a C4 hydrocarbon fraction (C4 raffinate II) obtained after the removal of butadiene and isobutene from crack gases, is processed into a mixture of *trans*-2,3-butene oxide (55%), *cis*-2,3-butene oxide (30%), and 1,2-butene oxide (15%) through chlorination followed by cyclization of chlorohydrins with sodium hydroxide. Then, the butene oxide mixture is hydrolysed at 160-220ºC, 50 bar, to produce a mixture of 2,3-BD that is subsequently be separated by vacuum fractionation (as shown in Figure 2.3) [22].

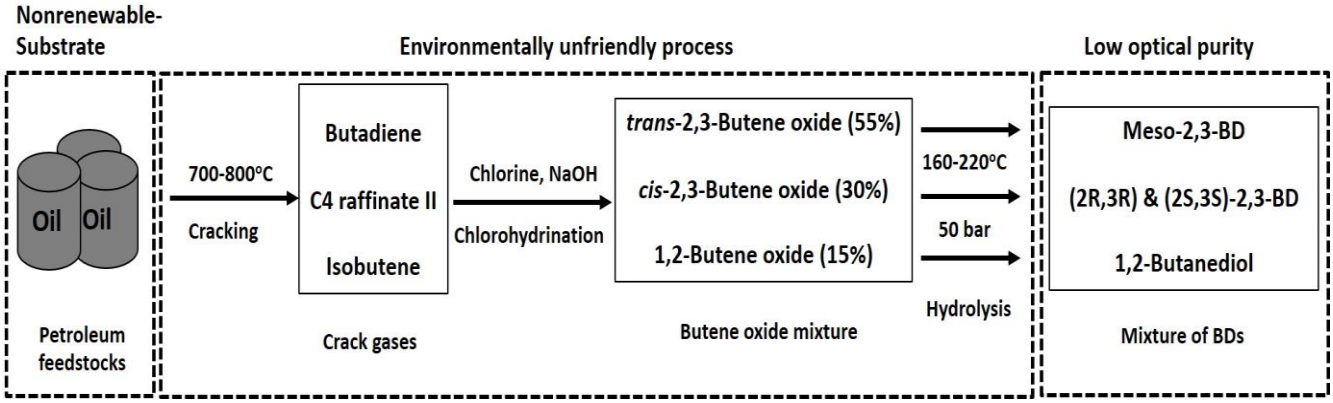



**Figure 2.3** Scheme of 2,3-BD production via chemical synthesis (adapted from Ge, 2016 [22]).

Meso- 2,3-BD is produced from *trans*-2,3-butene oxide, whilst (R,R)-2,3-BD and (S,S)-2,3-BD are produced from *cis*-2,3-butene oxide [22, 23]. The mixture of 2,3-BD comprises approximately 80% of meso-2,3-BD and 20% of racemic-2,3-BD. The selective synthesis of optically pure 2,3-BD via chemical route is consequently difficult to control and thus requires complicated and expensive processes [22]. Moreover, the

chemical synthesis of 2,3-BD is conducted under harsh conditions (160-220°C, 50 bar pressure), thus a large amount of energy is required. This leads to high production costs, which are the major barrier for the global market expansion of 2,3-BD [4]. Due to the high production cost as well as increasing concern about environmental sustainability, interest has shifted towards biotechnological 2,3-BD production which represents a much cleaner, more sustainable and environmentally-friendlier process compared to the chemical production [22] .

Petrochemical refineries and biorefineries are similar in that they are both required to refine raw materials and process to yield products with higher value for energy, chemicals, as well as materials manufacturing [24]. The most difference between them are raw materials and process used for production of products as shown in Figure 2.4. More specifically, petroleum refineries use crude oil, whereas biorefineries utilise biomass feedstock as raw materials [24].

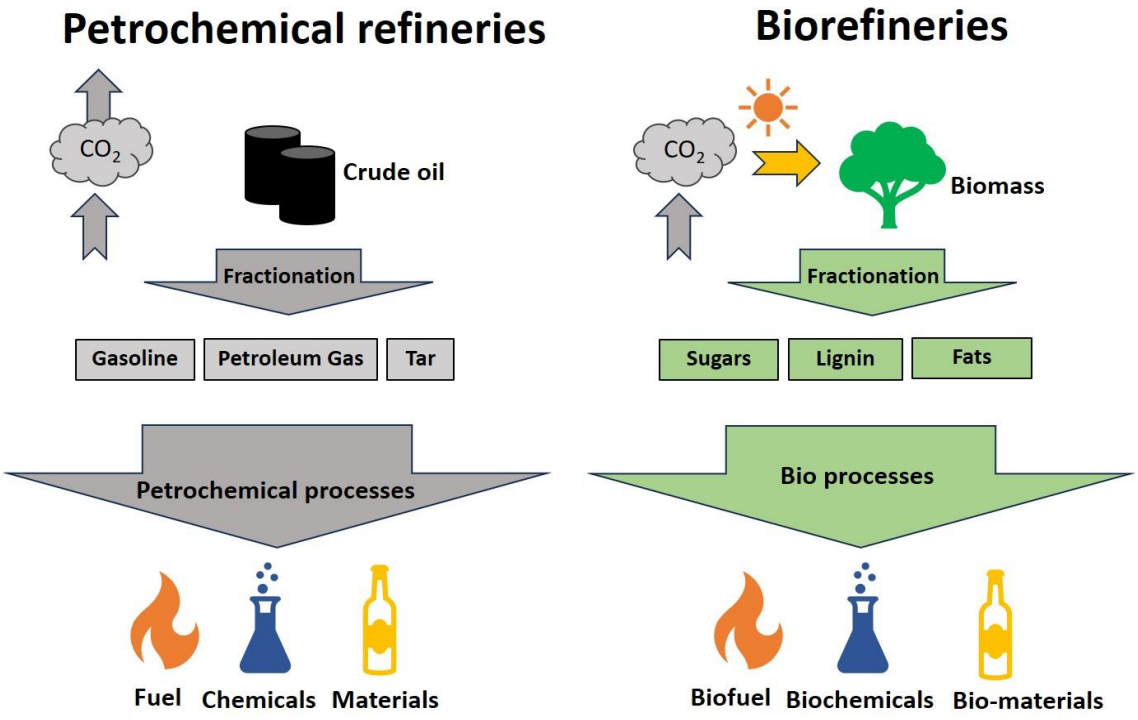



**Figure 2.4** Comparison between petrochemical refineries and biorefineries (Adapted from Hülsey, 2018 [24]).

## 2.4 Biotechnological Production and Biological Functions of 2,3-BD

Bio-based 2,3-BD is considered an eco-friendly approach [25, 26]. Rehman et al. [27] evaluated the footprint of bio-based 2,3-BD production from first generation feedstock and claimed that $CO_2$ emissions were 43.3% lower than that of petroleum-based ones. Higher volumes of optically pure 2,3-BD stereoisomers can be produced through microbial production compared to chemical synthesis. Moreover, one of the significant benefits of microbial 2,3-BD production is the lower inhibitory effect of 2,3-BD on the microbial cells, than other alcohols such as ethanol, and butanol exert, thus high 2,3-BD yields could be achieved under appropriate strain selection and optimum cultivation conditions [28].

2,3-BD production is taken place via the mixed acid fermentation pathway in microbial cells. Apart from 2,3-BD, other metabolites, like succinic acid, lactic acid, acetic acid could be generated leading to a decrease in the intracellular pH. Thus, 2,3-BD, a neutral compound, is produced with a vital role in preventing excessive acidification of the intracellular environment [9, 11]. Moreover, the metabolic pathway of 2,3-BD participates in the regulation of the intracellular NADH/$NAD^+$ ratio in microbial cell due to the reversible transformation between 2,3-BD and acetoin concomitant with the conversion between NADH/$NAD^+$. Additionally, when carbon and energy sources become depleted, 2,3-BD could be converted back into acetoin to regulate the carbon and energy balance in microbial cells. A number of microorganisms reutilise acetoin and 2,3-BD as carbon and energy storage reserves [29, 30].

## 2.5 Metabolic Pathways of 2,3 Production

A number of microbial species are able to metabolise different carbon sources for the production of 2,3-BD through fermentative processes [7, 8, 11, 31-33]. In the case of hexoses, sugars enter the microbial metabolism via phosphotransferase system (PTS) and convert into two moles of pyruvate through glycolysis pathway (Embden-Meyerhof pathway) concomitant with the generation of two moles of NADH and two moles of ATP. In contrast, pentoses proceed through a combination of the pentose phosphate and Embden-Meyerhof pathways to generate pyruvate [34]. In case of sucrose, sucrose is first hydrolysed into one mole of glucose and fructose, which are further converted into pyruvate. In general, 2,3-BD is produced from pyruvate via the mixed acid fermentation pathway through intermediate compounds, namely α-acetolactate, acetoin, and diacetyl. First, pyruvate from glycolysis is decarboxylated to form one molecule of α-acetolactate (catalysed by α-acetolactate synthase; α-ALS, 3 of Figure 2.5). Then, α-acetolactate could be converted into R-acetoin by acetolactate decarboxylase (ALDC, 4 of Figure 2.5) under oxygen limiting conditions. In the presence of oxygen, α-acetolactate can undergo spontaneous decarboxylation and converts into diacetyl. Then, diacetyl is converted to S-acetoin by diacetyl reductase (DAR) [35]. Finally, acetoin is reduced to 2,3-BD by 2,3-BD dehydrogenase (BDH; also known as acetoin reductase; ACR). It should be noted that the metabolic conversion from acetoin to 2,3-BD is reversible [8]. Acetoin is an intermediate compound prior to the generation of 2,3-BD. More specifically, from the two forms of acetoin (R- and S-acetoin) produced, three forms of 2,3-BD including (R,R), meso, and (S,S) are generated by different types of stereospecific BDH-dehydrogenases: (R,R)-, meso, and (S,S)-BDH.

Under fully aerobic conditions, α-ALS is rapidly and irreversibly inactivated, thus preventing 2,3-BD formation. In this case, pyruvate is converted into acetyl-CoA by the pyruvate dehydrogenase multienzyme complex, which cannot be synthesised under anaerobic conditions [36]. The generated acetyl-CoA is subsequently subjected to the tricarboxylic acid (TCA) cycle. Under microaerophilic conditions, lactate dehydrogenase (LDH), pyruvate-formate lyase (PFLB) and α-ALS act upon pyruvate in order to produce lactate, formate, and 2,3-BD, respectively. The produced formate can be further metabolised to carbon dioxide and hydrogen by the formate-hydrogen lyase (FDH) complex, which is not normally inhibited in aerobic environment, and in the presence of nitrate under anaerobic conditions [28]. These reactions help to maintain the NADH/NAD+ balance in microbial cells.

In bacterial metabolism, apart from 2,3-BD, pyruvate could be channelled towards the production of various metabolites, including lactate, acetate, succinate, formate, ethanol through the mixed acid fermentation pathway (reaction 15, (11 to 12), (16 to 19), 10, (13 to14) of Figure 2.5), respectively [8].

Apart from glucose, 2,3-BD can also be produced from glycerol through the oxidative pathway. Glycerol dehydrogenase (reaction 20 of Figure 2.5) is responsible for converting glycerol into dihydroxyacetone (DHA). The produced DHA is then converted into pyruvate by dihydroxyacetone kinase (reaction 21 of Figure 2.5). Pyruvate can be converted through different pathways to form 2,3-BD as describe previously. For the reductive pathway, glycerol can undergo dehydration to form hydroxypropionaldehyde and is further reduced to 1,3-propanediol (Figure 2.5) [37]. This pathway competes for a carbon source (glycerol) with 2,3-BD pathway [38].

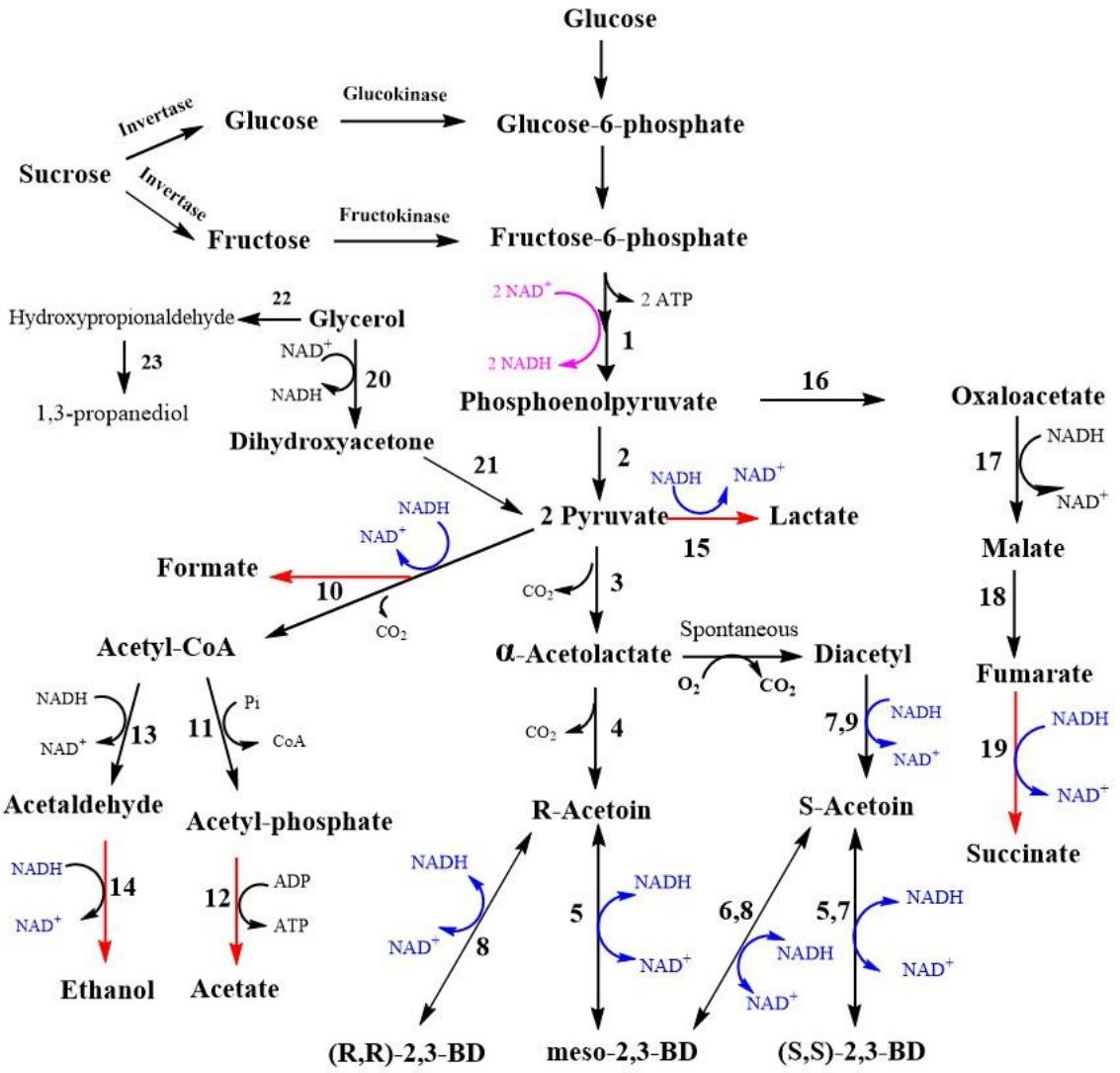



**Figure 2.5** The metabolic pathway of 2,3-butanediol synthesis in bacteria (Modified, based on [20, 28]). 1: Glycolysis and pentose phosphate pathway, 2: pyruvate kinase, 3: α-acetolactate synthase, 4: α-acetolactate decarboxylase, 5: meso-BD dehydrogenase (R-acetoin forming), 6: meso-BD dehydrogenase (S-acetoin forming), 7: (S,S)-BD dehydrogenase (S-acetoin forming), 8: (R,R)-BD dehydrogenase, 9: diacetyl reductase, 10: pyruvate-formate lyase, 11: phosphotransacetylase, 12: acetate kinase, 13: acetaldehyde dehydrogenase, 14: ethanol dehydrogenase, 15: lactate dehydrogenase, 16: phosphoenolpyruvate decarboxylase, 17: malate dehydrogenase, 18: fumarase,

19: succinate dehydrogenase, 20: glycerol dehydrogenase, 21: dihydroxyacetone kinase, 22: glycerol dehydratase, 23: 1,3-propanediol oxidoreductase.

The chemical equations for sucrose conversion to 2,3-BD are as follows:

Under anaerobic conditions

$$C_{12}H_{22}O_{11} + H_2O \longrightarrow C_6H_{12}O_6 + C_6H_{12}O_6 \longrightarrow 2C_4H_{10}O_2 + 4CO_2 + H_2O \quad (1)$$

Under aerobic conditions

$$C_{12}H_{22}O_{11} + H_2O \longrightarrow C_6H_{12}O_6 + C_6H_{12}O_6 + O_2 \longrightarrow 2C_4H_{10}O_2 + 4CO_2 + 2H_2O \quad (2)$$

The maximum theoretical yield for the conversion from sugar into 2,3-BD is 0.5 g/g sugar consumed [9], whereas the maximum theoretical yield of 2,3-BD from glycerol is 0.62 g/g [39].

## 2.6 Mechanisms Involved in the Formation of 2,3-BD Stereoisomers

Different stereoisomeric forms of acetoin are produced under different oxygen conditions and are also dependent on NADH availability [40]. The existence of different BDHs in native 2,3-BD producing strains leads to the production of diverse 2,3-BD stereoisomers. R-acetoin could be converted into either (R,R)-2,3-BD by R-BDH or into meso-2,3-BD by meso-BDH or S-BDH, whereas S-acetoin can be reduced to either (S,S)-2,3-BD by S-BDH or to meso-2,3-BD by meso-BDH or R-BDH. Moreover, 2,3-BD could be reversely converted into acetoin to regenerate NADH in order to maintain a balance of intracellular redox-state [19]. The key enzymes involved in the production of 2,3-BD are active under oxygen limiting conditions and it is likely that the target product (2,3-BD) is produced during late log phase or stationary growth phase [41]. It has been reported for some microorganisms that a single BDH could perform two

functions. For example, S-BDH could catalyse the conversion from diacetyl to S-acetoin, and from S-acetoin to (S,S)-2,3-BD. In contrast, in the case of *Brevibacterium saccharolyticum* and *K. pneumoniae*, the meso-BDH catalyses the conversion from acetoin to 2,3-BD and also exhibits a strong diacetyl reductase activity [42].

## 2.7 Butanediol Producing Microorganisms

### 2.7.1 Native 2,3-BD producing strains

A variety of bacterial species are able to produce 2,3-BD. However, only some species such as *Klebsiella* sp., *Enterobacter* sp., *Serratia* sp., *Bacillus* sp., and *Paenibacillus* sp. can produce 2,3-BD in considerable amounts [11]. Some studies obtained high yield of 2,3-BD by using native producers such as *Klebsiella oxytoca*, *Klebsiella pneumonia*, *Serratia marcescens*, and *Enterobacter aerogenes* [11, 43, 44]. Typically, different bacterial strains can synthesise different stereoisomeric forms of 2,3-BD through different pathways [9].

Native *Klebsiella* species have great potential for 2,3-BD production. Furthermore, they can be grown quickly in standard media and have the ability to utilise a wide variety of substrates [45]. However, the pathogenic features of *Klebsiella* species are belonging to risk class 2 (pathogenic) microorganisms, thus they are not favourable for industrial 2,3-BD production. [46]. *Klebsiella* species such as *K. pneumoniae* and *K. oxytoca*, can mainly produce two of 2,3-BD stereoisomers, including meso-2,3-BD and (S,S)-2,3-BD [47-49]. More specifically, meso-2,3-BD (can be produced from S-acetoin which is catalysed by meso-2,3-BD dehydrogenase or from R-acetoin catalysed by meso-2,3-BD dehydrogenase (*bud*C)); and (S,S)-2,3-BD can be produced from S-acetoin which is catalysed by (S,S)-2,3-BD dehydrogenase (*bud*C) [50]. Similarly,

*Enterobacter aerogenes* and *Enterbacter ludwigii* can produce meso-2,3-BD and (S,S)-2,3-BD [51-53], whereas *Enterobacter cloacae* can produce all three 2,3-BD isomers [54].

*Serratia marcescens* can produce meso-2,3-BD either from S-acetoin or R-acetoin which is catalysed by meso-2,3-BD dehydrogenase [8, 55].

*Bacillus* sp. is recognised as generally regarded as safe (GRAS) microorganisms and can perform satisfactory fermentative ability. Thus, this family has attracted great attention for 2,3-BD production. *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* have been reported to produce (R,R)-2,3-BD, catalysed by (R,R)-2,3-BD dehydrogenase and meso-2,3-BD which is catalysed by meso-2,3-dehydrogenase [56-61].

*Paenibacillus polymyxa* mainly produces (R,R)-2,3-BD with a small proportion of meso-2,3-BD by (R,R)-2,3-BD dehydrogenase [62, 63].

### 2.7.2 Strain improvement through genetic engineering

Recently, genetic tools have been applied for modifying microbes into efficient microbial cell factories. Thus, it is important to understand well the metabolic characteristics in order to optimise the metabolic pathways for improving yields of target products [21, 64, 65]. The deletion of some genes involved in the mixed acid pathway, particularly those requiring NADH for the synthesis of the by-products, has been introduced in order to improve 2,3-BD production. For example, the deletion of genes such as *ldh*A gene (encoding lactate dehydrogenase), *pfl*B gene (encoding pyruvate-formate lyase), *ack*A gene (encoding acetate kinase), and *adh*E gene (encoding alcohol dehydrogenase), responsible for converting pyruvate to lactate and formate, and for

converting acetyl Co-A to acetate and ethanol, respectively (the enzymes 15,10, 12, and 14, respectively of Figure 2.5). Consequently, pyruvate would then be directed towards the 2,3-BD pathway and thus enhance the production of 2,3-BD [31, 66, 67]. For instance, in *K. oxytoca* GSC 12206, the disruption of lactate dehydrogenase (*ldh*A) gene brought about a mutant with minimum lactic acid levels while improving the production of 2,3-BD up to 115 g/L with a yield of 0.41 g/g and 2.27 g/L/h productivity [68].

Moreover, enhancing 2,3-BD yield has been attempted by overexpressing genes regulated 2,3-BD synthesis [e.g., alpha-acetolactate synthase (*budB*), alpha-acetolactate decarboxylase (*budA*), and butanediol dehydrogenase (*budC*)] [69]. For example, co-overexpression of glyceraldehyde-3-phosphate dehydrogenase (encoded by *gap*A gene) and 2,3-BD dehydrogenase (encoded by *bud*C gene) in *B. amyloliquefaciens* enhanced the 2,3-BD yield by 22.7% compared to the parental strain. 2,3-BD concentration increased to 132.9 g/L with a productivity of 2.95 g/L/h in fed-batch fermentations [70]. Additionally, to improve the 2,3-BD yield in *B. subtilis*, the (R,R)-butanediol dehydrogenase gene was transformed with the butanediol dehydrogenase (*bud*C) gene that encoded by *K. pnuemoniae*. This led to the construction of a novel strain that could produce 103.7 g/L pure meso-2,3-BD with a yield of 0.487 g/g [71].

Even though genetic modification has shown to improve 2,3-BD production yields, requirements such as nutrient-rich medium, antibiotics, as well as chemical inducers to assure plasmid stability in host microbes, increase total costs for commercial production of 2,3-BD [72]. Moreover, several host strains such as *K. pneumoniae* and *E. aerogenes* belonging to risk group 2, which are not regarded as safe for industrial scale fermentation [9, 31, 72].

## 2.8 Feedstock for Microbial 2,3-BD Production

An important factor in achieving an economically feasible biobased process is the use of cost-effective resources[73]. So far, commercial substrates namely: refined sugars such glucose, xylose, and sucrose, starch, and pure glycerol, have been mainly used as substrates for microbial 2,3-BD production. However, the commercialisation for 2,3-BD production may not economically be viable due to the utilisation of relatively high cost of commercial sugars. Abundant, cheap, and easily available substrates such as starch-based, sugar-based, and lignocellulosic feedstocks have been recently employed as alternative feedstock for 2,3-BD production. In general, alternative low-cost substrate could be classified as two main types: non-lignocellulose and lignocellulose feedstocks.

### 2.8.1 Non-lignocellulosic substrates

Various non-lignocellulosic feedstocks have been investigated for 2,3-BD production by different microorganisms.

#### 2.8.1.1 Starchy feedstocks

Cassava has been investigated as a relatively cheap and abundant starch crop for 2,3-BD production. Wang et al. [54] reported that a newly isolated *E. cloacae* strain produced 93.9 g/L of 2,3-BD under optimum conditions during simultaneous saccharification and fermentation (SSF) of cassava powder. However, as cassava is edible, the utilisation of cassava powder as feedstock for biochemicals production competes with food crops, which conflicts with food security. In the study of Lee et al.

[74], cassava chip hydrolysate was employed as substrate in fed-batch fermentation using an engineered *Saccharomyces cerevisiae* producing 132 g/L of 2,3-BD with 1.92 g/L/h of productivity. Native 2,3-BD producers, such as *K. pneumoniae*, and *S. marcescens* are unable to utilise starch, thus enzymatic hydrolysis is required in order to convert starch into fermentable sugars. Besides *B. amyloliquefaciens*, other native 2,3-BD producing microbes cannot effectively produce 2,3-BD from starch through single step process [60].

2.8.1.2 Sugar-based substrates

Sugar-based feedstock such as sugar beet, sugarcane, and sweet sorghum could supply large proportions of readily fermentable sugars and thus have attracted considerable attention. Additionally, molasses, a by-product from sugar refinery processing, have been effectively employed as substrate for 2,3-BD production. Molasses consists of fermentable sugars, namely sucrose, glucose, and fructose, amino acids, vitamins, and trace elements, and could represent a promising substrate for 2,3-BD production. Generally, complex nutrients such as yeast extract are employed to promote microbial growth and fermentation efficiency, a fact which often contributes to higher substrate cost. As such, molasses, can reduce the cost of carbon source as well as nutrient supplementation, and thus leading to an overall reduction of 2,3-BD production costs. Deshmukh et al. [75] reported 50 g/L of 2,3-BD production from sugarcane molasses using *B. subtilis*. Likewise, a *B. amyloliquefaciens* strain was reported to produce mainly of (R,R)-BD and acetoin from sugarcane molasses, reaching 2,3-BD 48.79 g/L of 2,3-BD in fed-batch fermentations using sugarcane molasses as starting and feeding substrate [58]. Moreover, *Enterobacter* sp. achieved 90.3 g/L 2,3-BD and 10 g/L

acetoin in aerobic fed-batch fermentation using molasses and sucrose [76]. Additionally, Jung et al. [77] reported on an engineered *E. aerogenes* EMY-68 producing 98.7 g/L of 2,3-BD with yield of 0.366 g/g and productivity of 2.74 g/L/h in fed-batch process [77, 78]. Koutinas et al. [73] carried out a techno-economic assessment of 2,3-BD production through fermentation using glycerol, sucrose, and sugarcane molasses as raw materials. They concluded that the development of industrial 2,3-BD production is greatly dependent on the market price of feedstock and the fermentation nutrient utilised as well as the fermentation efficiency. Raw material cost normally accounts for 25-50% of the total production cost, thus utilising cheap and renewable resources, particularly food industry and agricultural wastes can help to increase the economic viability of biobased 2,3-BD production [79].

Sweet sorghum is an energy crop that is cultivated in tropical climate areas, and exhibits broad adaptability to various environmental conditions [80]. Furthermore, its stalks contain high amounts of fermentable sugars (sucrose, glucose, and fructose), vitamins, and amino acids [81]. However, the juice is obtained by extraction from sweet sorghum stalks and must be used immediately since it has a very short shelf life. An engineered *S. marcescens* H30 was cultivated in sweet sorghum juice, supplemented with nitrogen source and inorganic salts, in a two-stage agitation fed-batch process; 109 g/L of 2,3-BD were achieved with a productivity of 1.40 g/L/h, reaching 83% of the maximum theoretical yield [82].

The rationale of using pure sucrose as raw material for 2,3-BD production in this work is that its cost is lower pure glucose. Moreover, as its form is sugar; thus, no pretreatment process is required. In addition, sucrose is major component of other alternative sugars, such as sugarcane molasses, sugar beet molasses, and sweet sorghum juice. Thus, the use of pure sucrose as substrate for 2,3-BD production in this work could

be applied to employ other crude sucrose-based feedstocks for 2,3-BD production. As crude sucrose-based substrate contains vitamin, nitrogen source and mineral salts that might be beneficial for support microbial growth and 2,3-BD production. More specifically, sugarcane molasses and sugar beet molasses are by-product of sugar industry, which are generally used in the production of bioethanol and animal feed. The use of these by-products to produce 2,3-BD could add the value to waste or by-products. In addition, this can also be applied for using fruit wastes which are normally discharged to environment or used for anaerobic digestion. Thus, the use of low-cost and alternative source of sucrose for 2,3-BD production, can reduce production cost and make the process more sustainable. Table 2.1 provides details of sugar composition, annual production, and market price of different sources of sucrose.

**Table 2.1** Sugar compositions, annual production, and price of different sources of sucrose

| Type of sucrose | Feedstocks | Sugar composition | Global production (per year) | Market price (USD/kg) |
|---|---|---|---|---|
| Pure | Sucrose | Sucrose (>99%): glucose (50%), fructose (50%) | 180 million MT | 0.4 [b] |
| | Very high polarity | Sucrose (99%) | 180 million MT | 0.05-0.4 [b] |
| Crude | Sugarcane molasses | Sucrose (48.8%), glucose (5.29%), fructose (8.07%) [a] | 7.2 million MT | 0.05-0.2 [c] |
| | Sugar beet molasses | Sucrose (60.9%), glucose (0.28%), fructose (0.29%), raffinose (0.60%) [d] | 10 million MT | 0.1-0.2 [e] |
| | Sweet sorghum juice | Total sugar (20-30%), comprises of sucrose (53%–85%), glucose (9%–33%), fructose (6%–21%) | 21 million tonnes [f,g] | 0.5 [h] |

[a] Palmonari et al.[83]; [b] Maina et al.[51]; [c] Psaki et al. [53]; [d] Hemingway et al. [84]; [e] Urbaniec and Grabarczyk [85]; [f] Hamadou et al. [86]; [g] Kim et al. [87]; [h] Corado et al.[88]; MT: Metric tonne.

2.8.1.3 Biodiesel derived-glycerol

Biodiesel, an alternative renewable fuel, has attracted much interest in recent years. Biodiesel production generates approximately 10%(w/w) of glycerol as the main by-product [89]. In 2019, the global biodiesel production was approximately 45 million tonnes/year, and about 4.5 million tonnes of glycerol was produced as a by-product [90]. Glycerol could serve as a substrate for 2,3-BD, 1,3-propanediol (1,3-PD), and ethanol [91]. Petrov and Petrova [92] investigated 2,3-BD production by the natural strain of *K. pneumoniae* using glycerol as a carbon source. A 2,3-BD concentration of 52.5 g/L, yield of 0.303 g/g and productivity of 0.36 g/L/h were obtained in a fed-batch process without pH control. Likewise, a wild-type *K. oxytoca* M1 strain produced 73.8 g/L of 2,3-BD from crude glycerol-based media. Moreover, an engineered *K. oxytoca* M3 was developed by deleting glycerol dehydrogenase and lactate dehydrogenase; this led to a decrease of 1,3-PD and lactic acid formation, resulted in high production of 131.5 g/L 2,3-BD with yield of 0.44 g/g and 0.84 g/L/h of productivity [93]. However, through the reductive pathway, glycerol can also be converted to 1,3-PD as major by product; this could lead to challenges in downstream processes due to both compounds having close boiling point temperatures [94].

Table 2.2 shows the exploitation of various non-lignocellulosic feedstocks for the production of 2,3-BD by different microorganisms.

**Table 2.2** Production of 2,3-BD from various non-lignocellulosic substrates by different microbial strains.

| Substrate | Microorganism | Nitrogen source | Cultivation mode/ agitation and aeration rate | Temperature (ºC)/ pH | 2,3-BD concentration (g/L) | Yield (g/g) | Productivity (g/L/h) | References |
|---|---|---|---|---|---|---|---|---|
| Glucose | *K. pneumoniae* | CSLP+AMP | FB, 500 rpm,1.5 vvm | 37/ 6.0 | 150 | 0.475 | 4.21 | [49] |
| Glucose | *B. subtilis** | CSL | B, 350 rpm/0.66 vvm | 37 | 30.6 | 0.32 | 0.57 | [61] |
| Glucose | *B. licheniformis* | CSL | FB, (500 – 400 rpm), 1 vvm | 50/7.0 | 92 | 0.42 | 1.40 | [59] |
| Glucose | *B. licheniformis* | CSL+YE | FB, 400 rpm (0-10h) then 200 rpm | 50/7.0 | 115.7 | 0.47 | 2.4 | [57] |
| Glucose | *Raoultella ornithinolytica** | YE+CA+ AMS | FB, 400 rpm,1 vvm | 25/5.5 | 112.9 | 0.38 | 1.35 | [95] |
| Glucose | *Vibrio natriegens** | TP+YE | FB, cascade agitation 0-1%DO | 37/7.0 | 49.9 | 0.22 | 3.88 | [96] |
| Sucrose | *S. marcescens** | YE | FB, agitation control with RO, 0.5 vvm | 30/ 6.0 | 139.9 | 0.47 | 3.49 | [55] |
| Sucrose | *P. polymyxa* | YE | FB, 500-800 rpm,0.2-0.5 vvm | 37/ 6.0 | 72 (BD+AC) | - | - | [63] |
| Very high polarity sugar | *E. ludwigii* | AMS | FB, (150-400 rpm), 1 vvm | 33.9/6.3 | 86.8 | 0.37 | 3.95 | [51] |
| Sugarcane molasses | *E. ludwigii* | AMS+YE | FB, (150-400 rpm), 1 vvm | 33.9/6.3 | 50.6 | 0.31 | 2.66 | [53] |
| Sugarcane molasses | *B. amyloliquefaciens* | YE | FB,400 rpm,1 vvm | 33.9/6.3 | 48.7 | 0.40 | 0.83 | [58] |
| Sugar beet molasses | *B. subtilis** | YE+PT | FB, Shake flask, 130 rpm | - | 75.7 | 0.31 | 0.66 | [97] |
| Sugarcane molasses | *E. aerogenes** | AMS+YE+CA | FB, 280 rpm, 1.5 vvm | 37 | 98.7 | 0.37 | 2.74 | [78] |
| Sugarcane molasses | *B. subtilis* | CSL, SBM | B, 400 -250 rpm, (400 L/m- 40 L/m) | 34/6.0 | 50 | 0.33 | 0.52 | [75] |
| Sweet sorghum juice | *S. marcescens* | YE | FB, 300 rpm (0-12h) then 200 rpm | 30 | 109.4 | 0.42 | 1.40 | [82] |
| Potato starch | *K. pneumoniae** | YE+AMP | B-SSF, shake flask | 37/7.0 | 53.8 | 0.27 | 0.448 | [98] |
| Cassava hydrolysate | *S. cerevisiae** | YE+PT | FB, 400 rpm, 2 vvm | 30/5.5 | 132 | 0.32 | 1.92 | [74] |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cassava powder | *E. cloacae* | CSLP | FB-SSF, 500 rpm, 1 vvm | 30/6.0 | 93.9 | - | 2.0 | [54] |
| Glycerol | *K. pneumoniae* | AMS+YE | FB, 200 rpm, (1.1-2.2 vvm) | 37 | 52.5 | 0.30 | 0.36 | [92] |
| Raw glycerol | *R. terrigena* | YE | FB, 400 rpm, 1.5 vvm | 30/5.5 | 80.5 | - | 0.40 | [39] |
| Crude glycerol | *R. ornithinolytica** | YE+CA | FB, 400 rpm, 1 vvm | 25/5.5 | 78.1 | 0.42 | 0.62 | [99] |
| Biodiesel derived-crude Glycerol | *K. oxytoca** | YE+CA | FB, 400 rpm, 1 vvm | 30 | 131.5 | 0.44 | 0.84 | [93] |
| Whey powder | *K. oxytoca** | CSLP | FB, 400 rpm, 1 vvm | - | 65.5 | 0.44 | 2.73 | [100] |
| Bakery waste | *B. amyloliquefaciens* | YE | FB, ($K_La$ 110 $h^{-1}$), 1 vvm | 40/6 | 103.9(AC+BD) | 0.39 | 0.87 | [101] |
| Bread waste from enzyme hydrolysis | *E. ludwigii* * | YE | FB, 180 rpm, 1 vvm | 30/6.6 | 138.8 | 0.48 | 1.45 | [102] |

* Genetic engineered strains.

Nitrogen sources: CSLP: corn steep liquor powder; AMP: $(NH_4)_2HPO_4$; CSL: corn steep liquor; YE: yeast extract; CA: casamino acids; AMS: $(NH_4)_2SO_4$; TP: tryptone; PT: peptone; SBM: soybean meal.

Cultivation mode: B: batch process; FB: fed-batch process

**Table 2.3** Production of 2,3-BD from various lignocellulosic feedstocks by different microbial strains.

| Substrate | Microorganism | Nitrogen source** | Cultivation mode***, agitation and aeration rate | Temperature (°C)/ pH | 2,3-BD concentration (g/L) | Yield (g/g) | Productivity (g/L/h) | References |
|---|---|---|---|---|---|---|---|---|
| Corn stover | *B. licheniformis* | YE+CSLP | FB, 400 rpm, 1 vvm | 50/7.0 | 74 | 0.47 | 2.1 | [56] |
| Corn stover | *P. polymyxa* | YE+AMS | CT-cell recycling, 500 rpm,0.2 L/m | 37/6.5 | 18.8 | 0.31 | 1.13 | [62] |
| Corn stover | *E. cloacae** | CSLP | FB, (500-300 rpm),1 vvm | 30/7.0 | 119 | 0.475 | 2.3 | [103] |
| Sugarcane bagasse | *E. aerogenes** | YE+CA | FB, 200 rpm, 2 vvm | 37/7.0 | 114.3 | 0.44 | 1.49 | [104] |
| Apple pomace | *B. licheniformis* | YE | FB, 250 rpm, 1.2 vvm | 37/6.5 | 77.6 | 0.32 | 0.42 | [105] |
| Oat hull hydrolysate | *E. cloacae* | YE | Shake flask, 200 rpm | 37/6.5 | 37.6 | - | - | [106] |
| Fruit waste | *E. ludwigii* | YE | FB,cascade 5%DO(150-250rpm),1 vvm | 6.3 (pH) | 50 | 0.40 | 0.41 | [107] |
| vegetable waste | *E. ludwigii* | YE | FB,cascade 5%DO(150-250rpm),1 vvm | 6.3 (pH) | 17.6 | 0.32 | 0.39 | [107] |
| Jerusalem artichoke stalk | *K. pneumoniae* | - | FB-SSF, 450 rpm, stage shift aeration (0.2-0.5 vvm) | 37/5.8 | 80.5 | 0.26 | - | [108] |
| Corncob molasses | *K. pneumoniae* | CSL+AMP | FB, (500rpm,1 vvm), (400 rpm, 0.5 vvm) | 37/6.0 | 78.9 | 0.41 | 1.3 | [48] |
| Corncob acid hydrolysate | *K. oxytoca* | YE | FB, 300rpm | 37/6.3 | 35.7 | 0.5 | 0.59 | [47] |
| Spirit distillers' grain | *B. amyloliquefaciens* | - | Shake flask, 160 rpm | 37 | 40.7 | 0.29 | 0.81 | [60] |

* Genetic engineered strains.

**Nitrogen sources: CSLP: corn steep liquor powder; AMP: $(NH_4)_2HPO_4$; CSL: corn steep liquor; YE: yeast extract; CA: casamino acids; AMS: $(NH_4)_2SO_4$. ***Cultivation mode: B: batch process; FB: fed-batch process.

### 2.8.2 Lignocellulosic substrates

Lignocellulose is considered as the most abundant biomass available on Earth. It has attracted considerable attention as an alternative resource for biorefinery production due to its renewable nature and ready availability [109]. Lignocellulosic biomass has been used as an alternative feedstock for 2,3-BD production in order to minimize the raw material cost. However, the significant drawbacks of using this substrate for 2,3-BD production are low productivity and lignocellulosic feedstocks need to be pre-treated and hydrolysed by the enzymes to release the fermentable sugars. The pretreatment methods commonly require high temperature, pressure, and chemical whereas enzymatic hydrolysis require specific enzymes, contributing to high energy consumption, increasing production cost, and generating environmentally damaging liquid waste [4]. Moreover, some inhibitors such as furfural, hydroxymethyl furfural, and phenolic compounds are generated during the pretreatment of lignocellulosic materials, which can inhibit microbial growth and product biosynthesis. In addition, some organic compounds such as lignin need to be separated during downstream processing which adds complexity to the process. Therefore, the use of lignocellulosic substrate is generally considered as economically unfavourable for large-scale production [110]. However, the process optimisation could make a large-scale production of 2,3-BD economically viable. Different lignocellulosic feedstocks have been investigated for the production of 2,3-BD by diverse microorganisms are shown in Table 2.3.

Li et al. [98] developed a process using Jerusalem artichoke tubers for 2,3-BD production; 80.5 g/L of 2,3-BD were obtained at 68 h of the fed-batch SSF process. Furthermore, apple pomace hydrolysate was investigated as the main substrate for 2,3-

BD production by the strain of *B. licheniformis* NCIMB 8059, reaching 113 g/L with over 163 h of fed-batch cultivation [105]. Zang et al. [111] carried out a techno economic analysis to evaluate the co-production of 2,3-BD, furfural and lignin from switchgrass. It was reported that 2,3-BD production from lignocellulosic biomass contributed to higher fixed capital investment (FCI) and variable operating costs (VOC) than the three cases of non-lignocellulosic substrates from the study of Koutinas [73] due to the complex operation system and chemical costs, particularly associated with the pretreatment process.

## 2.9 Bioprocess Development for Microbial 2,3-BD Production

Diverse parameters such as nutrient requirements, oxygen concentration, pH, and temperature have been identified as crucial factors affecting fermentation efficiency of 2,3-BD production. Nutrient supplementation, pH, temperature as well as aeration and agitation at different stages of fermentation are major parameters that should be optimized to maximise 2,3-BD production.

### 2.9.1 Factors influencing cell growth and 2,3-BD production

#### 2.9.1.1 Oxygen supply (Aeration)

Oxygen supply is considered one of the most crucial parameters affecting the production yield and productivity of 2,3-BD as well as by-product formation during fermentation [9, 11]. In general, facultative anaerobic microorganisms can obtain energy via two different pathways, namely, respiration and fermentation. Under high oxygen

availability, NADH is generated through respiration from glycolysis therefore microbial growth and $CO_2$ formation are favoured. Under oxygen limiting conditions, 2,3-BD and other by-products are produced to maintain the intracellular redox balance of the pyridine nucleotide pairs ($NAD^+$/NADH) pool during glycolysis and cell biosynthesis [112]. At low oxygen or microaerobic conditions, the respiratory chain cannot effectively regenerate the excess reducing power (NADH) associated with glycolysis and thus NADH-consuming pathways are activated, leading to the formation of various by-products such as lactate, succinate, acetate, ethanol [112]. The NADH/$NAD^+$ balance could be maintained by the transformation between acetoin and 2,3-BD, regulated by the reversible action of butanediol dehydrogenase [113], since 2,3-BD and acetoin are produced concomitantly in most native 2,3-BD producers during fermentation [114]. The distribution of 2,3-BD and acetoin is impacted by dissolved oxygen concentration during fermentation [100]. It has been suggested that relatively low dissolved oxygen (DO) concentrations or micro-aeration favour 2,3-BD production, while relatively high DO saturations stimulate bacterial growth and acetoin production [41, 101, 115]. Lowering the air supply would enhance 2,3-BD yield, but the overall conversion rate would be decreased due to lower microbial cell formation [112, 116, 117]. Thus, proper oxygen supply control strategies are required for enhancing 2,3-BD production, whilst reducing the generation of by-products.

In case of *K. oxytoca*, the highest cell concentration and 2,3-BD formation was obtained under aerobic conditions. Optimum aeration rate and agitation speed were applied for partial suppression of by-products such as ethanol and lactic acid. However, acetoin and acetic acid may be generated at the expense of 2,3-BD under excessive aeration-agitation conditions [118]. Chan et al. [119] reported that low aeration rate of 0.8 vvm with agitation speed at 400 rpm was suitable for 2,3-BD production by

engineered *K. oxytoca* in fed-batch fermentation, giving 2,3-BD concentration of 88.1 g/L, yield of 0.412 g/g, and productivity of 1.13 g/L/h. Although *B. subtilis* is considered an aerobic bacterium, limited oxygen availability is preferred for the conversion of acetoin to 2,3-BD. Thus, microaerobic conditions with aeration rate of 0.02 vvm were employed to achieve high meso-2,3-BD concentrations of 103.7 g/L and yield of 0.487 g/g [71]. Moreover, a fed-batch under microaerobic cultivations using the engineered *Vibrio natriegens* yielded a combined diol (BD+AC) concentration of 49.9 g $L^{-1}$ and a productivity of 3.9 g $L^{-1}$ $h^{-1}$ [96]. On the other hand, higher aeration rate of 1.0 vvm was optimum for 2,3-BD production by *B. licheniformis* and *Enterobacter cloacae* [3, 120]. The optimum aeration rate used in numerous studies were varied in the range from 0.02 to 2.0 vvm, however, it also depends on the type of strains exploited as well as fermentation conditions, especially agitation speed [3, 22, 93, 120].

2.9.1.2 Agitation speed

Agitation offers proper mixing for maximum nutrient utilisation by microorganisms and promotes oxygen transfer in the culture medium. High stirring rate stimulates cell growth, acetoin and acetate accumulation resulting in a decrease of 2,3-BD yield, while low agitation speed favours 2,3-BD and some by-products like, succinate, lactate and ethanol [41, 58, 121]. Various agitation control strategies have been demonstrated to increase 2,3-BD production yield [9]. A constant agitation speed has been widely used for 2,3-BD production. Cho et al. [122] investigated agitation speed optimisation for high 2,3-BD production using *K. oxytoca* M1. The result showed that higher 2,3-BD concentrations of 78.8, 102.1, and 105.1 g/L were obtained by using agitation speeds at 200, 300, and 400 rpm, respectively.

A two-stage agitation speed control strategy was developed to enhance 2,3-BD production. Firstly, higher stirring rate was employed in order to increase oxygen supply for promoting cell growth. At the second stage of fermentation, lower agitation speed was applied to limit the oxygen availability, and support 2,3-BD formation [5, 123]. With this strategy, 103 g/L of 2,3-BD were obtained at 30 h of fed-batch fermentation by *B. licheniformis* ATCC 14580 [5]. The two-stage agitation speed control technique was also performed in *B. licheniformis* achieving 115.7 g/L of 2,3-BD from glucose [57]. Several research works are shown in Table 2.2 and 2.3.

Considering the above results, agitation rate optimization is essential for efficient 2,3-BD production, and it depends also on the type of strains employed and fermentation conditions used.

2.9.1.3 Temperature

Temperature is an essential parameter for industrial fermentation in terms of microbial and enzyme performance and operating costs. As 2,3-BD is a growth-associated metabolite, the optimum temperature for microbial growth and product biosynthesis should be within the same range [15]. Optimum temperatures for most of 2,3-BD producers varies between 25 and 50°C [11, 124]. It has been reported that lowering temperature from 35 to 30°C would not only stimulate a significant reduction in ethanol formation in favour of 2,3-BD biosynthesis, but also limit other side-products formation in *K. pneumoniae* strain. The optimal temperature for *K. pneumoniae* was identified to be 33°C [9]. Likewise, for *P. polymyxa*, 30°C was reported as the optimal temperature in both batch and fed-batch 2,3-BD fermentations [29]. In contrast, the maximum 2,3-BD concentration was achieved when cultivated a thermophilic

*B. licheniformis* strain at 50°C [22]. As observed in Table 2.2 and 2.3, in most case of *B. licheniformis*, the optimal temperature for 2,3-BD production is 50°C [57, 59], whereas temperatures between 30 and 37°C are suitable for other bacterial strains.

2.9.1.4 pH

The pH value influences intracellular enzyme activity in bacteria since it affects the formation of metabolic products in various strains and substrates [125]. The 2,3-BD biosynthesis from pyruvate is regulated by three key enzymes, namely, α-acetolactate synthase, α-acetolactate decarboxylase, and 2,3-BD dehydrogenase (BDH, also known as acetoin reductase). In the mixed acid fermentation pathway, alkaline conditions favour the biosynthesis of organic acids with a concomitant decrease in the yield of 2,3-BD. The α-acetolactate synthase has an optimum activity under slightly acidic conditions (pH 6). During fermentation, some microorganisms counteract the acidification of the culture medium by shifting the metabolism towards the production of neutral compounds such as 2,3-BD or alcohols; 2,3-BD is generated as a major product at a pH range from 5.0 to 6.5, lactic acid, ethanol, and 1,3-PD become the main products at pH range between 7.0 and 8.0 [126]. This indicates that the metabolic pathways could be diverted depending on the pH level, and thus 2,3-BD efficiency could be improved by controlling the pH of culture broth [21]. Nevertheless, the optimum pH for 2,3-BD production also depends on microorganisms and substrates utilised [9]. Many studies have revealed that the formation of 2,3-BD could occur in the pH range 4.5-9.0 with the optimal value of 6.0 [125, 127]. More specifically, the pH preference of the enzyme acetoin reductase (ACR) and 2,3-butanediol dehydrogenase (BDH) that regulate the transformation between acetoin and 2,3-BD in *B. subtilis* has

been investigated. The result showed the pH preferences for reduction is pH 6.5 and pH 8.5 for oxidation [128]. Wong et al. [127] reported the optimal pH value for 2,3-BD production by *Klebsiella* sp. Zmd30 was 6.0. Likewise, maximum 2,3-BD production was obtained at a pH 6.0 by *B. licheniformis* and *P. polymyxa*. Similarly, the optimum pH for 2,3-BD production by *E. aerogenes* was at pH 6.0 [9, 129].

2.9.1.5 Nitrogen source

Nitrogen source is essential for microbial growth and production of desired products. Complex organic nitrogen sources such as yeast extract, peptone, corn steep liquor (CSL), soybean meal (SBM) and casamino acid could promote growth and 2,3-BD formation. A great number of studies have used yeast extract as nitrogen source for 2,3-BD fermentation (Table 2.2 and 2.3). However, the use of this expensive nitrogen source contributes to high production costs. CSL, a by-product of corn wet-milling from starch production, has been employed as nitrogen source for various industrial fermentations. It contains vitamins, amino acids, trace elements, which are beneficial to microbial growth, and can substitute more expensive nitrogen sources such as yeast extract or peptone [41, 130]. Inorganic nitrogen sources, such as ammonium sulphate, can also be utilised for 2,3-BD production. Moreover, urea has been demonstrated as an effective nitrogen source for 2,3-BD production from lignocellulose derived glucose and xylose using *K. oxytoca* [130]. In addition, combinations of nitrogen sources including yeast extract, CSL and urea have been used in the fermentation medium for acetoin production by *B. subtilis* [114]. Yang et al. [131] demonstrated the utilization of co-substrates (biodiesel-derived glycerol and beet molasses), supplemented with a

mixture of CSL and SBM as nitrogen sources for production of 2,3-BD using *B. amyloliquefaciens*.

2.9.1.6 Media supplementation

Several studies have recently revealed that phosphate, acetate, $Mn^{2+}$, $Mg^{2+}$ and $K^{+}$ play key role on cellular metabolism, substrate consumption rate, and 2,3-BD production [49]. More specifically, it has been reported that potassium acetate at a concentration of 0.1 M could promote 2,3-BD production in *E. aerogenes* [132]. Ammonium citrate has been reported as a significant factor for promoting 2,3-BD production [133, 134]. It has been shown that various organic acids, and ammonium citrate, act as intermediate metabolites that can induce the formation of 2,3-BD [133].

### 2.9.2 Cultivation modes of 2,3-BD production

The effect of cultivation mode on 2,3-BD production is crucial to establish an optimal process design. For efficient 2,3-BD fermentation, different cultivation modes have been developed including batch, fed-batch, continuous culture, cell recycling as well as immobilised cell systems. A minimum 2,3-BD concentration of 80 g/L from fermentation broth should be obtained for an economically feasible product recovery [28]. Examples of different cultivation modes for 2,3-BD production are discussed below and summarised in Table 2.4.

#### 2.9.2.1 Batch process

Batch process has been employed in various microbial fermentations as it benefits from simple control and operation. Batch fermentation was used for 2,3-BD production by *E. aerogenes* SUMI014 strain; 93.75 g/L 2,3-BD was obtained with 0.49 g/g of yield and productivity of 1.74 g/L/h [132]. Yang et al. [61] also investigated the production of 2,3-BD from glucose using *B. subtilis* in batch fermentation; 2,3-BD concentration and yield of 39.2 g/L and 0.39 g/g was obtained, with a relatively low productivity of 0.68 g/L/h. The low productivity generally observed in batch fermentation is due to the fact that high carbon source concentrations could inhibit microbial growth and fermentation efficiency, resulting in a prolonged fermentation period and thus unsuitable for large scale 2,3-BD production.

#### 2.9.2.2 Fed-batch process

It is generally agreed that the fed-batch approach with intermittent feeding of substrates when residual nutrients are depleted, could be the most efficient cultivation mode for industrial 2,3-BD production. It can reduce the effect of initial substrate inhibition, thus higher 2,3-BD concentration would be achieved [127].

A fed-batch fermentation was realised for 2,3-BD production from glucose by *K. pneumoniae* SDM under aerobic conditions. A high concentration of 150 g/L 2,3-BD with a production yield of 0.475 g/g and productivity of 4.21 g/L/h was achieved with a constant residual glucose concentration feeding strategy [49]. A similar fed-batch strategy has also been successfully employed for 2,3-BD production using *S. marcescens* [55].

Likewise, Lee et al. [132] found that employing fed-batch fermentation with acetate feeding strategy for 2,3-BD production in *E. aerogenes* SUMI 014 strain, resulted in an increase of 2,3-BD concentration up to 126.1. g/L (compared to 93.75 g/L in batch process) with 0.38 g/g of yield and 2.10 g/L/h of productivity. Similarly, Chan et al. [119] optimised the process for 2,3-BD production from maltodextrin using engineered *K. oxytoca*. A maximum concentration of 88.10 g/L 2,3-BD with a yield of 0.412 g/g and productivity of 1.13 g/L/h was achieved.

2.9.2.3 Continuous process

Continuous fermentation is the cultivation process where fresh substrate is continuously added to the bioreactor, while the used medium, product, and cells are harvested at the same time. When the addition and removal are at the same rate, the culture volume would stay constant and steady state is achieved in the bioreactor. Several studies have reported that continuous cultivation could improve the productivity of biochemicals by reducing the inhibitory effect of end-product and by-products. Nevertheless, microbial contamination is still the limiting factor of this technique. Wong et al. [127] investigated the production of 2,3-BD in different cultivation mode from glucose using *Klebsiella* sp. Zmd30. A productivity of 2.81 g/L/h was obtained in continuous fermentation with concentration of 35 g/L 2,3-BD and 66% of theoretical yield.

2.9.2.4 Immobilised cell system

Cell immobilisation has been recognised as an attractive method for increasing fermentation productivity. This technique allows the possibility of cell reusing, increased cell stability, protection of cells from stress environment and shear force in the bioreactor; it can be operated continuously at high cell density cultures that usually lead to higher productivity, and the immobilised cells can easily be separated from the culture broth [11, 135]. Jurchescu et al. [135] conducted repeated batch/ fed-batch fermentations using immobilised *B. licheniformis*. More specifically, bacterial cells were entrapped in Lentikats® matrix, which is made of poly vinyl alcohol (PVA) gel. High stability and activity of the immobilised-cell system was observed even after several medium replacements; 118.3 g/L 2,3-BD was obtained with a productivity of 1.14 g/L/h. Moreover, the 2,3-BD concentration, yield, and productivity were similar to those obtained in free cell systems. In another study, Ca-alginate immobilised-cell culture of *K. pneumoniae* BLh1 was used for diol production, achieving 10.11 g/L 2,3-BD and 22 g/L of 1,3-propanediol. The immobilised cell system could be repeatedly used in several batches. The entrapped cells can maintain their biological activity and operational stability, with high 1,3-PD productivity of 4.48 g/L/h [136].

2.9.2.5 Cell-recycle systems

Batch or continuous bioreactor systems coupled with cell-recycle have been investigated as a way to enhance volumetric productivity; they usually consist of a bioreactor coupled with a microfiltration module, aiming to maintain high cell density during fermentation. 18.80 g/L of (R-R)-2,3-BD were obtained in continuous

fermentation with cell recycle system using *P. polymyxa* strain for 2,3-BD production from corn stover hydrolysate [62]. Likewise, a cell recycle method was developed with a microfiltration module for 2,3-BD production by *E. aerogenes*. Under optimum conditions, high productivity of 14.6 g/L/h was achieved, which was three-fold higher than that during conventional continuous process [52].

**Table 2.4** Different cultivation modes for 2,3-BD production

| Cultivation mode | Microorganism | Substrate | 2,3-BD concentration (g/L) | Yield (g/g) | Productivity (g/L/h) | References |
|---|---|---|---|---|---|---|
| Batch | *E. aerogenes* SUMI014 | Glucose | 93.75 | 0.49 | 1.74 | [132] |
| Batch | *B. subtilis* | Glucose | 39.2 | 0.39 | 0.68 | [61] |
| Fed-batch | *K. pneumoniae* SDM | Glucose | 150 | 0.475 | 4.21 | [49] |
| Fed-batch | *P. polymyxa* | Glucose | 68.54 | 0.34 | 0.70 | [137] |
| Fed-batch | *B.licheniformis* ATCC14580 | Inulin | 103 | NA | 3.4 | [5] |
| Fed-batch | *E. aerogenes* SUMI014 | Glucose | 126.10 | 0.38 | 2.10 | [132] |
| Fed-batch | *K. oxytoca* | Maltodextrin | 88.10 | 0.412 | 1.13 | [119] |
| Continuous | *Klebsiella* sp. Zmd30 | Glucose | 35 | 0.33 | 2.81 | [127] |
| Fed-batch+Immobilised cell system | *B. licheniformis* | Glucose | 118.3 | 0.41 | 1.14 | [135] |
| Immobilised cell system | *K. pneumoniae* BLh-1 | Biodiesel derived glycerol | 10.11 | 0.19 | 0.84 | [136] |
| Cell recycling +continuous | *P. polymyxa* | Corn stover hydrolysate | 18.80 | 0.313 | 1.13 | [62] |
| Cell recycling | *E. aerogenes* | Glucose | 54 | 0.44 | 14.6 | [52] |

NA = Not available

## 2.10 Separation and Recovery of 2,3-Butanediol from Fermentation Broth

Besides improving fermentation efficiency, the development of an economically sustainable method for separating 2,3-BD from the fermentation medium is crucial for rendering the microbial 2,3-BD production process competitive. The recovery of 2,3-BD from the fermentation broth is often challenging due to 2,3-BD's high boiling point (177-182ºC), high hydrophilicity, and the presence of impurities (e.g., nutrient residues or by-products of the microbial metabolism). Various separation methods have been investigated for downstream processing of 2,3-BD such as distillation [138], steam stripping [15], solvent extraction, pervaporation [118], and reverse osmosis [94]. More specifically, in the case of conventional steam stripping and vacuum distillation, a large amount of energy is required, thus their applications are still limited. Other techniques, such as solvent extraction, salting out [138] and counter current steam stripping [15] have been developed with some success, but mainly on a laboratory scale due to their complexity and high demand of extractant. For instance, anionic extraction has been investigated to separate and recover 2,3-BD, based on a reversible complexation with phenylboronate in an anionic complex. Under optimum conditions, 72-93% 2,3-BD could be extracted and up to 80-90% was recovered by back-extraction. However, the high demand of extractants still restricts the wider application of this method [139]. Recently, emerging methods have been evaluated for the efficient recovery of 2,3-BD, including integrated solvent extraction and pervaporation [140], aqueous two-phase extraction [141], *in situ* recovery [142], and reactive extraction [143]. For example, Li et al. [144] developed an aqueous two-phase extraction method for 2,3-BD separation. The aqueous two-phase systems consisted of hydrophilic solvents (ethanol) and inorganic salts (ammonium sulphate), which are low cost and allow recycling. The

highest partition coefficient (7.10) and 91.7% recovery of 2,3-BD were achieved by a system composed of 32% (w/w) ethanol and 16% (w/w) ammonium sulphate. Moreover, the removal ratio of cells and proteins from the fermentation broth reached 99.7% and 91.2%, respectively. In a recent study, the recovery and purification of 2,3-BD from the detoxified xylose-rich sugarcane bagasse fermentation broth was investigated by Narisetty et al. [145]. An aqueous two-phase system (ATPS) comprised of 30% (w/v) $(NH_4)_2SO_4$ and 50% (v/v) isopropanol, provided a high 2,3-BD recovery yield of 97.9% and partition coefficient of 45.5. So far, most of the liquid-liquid extraction methods for 2,3-BD separation have been carried out with conventional organic solvents. However, there are some drawbacks such as most organic solvents contribute to air pollution due to their high volatility. Moreover, most of organic solvents are toxic and flammable and their use is often associated with unavoidable waste water generation. Koutinas et al. [73] carried out a techno-economic assessment of 2,3-BD production and separation process from three types of substrate (glycerol, sucrose, and sugarcane molasses). Even though it was found that the fixed cost of a downstream product recovery using reactive extraction method is significantly lower than the cost of fermentation facility in all cases. However, it should be noted that the remaining aldehydes in reactive extraction method could negatively affect the applications of 2,3-BD as they may prohibit its subsequent polymerization. Using aldehydes as extractants is an expensive method and due to toxicity concerns and environmental issues associated with typical volatile organic solvents, ionic liquids (ILs), a novel and greener class of non-volatile alternative solvents, are being currently explored for extraction purposes. ILs exhibit extremely low volatility under atmospheric conditions, and commonly exhibit good thermal and chemical stability, and are non-flammable, attributes that have contributed to their extensive recognition as ambient-friendly compounds [146]. Dai et al. [147] investigated

the recovery of 2,3-BD using [1-butyl-3-methylimidazolium][chloride]-based salting out system. They reported that 35% [$C_4$mim][Cl] - 8% $K_2HPO_4$ (w/w) led to 96% recovery of 2,3-BD and a partition coefficient of 5.2. Thus, ILs based salting out extraction, could be applied as a promising system for the development of green separation processes for bio-based 2,3-BD.

## 2.11 Conclusions

There is considerable interest in 2,3-BD driven by its high value and extensive potential applications. However, a commercial scale production of bio-based 2,3-BD has not yet been consolidated due to relatively low fermentation efficiency. To achieve efficient and economically viable processes for bio-based 2,3-BD production, different strategies have been progressively developed. The novel technological developments in metabolic and genetic engineering approaches for enhancing target products and reducing by-products have been achieved. Various alternative low-cost substrates include lignocellulosic and non-lignocellulosic feedstocks have been utilised, aiming to offer economic benefits for bio-based 2,3-BD production. Also, bioprocess development for improving 2,3-BD concentration, yield, and productivity as well as advancements in downstream processes are simultaneously developed and are critically discussed in this review. The factors limiting its commercial scale production have been addressed. Further research and development on producing chiral pure 2,3-BD stereoisomers, minimising the production cost, improving cost-effectiveness of downstream process is required for the successful commercialisation of bio-based 2,3-BD production, as well as expanding their applications in various industries.

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# Chapter 3

# Biotechnological Production of Optically Pure 2,3-Butanediol by *Bacillus subtilis* Based on Dissolved Oxygen Control Strategy


**Abstract**

2,3-Butanediol (2,3-BD) is a promising platform chemical, produced from microbial cells. Oxygen availability is a crucial factor driving the formation and proportion of 2,3-BD and acetoin in 2,3-BD producing bacterial strains. In this study, the ability of *B. subtilis* to produce 2,3-BD in optimized sucrose-based media was evaluated, by investigating the impact of carbon to nitrogen (C/N) ratio and the effectiveness of alternative low-cost nitrogen sources (corn steep liquor, soybean meal, and ammonium sulphate). Subsequently, different dissolved oxygen (DO) controlling regimes were assessed in batch bioreactor fermentations. The best fermentation outcomes were obtained with uncontrolled DO, achieving 5.88 g/L of optically pure (R,R)-2,3-BD (~100% purity), accompanied by a production yield of 0.43 g/g, and a productivity of 0.2 g/L/h. Additionally, the influence of the DO controlling regime on *B. subtilis* key enzymes involved in the reverse activity of acetoin reductase was also monitored. The fed-batch process under the most suitable DO conditions was carried out to improve 2,3-BD production, achieving 42.31 g/L 2,3-BD with a production yield of 0.52 g/g. Thus, *B. subtilis* GD5 (FSBC 322) is a promising strain for the efficient

production of pure chiral (R,R)-2,3-BD under uncontrolled DO conditions, using alternative low-cost nitrogen sources.

**Keywords:** 2,3-butanediol; *Bacillus subtilis*; dissolved oxygen; optical purity; fermentation; fed-batch

## 3.1 Introduction

2,3-Butanediol (2,3-BD) is an important platform chemical that has potential applications in various industries such as in the production of antifreeze agents, printing inks, fuel additives, synthetic rubber, food flavour additives, and pharmaceutical products [1-3]. Currently, 2,3-BD is typically produced on an industrial scale from non-renewable petroleum feedstocks through chemical processes [4]. With the further development of green, sustainable technologies and continuous enhancement of environmental protection awareness, the production of bio-based chemicals inevitably has become a key trend of industrial development, even though bio-based chemicals are still generally more expensive than conventional petroleum-based ones [5]. A number of wild-type microorganisms, including those in the genera of *Klebsiella* sp., *Bacillus* sp., *Serratia* sp., *Enterobacter* sp. and *Paenibacillus* sp., have the ability to produce 2,3-BD [6-16]. Nevertheless, the pathogenic nature of *Klebsiella pneumoniae* limits its exploitation for industrial-scale production. Among those strains, *B. subtilis*, has been granted a generally regarded as safe (GRAS) status, and is considered an efficient 2,3-BD producing strain [17]. Much work has been performed using expensive nitrogen sources such as peptone, yeast extract, beef extract or tryptone to produce 2,3-BD;

however, these are often associated with higher production costs and could potentially limit the translation of the bioprocess on an industrial scale [18].

The biosynthesis of 2,3-BD is part of the mixed acid fermentation pathway in bacterial cells, which produces a variety of metabolic products including acetic, lactic and succinic acid, 2,3-BD, and ethanol. The metabolic pathways of 2,3-BD have been investigated intensively and involve three key steps: firstly, two molecules of pyruvate (synthesized from the glycolytic pathway) are converted into α-acetolactate and carbon dioxide by the catalysis of α-acetolactate synthase (ALS), then acetolactate decarboxylase (ALDC) transforms α-acetolactate to acetoin. Finally, acetoin is reduced into 2,3-BD by acetoin reductase (AR); however, 2,3-BD could be reversibly converted back into acetoin by 2,3-BD dehydrogenase (BDH) [2, 19, 20]. Acetoin has one chiral center, and exists as R-acetoin or S-acetoin. 2,3-BD has two chiral centers, resulting in three stereoisomers, including the optically active form of (R,R)-2,3-BD and (S,S)-2,3-BD, and the optically inactive form of (R,S), which also designated as meso- 2,3 BD [21]. In general, a mixture of 2,3-BD stereoisomers is produced by native 2,3-BD-producing strains, which possesses a challenge for the production of 2,3-BD with high chiral purity [4, 22]. On a biochemical level, 2,3-BD plays a significant role in bacterial growth, by preventing excessive acidification of the intracellular environment, regulating the balance ratio of reduced nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide ($NAD^+$), and serving as a carbon source for cell maintenance [23]. Since 2,3-BD, lactic acid and ethanol are NADH-dependent metabolites involved in the mixed acid fermentation pathway, NADH availability and quantity in its active form plays a critical role in directing the biosynthesis of 2,3-BD. Thus, 2,3-BD production could be enhanced by manipulating the NADH-dependent pathways [24]. Moreover, oxygen availability is a crucial factor driving the formation

and proportion of 2,3-BD and acetoin. It has been suggested that relatively low dissolved oxygen (DO) saturation favour 2,3-BD production, while relatively high DO concentrations promote cell growth and acetoin production [25-27]. Thus, an effective DO control strategy is required to reduce acetoin formation whilst enhance 2,3-BD production.

The overall aim of this study was to enhance 2,3-BD production in non-pathogenic wild-type of *B. subtilis*. Initially, the effects of alternative low-cost nitrogen sources and culture C/N ratio on growth and 2,3-BD production were explored. Then, optimized media were utilised in batch bioreactor processes to assess the effect of dissolved oxygen (DO) control strategies on 2,3-BD and acetoin production, by monitoring also key-enzyme activity involved in 2,3-BD metabolic pathway. Finally, optimized culture conditions were applied in a fed-batch process targeting enhanced 2,3-BD production.

## 3.2 Materials and Methods

### 3.2.1 Microorganisms

Five non-pathogenic, wild-type, bacterial strains from the Food and Nutritional Sciences culture collection, University of Reading, were used in this study, namely *Bacillus subtilis* GD2 (FSBC 151), *Bacillus subtilis* GD5 (FSBC 322), *Bacillus licheniformis* FSBC 320, *Serratia plymuthica* FSBC 401, and *Lactococcus lactis* FSBC 64. Those bacterial strains are equivalent to *Bacillus subtilis* NCIMB 10144, *Bacillus subtilis* ATCC® 21332, *Bacillus licheniformis* ATCC® 39307, *Serratia plymuthica* NCIMB 4612*, and Lactococcus lactis* ATCC® 13675, respectively. All the strains were classified according to the Advisory Committee on Dangerous Pathogens (ACDP) or the

U.S. Department of Health and Human Services, as Hazard Group 1 or Risk Group 1. Those strains are non-pathogenic strains, which are unlikely to cause disease in a healthy individual. *Bacillus* species and *S. plymuthica* were maintained on nutrient agar (NA) containing the following compounds: 2 g/L yeast extract, 1 g/L beef extract, 5 g/L peptone, 5 g/L sodium chloride, and 15 g/L bacterial agar. *L. lactis* was grown on M17 agar. All bacterial cultures were incubated at 30 ℃ for 18–24 h prior to inoculation.

### 3.2.2 Pre-culture and production culture media.

The pre-culture medium for *Bacillus* species and *S. plymuthica* was MRS medium, consisting of 20 g/L glucose, 10 g/L peptone, 8 g/L meat extract, 4 g/L yeast extract, 2 g/L di-potassium hydrogen phosphate, 5 g/L sodium acetate, 2 g/L tri-ammonium citrate, 0.2 g/L magnesium sulphate, and 0.05 g/L manganese sulphate. The pre-culture medium for *L. lactis* was M17 broth, comprising 5 g/L lactose, 5 g/L tryptone, 5 g/L soya pep-tone, 5 g/L meat digest, 2.5 g/L yeast extract, 19 g/L disodium glycerophosphate, 0.5 g/L ascorbic acid, 0.25 g/L magnesium sulphate. A single loop from agar Petri dish was inoculated into 50 mL of sterilized (121 °C, 15 min) pre-culture medium in 100 mL Erlenmeyer flasks. The seed culture was grown with agitation (180 rpm, orbital shaker) at 30 °C for 24 h. The inoculum was centrifuged at 11,627 × g, 4 °C for 10 min. The supernatant was removed, the cells were resuspended with sterilised 0.85% (*w/v*) NaCl and employed as inoculum. An initial optical density ($OD_{600}$) of 0.2–0.3 was used for the cultivation.

Modified MRS (MMRS) medium was used as production medium, containing 20 g/L sucrose instead of glucose. Initial screening of bacterial strains was done in aerobic and anaerobic conditions. Aerobic conditions were investigated in 250 mL

Erlenmeyer flasks with a working volume of 100 mL of MMRS medium, incubated in a shaking incubator with agitation speed of 180 rpm, at 30 ℃ for 48 h. The pH remained uncontrolled during the fermentation period.

In the case of anaerobic conditions, 250 mL Duran bottles with a working volume of 100 mL culture media were used. Duran bottles were placed in an orbital shaker with agitation speed of 80 rpm, at 30 ℃ for 48 h. The pH remained uncontrolled during the fermentation period.

### 3.2.3 Effect of different nitrogen sources on 2,3-BD production

Low-cost nitrogen sources, including corn steep liquor (CSL) (Sigma-Aldrich, Gillingham, UK) and soybean meal (SBM) (Target feed Ltd., Whitchurch, UK), were employed individually at 20 g/L to replace the original nitrogen sources in the MMRS medium. Ammonium sulphate (AMS) (10 g/L), representing an inorganic source of nitrogen, with and without vitamin supplementation, was also used for comparison reasons. The vitamin supplementation solution was prepared based on the vitamin composition of yeast extract [28], and was based on the amount of yeast extract in MRS medium, which contained 0.14 mg/L thiamine, 4.0 mg/L inositol, 0.07 mg/L panthothenic acid, 0.02 mg/L vitamin B6, 0.09 mg/L riboflavin, and 0.41 mg/L niacin. Fermentations were performed in 250 mL Erlenmeyer flasks, containing 100 mL of sterilised medium (121 ℃/ 15 min), at 30 ℃, 180 rpm.

### 3.2.4 Effect of carbon to nitrogen (C/N) molar ratio on 2,3-BD production

Various levels of initial C/N molar ratios were evaluated to establish a suitable ratio for bacterial growth and 2,3-BD production. In all experiments, sucrose concentration was constant at 20 g/L, while the concentration of CSL varied (from 50, 20, 10 to 5 g/L) corresponding to C/N ratios of 6, 11, 17, and 22, respectively. The contribution of other carbon- or nitrogen ingredients in MMRS was also taken into account when calculating the C/N molar ratio. The fermentations were conducted in 250 mL Erlenmeyer flasks, containing 100 mL of sterilised medium (121 ℃/ 15 min), at 30 ℃, 180 rpm.

### 3.2.5 Effect of dissolved oxygen control strategies in growth and 2,3-BD in 2-L bioreactor

Different DO controlling strategies were assessed during batch fermentations in a 2-L benchtop bioreactor (Biostat® B, Sartorius, Germany), in which sucrose (20 g/L) and CSL (20 g/L) were employed as carbon and nitrogen sources, respectively, at 30 ℃, pH 6, and aeration of 1.0 vvm. The DO controlling regimes included: (i) DO at 5% achieved through cascade agitation, (ii) DO at 10%, achieved through cascade agitation, (iii) uncontrolled DO at fixed stirring rate of 180 rpm, and (iv) a two-stage DO control, starting with DO at 5% with cascade agitation from 0–15 h, followed by anaerobic conditions (no oxygen input into the bioreactor). In the cases of controlling DO at 5% and 10% with cascade agitation, aeration was supplied to the bioreactor at flow rate of 1.0 vvm, while 5% and 10%DO was automatically adjusting by the system via agitation rate. DO and pH were measured using polarographic and potentiometric sensors,

respectively (Hamilton, Switzerland). 10% (*w/v*) Antifoam B (Sigma-Aldrich, Gillingham, UK) was fed into the bioreactor as needed. Samples were collected at various time intervals for measuring cell growth, sugar, and metabolites.

### 3.2.6 Fed batch cultivation for 2,3-BD production in 2-L bioreactor

The most effective DO control strategy from the previous experiment was selected for fed-batch fermentation in a 2 L benchtop bioreactor (Biostat® B, Sartorius, Germany), with an initial working volume of 1.2 L, in MMRS medium using sucrose (20 g/L) and CSL (20 g/L) as carbon and nitrogen sources, respectively. Fed-batch was conducted at 30 ℃, pH 6.0 and aeration of 1.0 vvm. Concentrated sucrose solution (500 g/L) was fed when residual sugar concentration dropped to 10 g/L. Samples were collected at various time intervals for the determination of cell growth, sugar, and metabolites.

### 3.2.7 Determination of Acetoin reductase/Butanediol dehydrogenase (AR/BDH) and NADH oxidase (NOX) Activities

3.2.7.1 Cell preparation

Bacterial cells were harvested from the fermentation broth by centrifugation at 9418 × *g* for 10 min at 4 ℃ and washed twice with 50 mM potassium phosphate buffer (pH 6.5). Then, the cell pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.5) containing a protease inhibitor cocktail (Sigma-Aldrich, Gillingham, UK). Then, the cell suspension was introduced to a Vibra cell™ ultra-sonicator (Sonics, Newtown, CT, USA) with 4 cycles of pulse for 30 s, intermittent for 5 s, on ice. Cell

debris was removed by centrifugation at 9418 × *g*, 4 ℃ for 10 min and a crude protein solution was obtained for enzyme assays.

3.2.7.2 Acetoin reductase and Butanediol dehydrogenase (AR/BDH) activities

The AR/ BDH activity was determined spectrophotometrically by monitoring the change of absorbance at 340 nm and 30 ℃ corresponding to the oxidation of NADH and reduction of $NAD^+$ [29-31]. The enzyme activity determination was performed in accordance with previously reported methods, with some modifications [17, 30, 32]. Briefly, the AR activity was determined in a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 25 mM acetoin, and 0.25 mM NADH. The BDH activity was determined using 50 mM glycine-NaOH buffer (pH 8.5) containing 25 mM 2,3-butanediol, and 0.25 mM $NAD^+$. In all assays, the reaction was initiated by the addition of the substrate after adding crude enzyme. One unit of activity (U) was defined as the amount of crude enzyme that reduced (for AR) or generated (for BDH) 1 μmol of NADH per minute. A correction was made for the spontaneous degradation of NADH. The protein concentration was measured using the Bradford protocol with bovine serum albumin as a standard [33]. The specific activity of AR/BDH was expressed as enzyme unit (U) per enzyme protein (mg).

3.2.7.3 NADH oxidase (NOX) activity

The NOX activity was determined using the assay kit (Fluorometric) ab273345 from Abcam (Cambridge, UK) according to the manufacturer's instructions. NOX activity couples the oxidation of NADH by NOX and the reduction of a colorless probe

to a brightly colored fluorescent product. The generated fluorescence corresponded to the NOX activity in the samples. The fluorescence was detected using an Infinite® 200 PRO microplate reader (Tecan, Männedorf, Switzerland). The reaction mechanism relies on the oxidation of lactate to generate NADH, which is in turn used by the NADH oxidase, catalyzing the reduction of the probe into a fluorescent molecule, which an emission can be read at 587 nm. One mole of oxidized lactate generates one mole of NADH from β-$NAD^+$. The NADH is subsequently used in the reduction of the substrate by NOX. One unit of NOX activity (U) can be defined as the amount of enzyme that catalyses the reduction of 1 μmol of substrate per minute under the assay conditions and the specific enzyme activity was expressed as μU per mg of protein.

### 3.2.8 Analytical Methods

Cell growth was determined by optical density at 600 nm ($OD_{600}$) using a spectrophotometer (Life Technologies Ltd., Paisley, UK). Dry cell weight (DCW) was estimated using a standard calibration curve established between optical density and dry cell weight data. Collected samples were centrifuged at 11,627 × *g* for 10 min, and the cell-free supernatant was hydrolysed with 10% (*v/v*) sulphuric acid at 100 ℃ for 30 min and filtered (0.2 μm, Whatman, SLS, Nottingham, UK)) before HPLC analysis [34]. The concentration of glucose, fructose, 2,3-BD, acetoin, succinic acid, lactic acid, acetic acid, and ethanol were determined by an Agilent 1260 high-performance liquid chromatography (HPLC) system (Agilent, Didcot, UK) with an aminex HPX-87H (300 mm x 7.8 mm, Biorad, Watford, UK) column coupled to refractive index (RI) and DAD detectors. The mobile phase consisted of 5 mM $H_2SO_4$ with a flow rate of 0.6 mL/min. The column temperature was controlled at 65 ℃. The concentrations of sugar and

metabolites were quantified using external calibration curves while the dilution of samples mixed with sulphuric acid was taken into consideration.

### 3.2.9 Identification of 2,3-BD and acetoin stereoisomeric forms by GC-FID

Supernatants were extracted with ethyl acetate using Fast prep 24™ homogenizer (MP Biomedicals, Eschwege, Germany), and the solvent was evaporated by nitrogen gas. The concentration of acetoin stereoisomers (R- and S- forms) and (R,R-), (S,S-), meso-2,3-BD were analysed in a GC system Agilent 7890B (Agilent, Denver, CO, USA). The system consisted of an FID detector and a chiral column (CP-Chirasil-Dex DB 25 m length, and 0.25 mm inner diameter, Agilent Technologies, Denver, CO, USA). The operating conditions were developed based on the method of Caligiani et al. [35], and were as follows: helium was used as a carrier gas at a flow rate of 1.2 mL/min; the injection port temperature and the detector temperature were set at 250 ℃; and the column temperature was maintained at 60 ℃ for 1 min, then raised to 120 ℃ at a rate of 10 ℃/min followed by a ramp of 40 ℃/min to 200 ℃. The concentrations of acetoin and 2,3-BD in the supernatant were determined using standard curves of known solutions of commercial samples (Sigma-Aldrich, Gillingham, UK).

## 3.3 Results and Discussion

### 3.3.1 Screening of bacterial strains for 2,3-BD production in sucrose-based media

The five bacterial strains were evaluated for their ability to convert sucrose into 2,3-BD under aerobic and anaerobic conditions, and the results are presented in Table 3.1. In terms of growth, aerobic conditions seemed to be favourable for most strains.

*B. subtilis* GD5 presented the highest growth, achieving an $OD_{600}$ of 5.34 (3.11 g/L DCW) at 24 h. Bacillus species are known as facultative anaerobic bacteria, and they can grow in either anaerobic or aerobic conditions. However, under the presence of oxygen, these species grow better compared to conditions where oxygen is limited or absent.

Figure 3.1 show the bacterial growth and pH profiles during 2,3-BD production with uncontrolled pH under aerobic and anaerobic fermentation by five bacterial strains tested. In the case of *B. subtilis* GD5 (the most effective strain for 2,3-BD production), the strain grew better under aerobic fermentation ($OD_{600}$ ~ 5) compared to anaerobic conditions ($OD_{600}$ ~1). For the pH trend under aerobic fermentation of this strain, a gradual decrease in pH (from 6.7 to 5.8) was observed during growth phase (0 h to 24 h), then the pH increased from 5.8 to 7.7 during stationary phase of growth (after 24 h to 48 h). This could indicate that the strain produced neutral compounds (2,3-BD and acetoin) to prevent excessive acidification of its environment. In general, *Serratia* sp. produces acid products during growth phase, leading to a decrease in the pH of fermentation medium. Thus, the strain uses defensive abilities to prevent excessive acidification of internal environment by switching from acidogenesis to solventogenesis.

In the case of anaerobic fermentation (Figure 3.1c), lower growth was obtained for most of bacterial strains tested. However, *S. plymuthica* exhibited best growth under this condition ($OD_{600}$ ~2 to 3). In terms of pH trend of this strain (Figure 3.1d), the pH decreased from 6.7 to 6.0 during growth phase, then slightly increased to 6.3 during stationary phase of growth.

**Table 3.1** Cell growth, pH and 2,3-BD production in sucrose-based media by various bacterial strains under anaerobic (Duran bottles) and aerobic (shake-flask) conditions.

| Growth conditions | Bacterial strain | Time (h) | Substrate consumed (g/L) | $OD_{600\ nm}$ (Abs) | pH | 2,3-BD (g/L) | 2,3-BD production yield (g/g) | Acetoin (g/L) | Lactic acid (g/L) |
|---|---|---|---|---|---|---|---|---|---|
| Aerobic | *B. subtilis* GD2 | 24 | 1.45 ± 0.03 | 1.27 ± 0.19 | 6.31 ± 0.05 | 0.87 ± 0.13 | 0.6±0.01 | ND | ND |
| | | 48 | 10.23 ± 0.03 | 2.41 ± 0.07 | 5.94 ± 0.03 | 3.78 ± 0.00 | 0.37±0.01 | ND | ND |
| | *B. subtilis* GD5 | 24 | 16.10 ± 0.01 | 5.34 ± 0.15 | 5.82 ± 0.07 | 7.28 ± 0.25 | 0.45±0.02 | 0.17 ± 0.05 | 0.96 ± 0.00 |
| | | 48 | 18.18 ± 0.01 | 5.28 ± 0.26 | 7.54 ± 0.05 | 3.46 ± 0.01 | 0.19±0.01 | 3.21 ± 0.01 | ND |
| | *B. licheniformis* | 24 | 17.55 ± 0.00 | 5.18 ± 0.30 | 6.40 ± 0.06 | 6.27 ± 0.00 | 0.36±0.00 | ND | 0.86 ± 0.00 |
| | | 48 | 18.19 ± 0.00 | 3.53 ± 0.37 | 8.04 ± 0.11 | 1.77 ± 0.01 | 0.10±0.00 | 5.32 ± 0.01 | ND |
| | *S. plymuthica* | 24 | 18.50 ± 0.00 | 4.04 ± 0.03 | 5.87 ± 0.01 | 5.88 ± 0.03 | 0.32±0.01 | 1.74 ± 0.04 | 0.15 ± 0.00 |
| | | 48 | 18.73 ± 0.00 | 5.78 ± 0.10 | 7.63 ± 0.03 | 2.63 ± 0.01 | 0.14±0.00 | 4.17 ± 0.02 | ND |
| | *L. lactis* | 24 | 12.92 ± 0.14 | 4.16 ± 0.08 | 4.90 ± 0.06 | 0.46 ± 0.00 | 0.04±0.00 | 0.48 ± 0.00 | 3.47 ± 0.00 |
| | | 48 | 14.68 ± 0.14 | 4.50 ± 0.38 | 4.46 ± 0.03 | ND | ND | 1.20 ± 0.01 | 4.46 ± 0.01 |
| Anaerobic | *B. subtilis* GD2 | 24 | 2.63 ± 0.01 | 0.68 ± 0.04 | 6.11 ± 0.01 | 0.21 ± 0.03 | 0.08±0.01 | ND | ND |
| | | 48 | 3.17 ± 0.28 | 0.84 ± 0.08 | 6.33 ± 0.03 | 0.19 ± 0.00 | 0.06±0.00 | ND | ND |
| | *B. subtilis* GD5 | 24 | 3.44 ± 0.09 | 1.16 ± 0.04 | 5.94 ± 0.02 | 1.73 ± 0.01 | 0.50±0.01 | ND | 0.28 ± 0.00 |
| | | 48 | 7.15 ± 0.27 | 1.16 ± 0.07 | 5.86 ± 0.04 | 3.02 ± 0.02 | 0.42±0.02 | ND | 1.30 ± 0.02 |
| | *B. licheniformis* | 24 | 3.30 ± 0.25 | 0.91 ± 0.13 | 5.73 ± 0.03 | 1.32 ± 0.01 | 0.40±0.03 | ND | 0.66 ± 0.03 |
| | | 48 | 5.68 ± 0.88 | 0.71 ± 0.30 | 5.64 ± 0.09 | 2.15 ± 0.04 | 0.38±0.07 | ND | 1.66 ± 0.07 |
| | *S. plymuthica* | 24 | 17.56 ± 0.18 | 2.61 ± 0.08 | 6.00 ± 0.04 | 7.08 ± 0.12 | 0.40±0.01 | ND | 1.47 ± 0.04 |
| | | 48 | 17.61 ± 0.23 | 2.08 ± 0.27 | 6.18 ± 0.19 | 6.96 ± 0.02 | 0.40±0.01 | ND | 1.29 ± 0.01 |
| | *L. lactis* | 24 | 7.97 ± 0.51 | 0.96 ± 0.01 | 6.21 ± 0.07 | 0.16 ± 0.01 | 0.02±0.00 | ND | 1.82 ± 0.11 |
| | | 48 | 13.61 ± 0.04 | 2.11 ± 0.27 | 5.17 ± 0.17 | 1.56 ± 0.02 | 0.11±0.00 | ND | 6.57 ± 0.10 |

Culture conditions: Temperature 30 ºC, agitation rate 180 rpm, initial pH 6.8–7.0, initial sucrose concentration 20 g/L. ND: Not detected. Data shown are means ± SD of duplicate independent experiments.

As observed in Table 3.1, under anaerobic conditions, *S. plymuthica* exhibited the highest growth, reaching an $OD_{600}$ of 2.61 (1.95 g/L DCW) at 24 h. *Serratia* spp., like other members of Enterobacteriaceae, can grow well in synthetic media under both anaerobic and aerobic conditions. Moreover, it can be seen in Table 3.1 that high amount of substrate (sucrose) (~17 -18 g/L) was consumed by *S. plymuthica* under both aerobic and anaerobic fermentation. This could be implied that *S. plymuthica* utilised high amount of sugar for producing high 2,3-BD concentrations (5.88 and 7.08 g/L) under aerobic and anaerobic fermentation, respectively. On the other hand, other bacterial strains consumed lower amounts of sugars, thus lower concentrations of 2,3-BD were obtained under anaerobic condition compared to aerobic fermentation. As observed in Table 3.1, most bacterial strains produced higher concentrations of 2,3-BD under aerobic compared to anaerobic conditions. Interestingly, *B. subtilis* GD5 produced the highest 2,3-BD concentration (7.28 g/L) under aerobic conditions, whereas *S. plymuthica* produced relatively high 2,3-BD (> 5 g/L) under both conditions. In the case of *B. subtilis* GD5, *B. licheniformis* and *S. plymuthica* strains all of which grew very well under aerobic conditions, maximum concentrations of 2,3-BD were obtained during the growth phase (24 h), indicating that BD is a growth-associated metabolite. Subsequently, 2,3-BD declined, indicating the reversible conversion of 2,3-BD to acetoin under conditions of carbon depletion. 2,3-BD production is favoured under slightly acidic conditions (pH 5.0–6.5) and is a mechanism for bacteria to limit the decrease of external pH that is caused by the formation of organic acids such as acetic, lactic and succinic acid, from pyruvate. The reverse conversion of 2,3-BD to acetoin occurred in the stationary phase (24 h–48 h), when the pH of the medium increased to higher than 7. Specifically, under aerobic fermentation, 2,3-BD were high produced by most strains at 24 h, then 2,3-BD converted into acetoin at 48 h, concomitant with

increase in pH values. This might be that the reversible conversion from 2,3-BD back into acetoin was taken place after sugar depletion. Then, other carbon sources in culture medium such as sodium acetate could be used to maintain cell growth, thus contributing to a gradual increase in pH value to higher than 7 [36]. As the pH preference for reversible conversion of 2,3-BD to acetoin is around 8-9 [37], thus this would activate BDH that catalysed the conversion from 2,3-BD back to acetoin concentrations (whilst decreased of 2,3-BD) at 48 h of most strains under aerobic fermentation. In addition, the problem of reversely conversion from 2,3-BD to acetoin after sugar depletion could be overcome by supplying sufficient sugar to cover NADH requirement for cell metabolism. On the other hand, acetoin was not produced by any strain under anaerobic fermentation as sugar was sufficient to cover NADH requirement for cell metabolism. Therefore, the pathway for conversion from 2,3-BD back to acetoin was not active. This could also be observed that the pH trend was relatively stable and did not increase throughout anaerobic fermentation. *L. lactis* produced lactic acid as a major metabolite under both conditions and led to a decrease in pH throughout the fermentation under both conditions. Moreover, acetoin was not produced by any strain under anaerobic conditions.

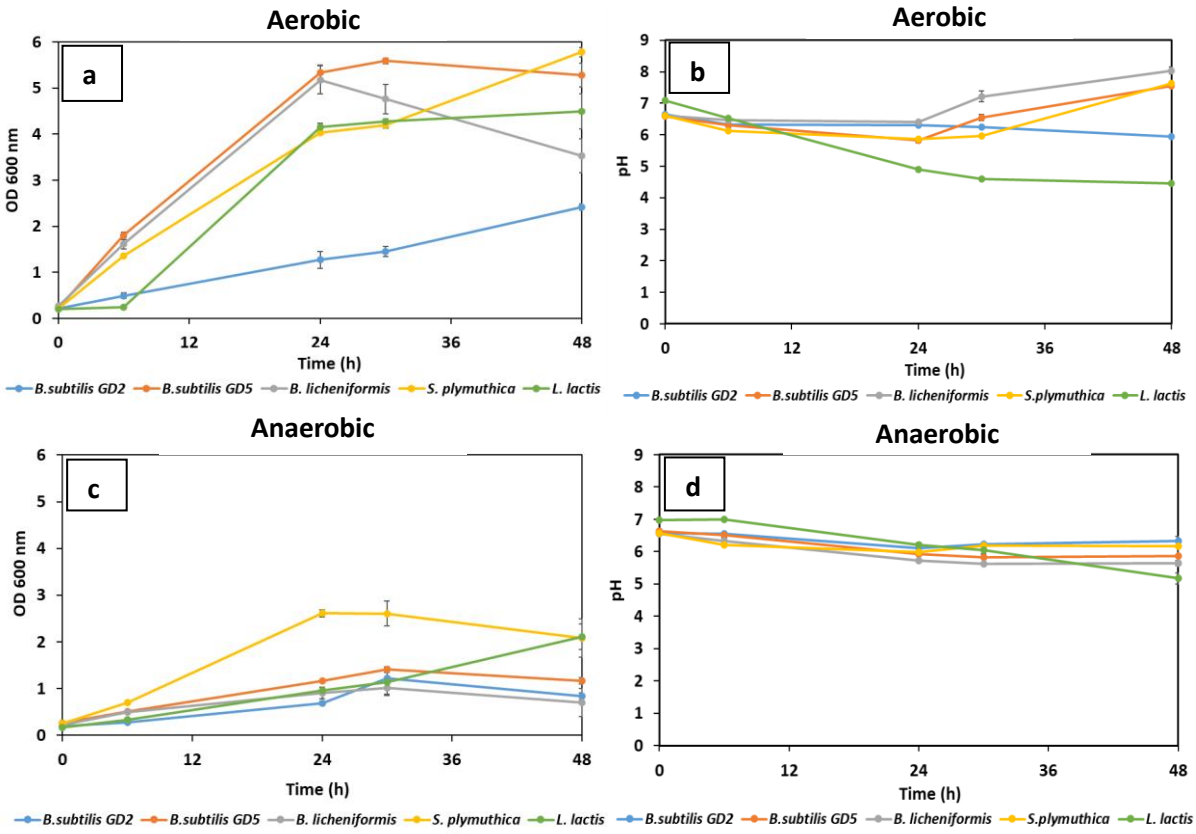


**Figure 3.1** Time course of cell growth and pH profiles during 2,3-BD production with uncontrolled pH, under aerobic (a, b) and anaerobic (c, d) fermentation by five bacterial strains. Data shown are means ± SD of duplicate independent experiments.

With regard to 2,3-BD stereoisomers, *B. subtilis* GD5 produced pure (R,R)-2,3-BD after 24 h of fermentation. *B. licheniformis* also exhibited notable production of pure (R,R) 2,3-BD (5.70 g/L), whereas *S. plymuthica* produced 95% meso-2,3-BD and 5% (S,S)-2,3-BD. Moreover, it was observed that *B. subtilis* GD5 and *S. plymuthica* produced R and S-acetoin, *L. lactis* generated only R-acetoin, whereas no detection of acetoin in *B. licheniformis* at 24 h was noted. Considering the above results and the criterion of highest 2,3 BD concentrations and bacterial growth from all screened bacterial strains, *B. subtilis* GD5 and aerobic fermentation were selected as the most suitable strain and conditions to be used in further experiments.

### 3.3.2 Effect of alternative low-cost nitrogen sources on cell growth and 2,3-BD production

Low-cost nitrogen sources, including corn steep liquor (CSL), soybean meal (SBM), and ammonium sulphate (AMS) with and without vitamin addition, were employed individually to replace more costly nitrogen sources, such as peptone, yeast extract and meat extract which are found in MRS medium. As shown in Table 3.2, relatively high bacterial growth was observed when organic sources of nitrogen were employed compared to inorganic nitrogen sources (i.e., ammonium sulphate). CSL was the most suitable nitrogen source, as it supported adequate cell growth and 2,3-BD production from *B. subtilis* GD5, reaching 3.52 g/L at 24 h. It was noted that less acetoin was produced when CSL was used as nitrogen source compared to the control (unmodified MRS medium). The highest 2,3-BD production yield of 0.39 g/g was achieved after 24 h of fermentation when CSL was employed as nitrogen source. CSL is a major by-product of the wet-milling process of corn starch production [38]. It is an inexpensive nutrient-rich source and has been employed as a supplement in various microbial fermentations [39, 40]. Apart from amino acids, vitamins, and minerals, 2.5% (*w/w*) invert sugars are also present in CSL composition [41]. Yang et al. [42] also reported that CSL exhibited a positive effect on 2,3-BD production over other N-sources, such as soy peptone and beef extract. They also demonstrated that organic nitrogen sources such as CSL and yeast extract not only stimulate the conversion of acetoin to 2,3-BD, but also promote cell growth in *B. subtilis*. Soybean meal has been reported as a suitable nitrogen source to replace yeast extract for 2,3-BD and acetoin production [9]. However, it was not comparable to the results of this study; CSL seemed to be more easily assimilated than SBM by *B. subtilis* GD5. In the case of inorganic

nitrogen, the results showed that even with vitamin supplementation, 2,3 BD production was not improved. Thus, corn steep liquor was selected as a promising alternative nitrogen source for further studies.

**Table 3.2** Effect of different nitrogen sources on biomass production and 2,3-BD production by *B. subtilis* at 24 h cultivated under aerobic conditions in MMRS medium.

| N-source [a] | DCW (g/L) | pH | 2,3-BD (g/L) | Acetoin (g/L) | Lactic acid (g/L) | 2,3-BD production yield (g/g) |
|---|---|---|---|---|---|---|
| Control | 3.14 ± 0.09 | 6.14 ± 0.02 | 3.58 ± 0.93 | 2.24 ± 0.69 | 0.23 ± 0.03 | 0.27 ± 0.08 |
| CSL | 3.52± 0.04 | 6.97 ± 0.05 | 5.42 ± 0.16 | 1.32 ± 0.25 | 0.51 ± 0.03 | 0.39 ± 0.01 |
| SBM | 2.70 ± 0.34 | 6.78 ± 0.02 | 4.38 ± 0.10 | 0.59 ± 0.00 | 0.37 ± 0.03 | 0.34 ± 0.02 |
| AMS 20 g/L | 1.35 ± 0.03 | 7.08 ± 0.01 | 0.15 ± 0.01 | 1.02 ± 0.08 | ND | 0.03 ± 0.01 |
| AMS 10 g/L | 1.50 ± 0.07 | 7.13 ± 0.07 | 0.20 ± 0.01 | 0.84 ± 0.04 | ND | 0.04 ± 0.01 |
| AMS 10 g/L + Vitamins | 1.56 ± 0.04 | 7.11 ± 0.00 | 0.14 ± 0.02 | 0.85 ± 0.02 | ND | 0.03 ± 0.01 |

Culture conditions: T = 30◦C; initial pH = 7.0; agitation rate = 180 rpm.

[a] Control: unmodified MRS, CSL: Corn steep liquor, SBM: Soybean meal, AMS: Ammonium sulphate.

ND: Not detected. Data shown are means ± SD of duplicate independent fermentations.

### 3.3.3 Effect of different carbon-to-nitrogen (C/N) molar ratios on cell growth and 2,3-BD production

Microbial metabolism requires carbon for the provision of energy and carbon and nitrogen for cell synthesis. In order to investigate the impact of the C/N ratio on 2,3-

BD production, media with varying concentrations of CSL were used, whilst other ingredients, as well as sucrose concentration in the culture medium, were fixed.

As seen in Table 3.3, the lowest dry cell mass (2.2 g/L) and lowest specific growth rate (0.111 $h^{-1}$) were obtained in cultures with a C/N ratio of 6. Moreover, trials with C/N ratios of 6 and 22 provided the lowest 2,3-BD production yield and productivity. This might reflect an imbalanced C/N ratio in the media for *B. subtilis* GD5, either an overabundant (C/N 6) or inadequate (C/N 22) provision of nitrogen. Maximum biomass (3.5 g/L), specific growth rate (0.125 $h^{-1}$), 2,3-BD production yield (0.35 g/g) and productivity (0.22 g/L/h) were obtained at 24 h in media with a C/N ratio of 11. Considering those parameters, a C/N ratio of 11, provided an excellent balance between carbon and nitrogen provision during batch fermentation, and thus was therefore selected for further study. It has been reported that the C/N ratio has a great impact on cell growth and metabolite production in various microorganisms [43, 44]. Thus, it should be finely adjusted in fermentation media to enhance microbial growth and promote the biosynthesis of desired products. Yang et al. [42] reported that the initial concentration of CSL affected 2,3-BD and acetoin production and also the ratio of 2,3-BD to acetoin. They claimed that with an increase in CSL concentration, cell growth of *B. subtilis* was increased, acetoin reductase (AR) was stimulated, 2,3-BD concentrations improved by 78.6%, acetoin concentration decreased by 61.9%, and the ratio of 2,3-BD to acetoin enhanced by 3.69-fold.

**Table 3.3** Effect of different carbon to nitrogen (C/N) molar ratios on bacterial growth and 2,3- BD production by *B. subtilis* at 24 h cultivated under aerobic conditions in MMRS medium.

| C/N ratio | DCW (g/L) | Specific growth rate ($h^{-1}$) | 2,3-BD (g/L) | 2,3-BD production yield (g/g) | 2,3-BD productivity (g/L/h) |
|---|---|---|---|---|---|
| 6 | 2.24 ± 0.15 | 0.111± 0.01 | 3.09 ± 0.04 | 0.27 ± 0.02 | 0.13 ± 0.00 |
| 11 | 3.53 ± 0.04 | 0.125 ± 0.02 | 5.39 ± 0.01 | 0.35 ± 0.02 | 0.22 ± 0.02 |
| 17 | 3.39 ± 0.02 | 0.120 ± 0.01 | 4.65 ± 0.01 | 0.33 ± 0.01 | 0.19 ± 0.01 |
| 22 | 2.98 ± 0.01 | 0.115 ± 0.01 | 3.25 ± 0.15 | 0.27 ± 0.03 | 0.14 ± 0.01 |

Data shown are means ± SD of duplicate independent experiments.

The research work using pure sucrose as substrate has rarely been conducted for 2,3-BD production compared to the use of pure glucose. Moreover, commercial sucrose is cheaper than a commercial glucose. As sucrose can be derived from a variety of cost-effective feedstocks such as sugarcane molasses, sugar beet molasses, sweet sorghum juice or even fruit waste; thus, the knowledge from this study can be applied for 2,3-BD production in other renewable and low-cost sucrose-based feedstocks. In addition, for the investigation of alternative low-cost nitrogen sources, corn steep liquor is the most effective nitrogen source substitution of high-cost nitrogen sources (peptone, yeast extract, and meat extract) for 2,3-BD production. Moreover, the most effective C/N ratio of culture medium for 2,3-BD production by this strain is 11. Thus, an optimized culture medium containing lower-cost ingredients compared to the original MRS medium could lowering the production cost. A comparison of composition of original MRS medium and optimized culture medium are shown in Table 3.4.

**Table 3.4** Composition of the original MRS medium and optimized culture medium used in this study

| Composition of original MRS medium | Composition of optimized culture medium for *B. subtilis* |
|---|---|
| 20 g/L glucose, 10 g/L peptone, 8 g/L meat extract, 4 g/L yeast extract, 2 g/L di-potassium hydrogen phosphate, 5 g/L sodium acetate, 2 g/L tri-ammonium citrate, 0.2 g/L magnesium sulphate, and 0.05 g/L manganese sulphate. | 20 g/L sucrose, 20 g/L corn steep liquor, 2 g/L di-potassium hydrogen phosphate, 5 g/L sodium acetate, 2 g/L tri-ammonium, 0.2 g/L magnesium sulphate, and 0.05 g/L manganese sulphate. |

### 3.3.4 Effect of dissolved oxygen control strategy on cell growth and 2,3-BD production in 2-L batch bioreactor

The impact of four DO control strategies on *B. subtilis* GD5 cell growth and 2,3-BD production was investigated in a 2-L bioreactor operated under batch mode (Figure 3.2). Unless stated otherwise, the fermentations were carried out at 30 ℃, pH 6.0 and an aeration rate of 1.0 vvm throughout the cultivation. Higher growth levels were obtained when DO concentrations were maintained at 5 and 10% with cascade agitation throughout the fermentation, compared to fermentations with uncontrolled DO. This indicated that higher stirrer speed generated higher oxygen saturation and provided better mixing of bacteria and medium in the bioreactor, stimulating bacterial growth. It is noted that *B. subtilis* entered the exponential growth phase after 6 h and maximum DCW (5–6 g/L) was reached within 24 h in both DO control levels. In uncontrolled DO

fermentations, cell growth increased gradually up until 30 h. In the case of the two-stage DO control strategy, higher growth (maximum DCW 2.1 g/L) was observed during the first stage with cascade agitation at 5% until 15 h. However, during anaerobic conditions (after 15 h), bacterial growth decreased slightly, possibly due to the absence of dissolved oxygen in the medium. In terms of maximum specific growth rate ($\mu_{max}$), the highest $\mu_{max}$ of 0.37 $h^{-1}$ was achieved in the runs with controlling DO at 10% followed by a $\mu_{max}$ of 0.23 $h^{-1}$ when controlling DO at 5%, whereas $\mu_{max}$ values of 0.14 and 0.16 $h^{-1}$ were obtained in cultures with uncontrolled %DO and the two-stage DO control regimes, respectively.

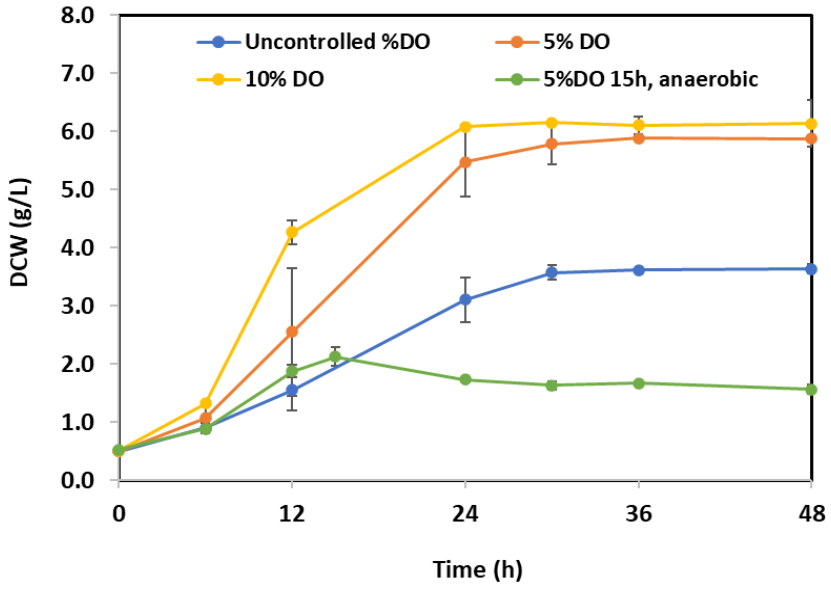


**Figure 3.2** Cell growth of *B. subtilis* at 30 °C, pH 6 in a 2 L benchtop bioreactor under different DO control strategies: controlling DO with cascade agitation at 5%, controlling DO with cascade agitation at 10%, uncontrolled DO at fixed stirrer speed at 180 rpm throughout fermentation, controlling DO with cascade agitation at 5% during the first 15 h, followed by anaerobic conditions.

As shown in Figure 3.3, the cells consumed sucrose faster under high agitation and high DO conditions (Figure 3.3a and 3.3b) compared to the fermentations with uncontrol DO (Figure 3.3c) and the two-stage DO control strategy (Figure 3.3d).

Additionally, the effect of different DO control strategies on 2,3-BD and acetoin production was evaluated. Controlling the DO at 5 or 10% contributed to the high oxygen concentration in the bioreactor, and resulted in the accumulation of acetoin (approximately 5 g/L), whereas very low levels of 2,3-BD (≤ 1 g/L) were obtained. This finding is in agreement with the study of Qureshi et al. who reported that excessive aeration-agitation led to the production of acetoin at the expense of 2,3-BD [45]. Moreover, after sucrose was depleted, acetoin concentration decreased to nearly zero (Figure 3.3a and 3.3b). This phenomenon was in agreement with Xiao [46] who claimed that acetoin could be reutilised during the stationary phase after the depletion of other carbon sources, providing further evidence that microorganisms produce 2,3-BD and acetoin as carbon and energy storage [46].

On the other hand, uncontrolled DO at a fixed agitation speed of 180 rpm (Figure 3.3c), led to DO reduction at 0% within 5 h, and favoured 2,3-BD production, resulting in a maximum 5.88 g/L of 2,3-BD, with a yield of 0.35 g/g sucrose, and productivity of 0.20 g/L/h after 30 h of cultivation. The enzymes involved in the 2,3-BD production pathway (α-acetolactate synthase, α-acetolactate decarboxylase, acetoin reductase) are generally activated under micro-aerobic conditions, and can be inactivated by high oxygen supply under fully aerobic conditions [47]. To this end, it has been suggested that 2,3-BD production yield could be maximised by regulating a suitable oxygen supply in order to limit microbial respiration [48]. The levels of produced 2,3-BD and acetoin seem to be biochemically driven by oxygen requirement. As shown in Figure 3.3c, notable concentrations of 2,3-BD were obtained during growth phase (0–30 h),

indicating that 2,3-BD is a growth associated metabolite for this particular strain. The growth-associated product is indicated by that the product or metabolite (2,3-BD) is produced at the same period of time with cell growth or during log phase. It should be noted that 2,3-BD concentrations might not be related to biomass concentrations. Thereafter, 2,3-BD steadily declined and was reversibly converted to acetoin, providing further evidence on the reverse activity of acetoin reductase in the *B. subtilis* GD5 strain. Considering the metabolic pathways of acetoin and 2,3-BD production in bacteria cells, acetoin and 2,3-BD could be transformed into each other directly [8, 27, 49, 50]. Initially, the produced acetoin is converted into 2,3-BD by acetoin reductase (AR), then 2,3-BD is reversibly transformed to acetoin by butanediol dehydrogenase (BDH) [51]. In this reaction, one molecule of NADH is regenerated through the BDH activity to maintain bacterial growth [27]. Zhang [37] reported that the reverse activity of AR/BDH takes place when *B. subtilis* enters the late stationary phase. They showed that a pH around 6 was optimum for the reduction of acetoin into 2,3-BD. The reverse activity of acetoin reductase can be also explained by cell metabolism. When the carbon source is depleted, the NADH required for microbial metabolism is generated through the conversion of 2,3-BD to acetoin for maintaining a constant redox state [27, 52, 53]. This phenomenon has also been reported by Tian [27] who produced acetoin from sugar-based substrates using *B. subtilis*, but also for other 2,3-BD-producing strains such as *B. licheniformis* and *Serratia marcescens* [27, 52, 54].

In order to further verify the effect of dissolved oxygen on 2,3-BD and acetoin production, a two-stage DO control strategy was investigated (Figure 3.3d). The DO was controlled with a cascade agitation at 5% for 15 h, followed by anaerobic conditions. Under these conditions, the strain consumed less sucrose and at a slower rate, particularly after anaerobic conditions were applied (no oxygen input to the

bioreactor), compared to the other DO control strategies. Moreover, during the first 15 h, acetoin production was superior to 2,3-BD as more DO was present in the culture. In this phase, the NADH generated from the glycolytic pathway was mostly oxidised through the electron transport chain to regenerate $NAD^+$, thus the pathway of acetoin to 2,3-BD conversion was not active [27, 55]. On the other hand, when the cultivation mode switched to anaerobic conditions, the pathway for transformation from acetoin to 2,3-BD was active. As a result, 2,3-BD concentration reached a peak at 30 h (4.98 g/L), and then remained steady until the end of fermentation. Moreover, 2 g/L of lactic acid was produced, whereas acetoin decreased to zero at the end of cultivation. Besides acetoin and lactic acid, traces of succinic acid (less than 0.2 g/L) were also detected in the culture medium.

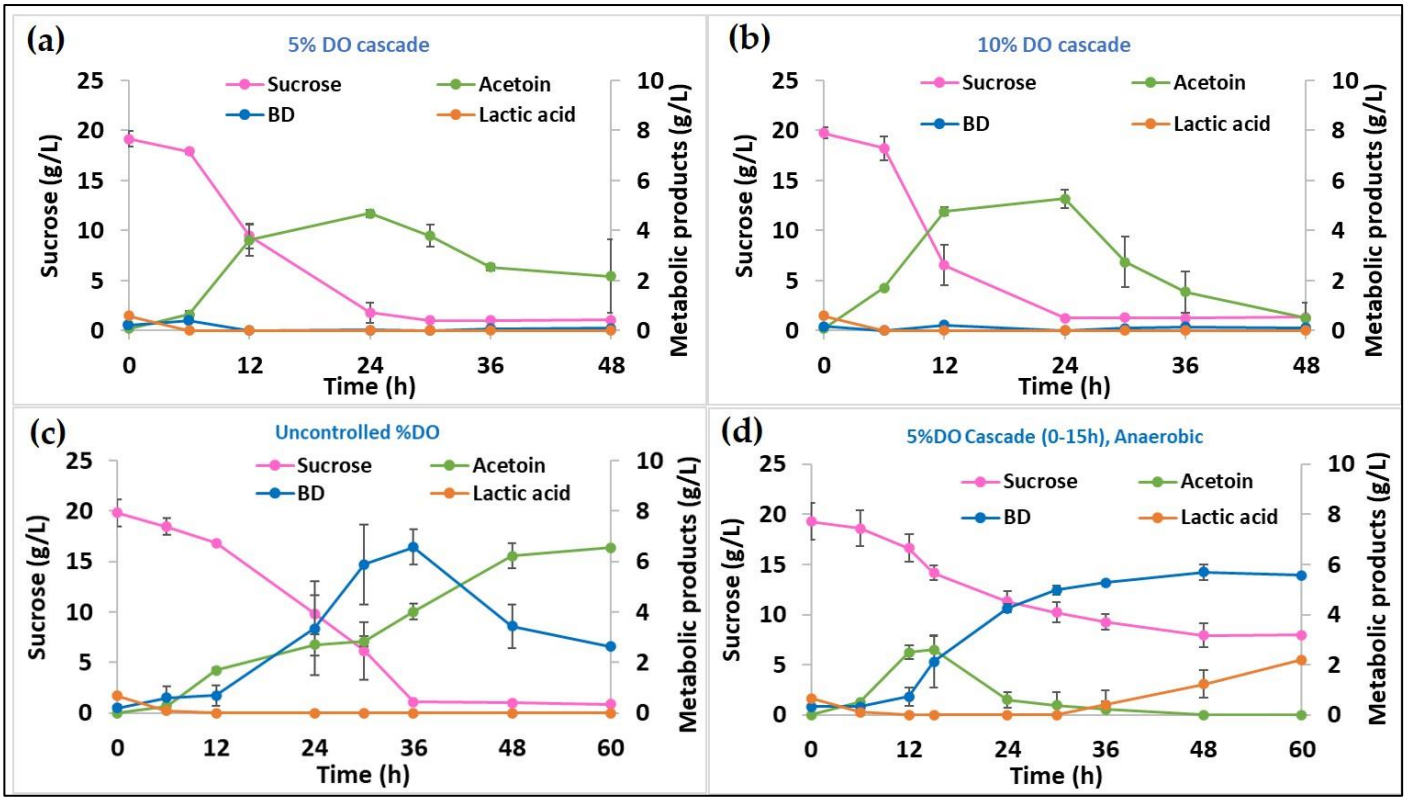


**Figure 3.3** Time courses of substrate utilisation and production of metabolic products by *B. subtilis* from sucrose-based medium in 2 L bioreactor at 30 °C pH 6, aeration rate 1.0 vvm under **(a)** controlling DO with cascade agitation at 5%, **(b)** controlling DO with

cascade agitation at 10%, **(c)** uncontrolled DO at fixed stirrer speed at 180 rpm throughout fermentation, **(d)** controlling DO with cascade agitation at 5% during the first 15 h, followed by anaerobic conditions.

### 3.3.5 Determination of 2,3-BD and acetoin stereoisomeric forms

To further understand the mechanism of AR/BDH enzymes involved in the metabolic pathway of 2,3-BD production, the isomers of the produced acetoin and 2,3-BD were identified. R-acetoin is produced under anaerobic conditions from acetolactate, whilst S-acetoin is the product of aerobic fermentations from diacetyl. The existence of various AR/BDHs in native 2,3-BD producers leads to different 2,3-BD isomers [56]. Interestingly, *B. subtilis* produced high optical purity of R- acetoin (100%) and (R,R)-2,3-BD (100%) throughout the cultivation under uncontrolled DO regime (Figure 3.4). This could imply that the limited presence of oxygen promoted the (R,R)-AR enzyme that catalyses the conversion of R-acetoin to (R,R)-2,3-BD during the exponential growth phase. On the contrary, during the late stationary phase and after sugar depletion, (R,R)-BDH seemed to be active and converted (R,R)-2,3-BD to R-acetoin. This is the first study that reports on optically pure (~100% purity) R-acetoin and (R,R)-2,3-BD isomers produced by the non-pathogenic wild-type strain *B. subtilis* DG5. This finding was consistent with the work of Fu et al. [57] who revealed that the wild-type *B. subtilis* 168 generated only (R,R)-2,3-butanediol (purity > 99%) under low oxygen conditions [57]. Most studies in the literature have reported that wild-type strains of *B. subtilis* produce a mixture of (R,R) and meso-2,3-BD with a ratio of 3:2 [1, 2, 58-60]. Optically active forms of 2,3-BD are industrially important as chiral groups for the production of high-value chemicals and pharmaceutical products [61].

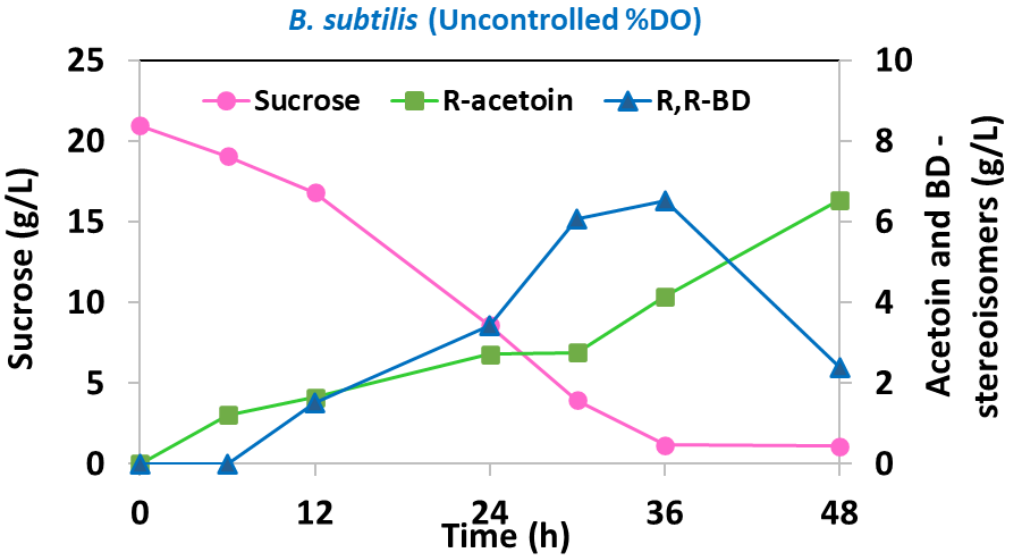


**Figure 3.4** Time courses of acetoin and 2,3-BD isomers produced by *B. subtilis* from sucrose-based medium in 2 L bioreactor with uncontrolled DO at 30 ℃, pH 6, aeration rate 1.0 vvm.

### 3.3.6 Investigation of metabolic pathways in *B. subtilis* FSBC 322 by enzyme activity assay

Acetoin reductase/2,3-butanediol dehydrogenase (AR/BDH) catalyses the interconversion between acetoin and 2,3-BD. More specifically, based on substrate and reaction used for the conversion, acetoin reductase (AR; reductive reaction) is responsible for conversion from acetoin into 2,3-BD, whereas 2,3-butanediol dehydrogenase (BDH; oxidative reaction) is responsible for conversion from 2,3-BD back into acetoin [17, 37].

As one pair of cofactors, NADH and $NAD^+$ play a vital role in more than 300 biochemical reactions including oxidation and reduction [62, 63], which help maintain a

constant redox state for microbial cell metabolism [64]. The 2,3-BD pathway participates in the regulation of $NAD^+$/NADH ratio in bacterial cells. As shown in Figure 3.5, acetoin can be converted into 2,3-BD by acetoin reductase (AR) with the concomitant oxidation of NADH to $NAD^+$. Thus, the biosynthesis of 2,3-BD is likely driven by the proportion of a rate-limiting factor, particularly AR activity, and/or NADH levels [24]. During the catalytic reaction of butanediol dehydrogenase (BDH), 2,3-BD is reversibly transformed to acetoin concomitantly with the reduction of $NAD^+$ to NADH. Consequently, $NAD^+$ is depleted, whilst NADH and acetoin are produced [55]. Thus, a driving force carried out by NADH oxidase (NOX) is required to regenerate $NAD^+$ from NADH to maintain a constant redox state in the bacterial cell. NOX reduces oxygen to produce either water or hydrogen peroxide [65].

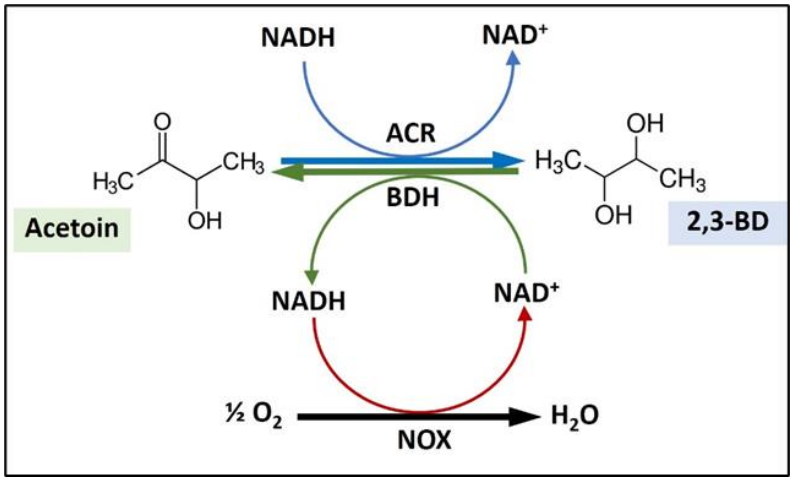


**Figure 3.5** Enzymatic NAD regeneration system in microbial cell for the production of 2,3-BD and acetoin [5]. AR: acetoin reductase; BDH: 2,3-butanediol dehydrogenase; NOX: NADH oxidase.

Considering the above, in order to evaluate the enzyme activities involved in *B. subtilis* acetoin reduction and 2,3-BD oxidation, the reductive reaction utilising acetoin and NADH as well as the oxidative reaction using 2,3-BD and $NAD^+$ were

determined in crude extracts of *B. subtilis* cultures obtained from various DO control patterns. As shown in Table 3.5, particularly, at 36 h, the highest specific activity of AR (13.79 U/mg) was obtained with uncontrolled DO, followed by the two-stage DO control (10.17 U/mg), whereas only 0.97 U/mg of AR was detected with controlling DO at 5% with cascade agitation throughout the cultivation. These results were correlated to the concentrations of the produced 2,3-BD in each DO control strategy at 36 h (Figure 3.6), where higher concentrations of 2,3-BD (~6 g/L) were produced under uncontrolled DO and during the anaerobic phases of the two-stage DO control techniques. On the other hand, very low concentrations of 2,3-BD (< 0.5 g/L) were detected when controlling DO at 5% throughout cultivation.

**Table 3.5** Specific activities of AR, BDH, and NOX obtained in *B. subtilis* during batch fermentations with different DO control strategies.

| **Cultivation conditions** | **Fermentation time (h)** | **Specific enzyme activity** | | |
|---|---|---|---|---|
| | | **AR (U/mg)** | **BDH (U/mg)** | **NOX (mU/mg)** |
| **Uncontrolled DO** | 12 | 2.51 ± 0.40 $^{ab}$ | 0.00 ± 0.00 $^{a}$ | 2.69 ± 0.39 $^{a}$ |
| | 36 | 13.79 ± 0.51 $^{A}$ | 5.92 ± 0.81 $^{A}$ | 4.01 ± 0.46 $^{A}$ |
| Cascade 5%DO (15 h), Anaerobic | 12 | 4.19 ± 0.06 $^{a}$ | 0.28 ± 0.12 $^{a}$ | 2.00 ± 0.04 $^{a}$ |
| | 36 | 10.17 ± 0.59 $^{B}$ | 0.00 ± 0.00 $^{B}$ | 0.92 ± 0.00 $^{B}$ |
| Cascade 5%DO throughout | 12 | 1.29 ± 0.61 $^{b}$ | 0.00 ± 0.00 $^{a}$ | 14.69 ± 0.22 $^{b}$ |
| | 36 | 0.97 ± 0.13 $^{C}$ | 0.33 ± 0.47 $^{B}$ | 12.79 ± 0.14 $^{C}$ |

Indicated values are reported as means ± standard deviation. Values with the different superscript letters (within same column) are significantly different ($p < 0.05$).
Data are average values and standard deviations of duplicate measurements.
All culture conditions were conducted in 5-L bioreactors at 30 °C, pH 6.0 using MMRS with sucrose 20 g/L and corn steep liquor 20 g/L.

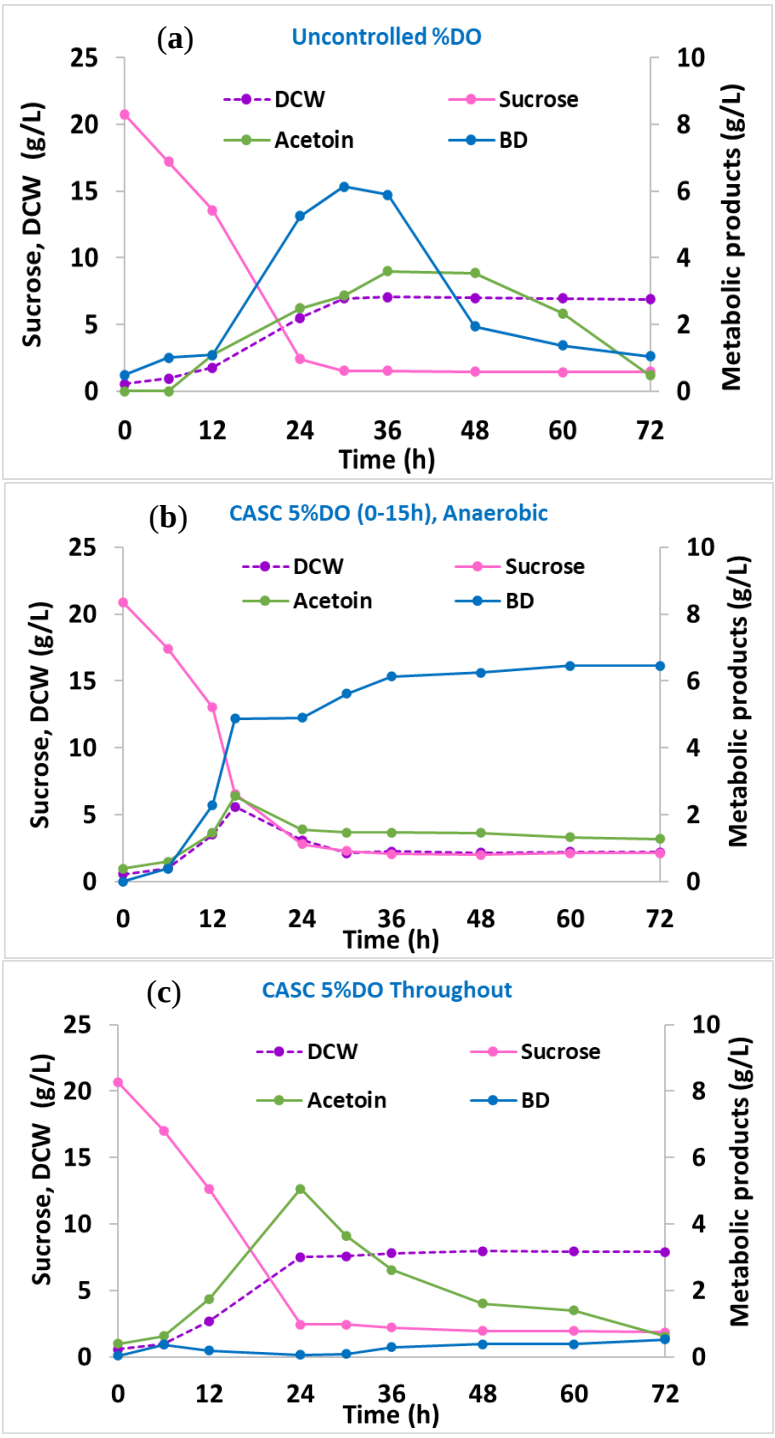


**Figure 3.6** Metabolite production during cultivation with (**a**) uncontrolled DO, (**b**) two-stage DO regime, and (**c**) ontrolling DO at 5% throughout using *B. subtilis* in MMRS medium in 5 L benchtop bioreactor at 30 ℃, pH 6.0, agitation speed 180 rpm, aeration 1.0 vvm.

In terms of the specific BDH activities, the highest BDH activity of 5.92 U/mg was detected in uncontrolled DO cultures and was 2-fold lower than the specific AR activity (13.79 U/mg) at 36 h of cultivation, indicating that the reversible reaction of BDH was also active during this period of time; however, the reductive reaction was more favourable than the oxidative reaction. Thus, the strain produced reasonable concentrations (6 g/L) of 2,3-BD, with lower concentrations of acetoin (3.9 g/L). These results were consistent with Cho et al. [66], in which the AR activity of *Klebsiella oxytoca* M1 was higher than BDH activity, leading to higher 2,3-BD concentrations compared to acetoin. This demonstrates that AR is a key enzyme for acetoin conversion to 2,3-BD [17, 67]. Moreover, the reversible reaction of BDHs was not active in the other two DO control strategies as no BDH activity was detected. The rationale could be that under oxic conditions in the case of controlling the DO at 5% throughout cultivation, relatively high acetoin was accumulated and was not reduced to 2,3-BD, thus $NAD^+$ was not generated. Consequently, the BDH enzyme was not active due to the limited presence of substrate (2,3-BD) and coenzyme ($NAD^+$). During anaerobic conditions (36 h), the AR enzyme was much more active (10.17 U/mg), than BDH (0.0 mU/mg). This indicated that *B. subtilis* could not effectively convert 2,3-BD to acetoin with low or no BDH activity and the limited presence of $NAD^+$ [5].

Considering NADH oxidase (NOX), it should be mentioned that both AR and NOX are NADH-dependent enzymes; AR is activated under low oxygen levels, whilst NOX is activated under high oxygen availability. At low DO levels, considerably lower levels of NOX activity were noted (4.01 mU/mg for uncontrolled DO and 0.92 mU/mg during the anaerobic phases of the two-stage DO control strategies), resulting in less competition for NADH in the cell and redirection of the carbon flux towards 2,3-BD production. A possible reason for this was that NOX was not substantially active under

low oxygen conditions [68]. High levels of NOX activity (12.79 mU/mg) were noted at cultures where DO was controlled at 5%, at 36 h. This could imply that intracellular NADH was oxidised and that $NAD^+$ was continuously regenerated by NOX activity. Thus, the intracellular $NAD^+$ pools in *B. subtilis* increased. In the meantime, NADH was regenerated by BDH enzyme for maintaining a constant redox state, thus kept persisting on acetoin production to 3 g/L. This result is in consistent with Bao [5] who demonstrated that $NAD^+$ was regenerated by over-expression of NOX. In general, $NAD^+$ is required for cell metabolism. To regenerate $NAD^+$ for cellular respiration under aerobic conditions, the NOX was active to oxidise NADH into $NAD^+$ using oxygen. Thus, the pathway for conversion from acetoin to 2,3-BD was not active or not required to regenerate $NAD^+$, leading to the accumulation of acetoin, whereas very low amount of 2,3-BD was produced [69].

### 3.3.7 Enhancing 2,3-BD production by fed-batch fermentation

The results from batch bioreactor process showed that highest 2,3-BD and low amounts of other metabolites such as lactic acid, was achieved from uncontrolled DO regime. Even though aerobic-anaerobic regime also exhibited high 2,3-BD production, but high lactic acid was also produced during anaerobic stage. Thus, uncontrolled DO method was chosen for next experiment of fed-batch fermentation. Sugar depletion appeared to have a critical role in influencing the conversion of 2,3-BD back into acetoin under uncontrolled DO regime. To further improve the production of 2,3-BD and reduce the formation of acetoin, a fed-batch process was performed with a constant residual sugar feeding strategy. Sucrose was fed into the bioreactor when sucrose dropped to around 10 g/L until no further increase in 2,3-BD production or sucrose

consumption was observed. As shown in Figure 3.7, maximum cell biomass of 8.64 g/L was obtained at 108 h. The maximum concentration of 2,3-BD obtained from fed-batch fermentations was 42.31 g/L with a yield of 0.52 g/g and a productivity of 0.33 g/L/h at 130 h of fermentation. Interestingly, even though acetoin was still observed and seemed to increase towards the end of fermentation, the ratio of acetoin to 2,3-BD (0.20) in the fed batch fermentation was considerably reduced (~60%) compared to its ratio (0.48) in the batch fermentations. It is likely that high sugar provision was enough to cover NADH demand for microbial cell metabolism, therefore the reverse transformation of 2,3-BD to acetoin was no longer necessary for NADH regeneration [46]. Other by-products, i.e., ethanol, lactic acid, and succinic acid were only produced in traces. The increase of acetoin towards the end of cultivation, concurring with a decline in 2,3-BD, could be the reason that the sucrose consumption rate seemed to reduce after 96 h and hence, the BDH that converts 2,3-BD to acetoin was activated to regenerate NADH for cell maintenance.

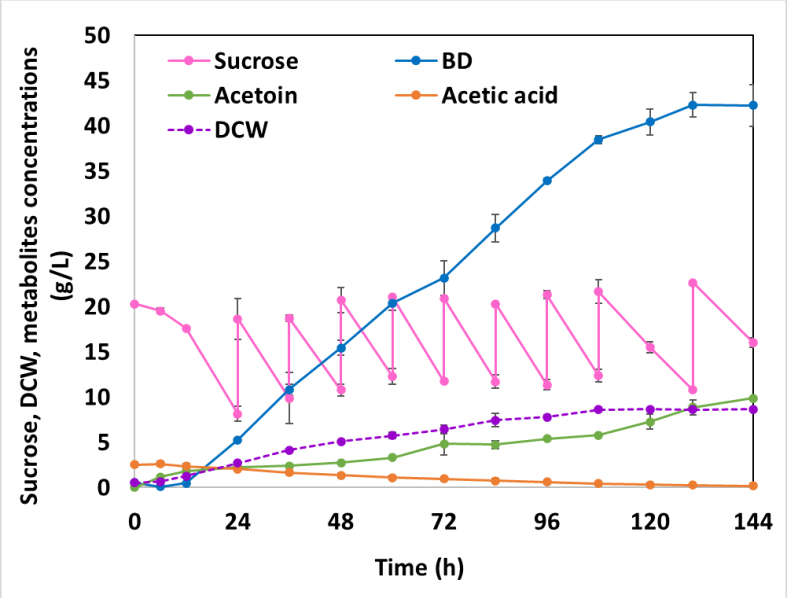


**Figure 3.7** Fed-batch fermentation by *B. subtilis* in MMRS medium in 2 L benchtop bioreactor with uncontrolled %DO and a constant residual sugar feeding method, at 30 °C, pH 6.0, agitation speed 180 rpm, aeration 1.0 vvm.

The production of 2,3-BD by *B. subtilis* strain of this work in comparison with other literature reports is shown in Table 3.6.

**Table 3.6** Comparison of 2,3-BD production by *B. subtilis* strains.

| Bacterial strains | Substrate/ fermentation process | 2,3-BD concentrations (g/L) | 2,3-BD production yield (g/g) | 2,3-BD productivity (g/L/h) | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|
| *B. subtilis** | Glucose/Batch | 44.29 | 0.47 | 0.22 | - High production yield | - Engineered strain<br>- Pure sugar substrate | [57] |
| *B. subtilis** | Glucose/ Fed-batch | 30.6 | 0.32 | 0.57 | - Low-cost nitrogen source (CSL) | - Engineered strain<br>- Low production yield | [42] |
| *B. subtilis** | Sugarcane molasses/ Fed-batch | 75.7 | 0.31 | 0.66 | - Low-cost feedstock | - Engineered strain<br>- Low production yield | [70] |
| *B. subtilis* | Sugarcane molasses/ Fed-batch | 50 | 0.33 | 0.52 | - Low-cost feedstock<br>- Non-engineered strain | - Low production yield | [9] |
| *B. subtilis** | Glucose/ Fed-batch | 102.6 | - | 0.93 | - High product concentration | - Engineered strain<br>- High-cost N source (yeast extract)<br>- Pure sugar substrate | [71] |
| *B. subtilis* | Sucrose/ Fed-batch | 42.31 | 0.52 | 0.33 | - Non-pathogenic and non-engineered strain<br>- Low-cost N-source (CSL)<br>- High production yield<br>- Highly pure (R,R)-2,3-BD | - Pure sugar substrate<br>- Low productivity | This study |

* Engineered or mutant strain

### 3.4 Conclusions

In the present study, *B. subtilis*, a non-pathogenic wild-type strain was used for evaluating 2,3-BD production in sucrose-based media. The result showed that corn steep liquor could be employed as an alternative low-cost nitrogen source to replace more expensive nitrogen (yeast extract, meat extract, and peptone) in Modified MRS medium. This could contribute to the reduction of production cost on large scale 2,3-BD production. Moreover, the evaluation of dissolved oxygen concentrations showed that the limited presence of oxygen produced a higher concentration of 2,3-BD, whilst the presence of oxygen or microaerophilic conditions promoted bacterial growth and acetoin (by-product) formation. Thus, batch cultures with uncontrolled DO have the potential for developing an efficient and economically viable process for the production of high optically pure (R,R)-2,3-BD (purity ~ 100%). Fed batch processes can further enhance 2,3-BD production, by overcoming metabolic changes related to substrate depletion and reverse activity of acetoin reductase.

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# Chapter 4

# *Serratia plymuthica* as a Novel Candidate for meso-2,3-Butanediol Production


**Abstract**

In this work, the ability of *S. plymuthica* to produce 2,3-BD in optimized sucrose-based media was assessed, by investigating the efficacy of alternative low-cost nitrogen sources (corn steep liquor, soybean meal, and ammonium sulphate) and the impact of carbon to nitrogen (C/N) molar ratio. Thereafter, different dissolved oxygen (DO) controlling strategies were evaluated in batch bioreactor processes. In uncontrolled DO conditions, 7.41 g/L of 2,3-BD were produced, (95% of meso-2,3-BD and 5% of (S,S)-2,3-BD), with a production yield of 0.38 g/g. This is the first report on *S. plymuthica* producing chiral meso-2,3-BD with high purity (~95%) under oxygen-limiting conditions. In addition, the influence of the DO controlling regime on *S. plymuthica* key enzymes involved in the reverse activity of acetoin reductase were also determined. Ultimately, a fed batch fermentation under the most suitable DO conditions was performed to enhance 2,3-BD production, achieving 26.23 g/L of 2,3-BD with a production yield of 0.34 g/g. This work demonstrated that *S. plymuthica* is a propitious strain specifically for high chiral meso-2,3-BD production, utilising alternative low-cost nitrogen source.

## 4.1 Introduction

Concerns over climate change and depletion of fossil fuels have driven the development of environmentally friendly and sustainable processes for bio-based chemicals production. 2,3-Butanediol (2,3-BD) is a high-value chemical [1, 2]. A global market of 2,3-BD is expected to reach 300 million US dollars in 2030, growing at a compound annual growth rate of 3.5% from 2020 to 2030 [3]. The 2,3-BD and its derivatives had a potential global market of over 32 million tonnes annually, accounting for 43 billion US dollars in 2018 [2, 4, 5]. 2,3-BD is produced either via chemical synthesis or through biological routes. The chemical synthesis of 2,3-BD involves non-renewable petroleum feedstock as raw material and processes under extreme reactions. The final step is hydrolysis at 160-220$^{o}$C at 50 bar, which usually results in a mixture of 2,3-BD isomers with low optical purity [6]. On the other hand, biotechnological route could offer a greener, sustainable and eco-friendly process compared to the traditional chemical synthesis [7, 8]. Biosynthesis of 2,3-BD involves three enzymatic conversion steps through α-acetolactate and acetoin [9]. Depending on the isomer form of acetoin used, three different stereoisomers of 2,3-BD are produced, namely (S,S), (R,R), and meso-2,3-BD [2, 6, 10-13]. Each stereoisomer of 2,3-BD comes with different physicochemical properties which are linked with different industrial applications. For instance, (R,R)-2,3-BD could be used as antifreeze agent due to its low freezing point. Meso and (R,R)-2,3-BD can serve as precursors for production of 1,3-butadiene, methyl ethyl ketone (MEK), printing ink, and softening agents [1, 14, 15]. More specifically, meso-2,3-BD also finds applications in the cosmetic industry; it was found that the addition of meso-2,3-BD in skincare products could improve their storage stability, owing to its antibacterial properties compared with the chemical preservatives

(phenoxyethanol and paraben), which are considered potential allergens. Thus, meso-2,3-BD could be applied as an antiseptic, humectant, and emollience in powder, lotions, and hand sanitisers [16]. Moreover, due to its excellent antibacterial properties, meso-2,3-BD can be used for relieving and improving human skin diseases, like various eczema caused by bacterial and fungal infections [17]. Additionally, meso-2,3-BD can induce plant resistance against pathogenic virus, thereby denoting its application in agriculture [18]. Most of native 2,3-BD producing strains generally produce a mixture of 2,3-BD stereoisomers. There are several possible explanations for the mixed formation of 2,3-BD stereoisomers, including aeration supply conditions (redox balance), different stereospecific dehydrogenases and metabolic pathways [19, 20]. Among all 2,3-BD producing strains, *Serratia* sp. has been rarely reported as 2,3-BD producing species. Moreover, it has been reported that mainly the meso-2,3-BD can be produced from *S. marcescens* [9]. The separation and purification of the isomers mixtures could be difficult as a consequence of their similar physicochemical characteristics, such as boiling point [1]. This problem is a crucial factor, shifting research interest into strategies such as fermentation optimisation and genetic engineering for the production of 2,3-BD isomers with high purity [6, 21].

Several microorganisms, such as *Klebsiella* sp., *Enterobacter* sp., and *Bacillus* sp. have been known to naturally produce 2,3-BD. Nevertheless, high 2,3-BD concentrations are usually produced by risk group 2 microbes such as *K. pneumoniae* and therefore are not desirable for commercial scale production [16, 22]. Several efforts have been made to produce high purity of 2,3-BD isomers, particularly using genetic engineering to modify genes in bacteria responsible for the production of high optically pure 2,3-BD [23, 24].

The overall aim of this work was to evaluate and improve 2,3-BD production in a non-pathogenic, wild-type *S. plymuthica* strain. The strain exhibited good abilities in producing 2,3-BD under both aerobic and anaerobic conditions in the screening stage of Chapter 3. Initially, the impact of alternative low-cost nitrogen sources and C/N ratios on cell growth and 2,3-BD production were investigated. Then, batch bioreactor cultures were employed in optimised media to assess the influence of dissolved oxygen (DO) on 2,3-BD production by *S. plymuthica*, monitoring also key enzyme activities involved in 2,3-BD metabolic pathway. Ultimately, optimised culture conditions were validated in a fed batch process aiming to improve overall 2,3-BD production by the strain.

## 4.2 Materials and Methods

### 4.2.1 Microorganisms and media

A wild type *S. plymuthica* strain (FSBC 401) from the Food and Nutritional Sciences culture collection, University of Reading, was used in this study. This bacterial strain is equivalent to *S. plymuthica* NCIMB 4612, which is classified by the Advisory Committee on Dangerous Pathogens (ACDP) as a hazard group 1 or non-pathogenic strain. The strain was cultured on Nutrient Agar (NA) containing (per liter): 2 g yeast extract, 1 g beef extract, 5 g peptone, 5 g NaCl, and 15 g bacterial agar. The strain was incubated for 24 h at 30°C, 180 rpm agitation and was sub-cultured into fresh media every week, to maintain its viability. The seed medium composed of (per liter): 20 g glucose, 10 g peptone, 8 g beef extract, 4 g yeast extract, 5 g $CH_3COONa$, 2 g $K_2HPO_4$, 2 g $C_6H_{17}N_3O_7$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.05 g $MnSO_4 \cdot 4H_2O$. A loopful of culture from NA plate was inoculated into 100 mL Erlenmeyer flasks containing 50 mL of sterilised seed medium. The inoculum was grown at 30°C for 24 h in a shaking incubator, whereby

the rpm was maintained at 180 rpm. The cells were harvested by centrifugation at 11,627 x g for 10 min at 4°C, and were suspended in sterilised 0.85% (w/v) NaCl solution. The fermentation medium was a modified MRS (MMRS) medium contained per liter: 20 g sucrose, 10 g peptone, 4 g yeast extract, 8 g beef extract, 5 g $CH_3COONa$, 2 g $K_2HPO_4$, 2 g $C_6H_{17}N_3O_7$, 0.2 g $MgSO_4 \cdot 7H_2O$, and 0.05 g $MnSO_4 \cdot 4H_2O$. All culture media were sterilised by autoclaving at 121°C for 15 min.

### 4.2.2 Batch-flask fermentations

The impact of low-cost nitrogen sources and initial carbon to nitrogen (C/N) molar ratios on 2,3-BD production were evaluated in batch-flask fermentations. The fermentations were carried out in 250 mL Erlenmeyer flasks containing 100 mL of sterilised fermentation medium (described in 4.2.1). After inoculation, flasks were placed a shaking incubator (Incu-shake Midi, SciQuip Ltd, UK) at 30°C and 180 rpm for 48 h. The pH of the media was not controlled throughout the cultivation. All batch-flask fermentations were conducted in duplicate and the results are presented as average ± standard deviation.

The impact of alternative low-cost nitrogen sources was evaluated by replacing costly nitrogen sources (yeast extract, peptone, and beef extract) in the original MMRS medium with less expensive nitrogen sources, namely corn steep liquor (CSL) (Sigma-Aldrich, Gillingham, UK) and soybean meal (SBM) (Target feed Ltd., Whitchurch, UK), which were used separately at concentration of 20 g/L. Ammonium sulphate with and without the addition of vitamins, were also employed as inorganic nitrogen source to replace those expensive nitrogen components. Based on the vitamin content of yeast extract [25], and the concentration of yeast extract in the original MMRS medium, the

vitamin supplement consisted of (per liter): 4.0 mg inositol, 0.41 mg niacin, 0.14 mg thiamine, 0.09 mg riboflavin, 0.07 mg panthothenic acid, and 0.02 mg vitamin B6.

To design a proper C/N molar ratio of fermentation medium for supporting 2,3-BD production and bacterial growth, different initial C/N ratios were also assessed in this study. In all trials, other components of the MMRS medium were kept unchanged, whilst the amount of CSL varied from 5, 10, 20 to 50 g/L, corresponding to the C/N ratios of 22, 17, 11, and 6, respectively. The total nitrogen content of CSL was determined by Kjeldahl method [26]. In the calculation of C/N molar ratios, the contributions of all carbon and nitrogen components in fermentation medium were taken into account.

### 4.2.3 Investigate oxygen limiting fermentation conditions for 2,3-BD production

Different DO control conditions were compared to evaluate the impact of oxygen saturation on 2,3-BD production through batch fermentations in a 2-L Biostat® B bioreactor (Sartorius, Germany). A 24-h culture was inoculated into the developed fermentation medium obtained from 4.2.2. The fermentations were conducted at 30 ℃, and the pH was controlled at 6.0 by automatic addition of 4 M NaOH or 4 M HCl. The medium was sparged with air at a rate of 1.0 vvm throughout the whole fermentation unless otherwise specified. The DO controlling conditions included: (a) uncontrolled DO culture at a constant agitation rate of 180 rpm, (b) a two-stage DO control, the DO level was first controlled at 5% via cascade agitation mode from 0–30 h, followed by anaerobic conditions (no oxygen was supplied to the bioreactor), and (c) 5% DO control via cascade agitation (180 – 500 rpm) throughout the cultivation. During cascade agitation mode, the culture was aerated at 1.0 vvm while the DO was kept constantly at

~5%, achieved by automatically adjusting the agitation rate between 180 and 500 rpm. The pH and DO values were monitored using potentiometric and polarographic sensors, respectively (Hamilton, Switzerland). Foaming was prevented with 10% (*w/v*) antifoam B (Sigma-Aldrich, Gillingham, UK). Samples were taken at random intervals to determine bacterial growth, sugar, and metabolites concentrations.

### 4.2.4 Assay of Acetoin reductase/Butanediol dehydrogenase (AR/BDH) and NADH oxidase (NOX) activities.

Crude cell extracts were prepared for determination of AR/BDH and NOX activities in accordance with our previous work [27]. In short, the fermentation broth was centrifuged for 10 min (9418 × *g*; 4 ℃) to remove supernatant, then the cell pellet was resuspended in 50 mM potassium phosphate buffer, pH 6.5. After ultrasonic disruption for 4 cycles of 30 s pulse, intermittent for 5 s, the samples were centrifuged for 10 min at 9418 × *g* and 4 ℃ to remove cell debris. Crude protein solution was obtained and used for determination of enzyme activities.

The determination of AR/ BDH activities was carried out by measuring the change of absorbance at 340 nm using spectrophotometer. Acetoin reduction and 2,3-BD oxidation were determined by following the oxidation of NADH and reduction of $NAD^+$ over time [28-30]. In brief, the AR activity was assayed in a reaction mixture containing crude enzyme, 50 mM potassium phosphate buffer, pH 6.5, 0.25 mM NADH, and 25 mM acetoin. The BDH activity was measured in the mixture consisting crude enzyme, 50 mM glycine-NaOH buffer, pH 8.5, 0.25 mM $NAD^+$, and 25 mM 2,3-BD. After adding crude enzyme extracts, the reaction was started by the addition of the substrates [27, 29, 31, 32]. One unit (U) of enzyme activity was defined as the amount

of enzyme that consumed (in case of AR) or produced (in case of BDH) one micromole of NADH per minute. The Bradford method was used to quantify protein content of crude enzyme by measuring the absorbance at 595 nm, comparing it to standard curve of bovine serum albumin [33]. The specific (AR/BDH) activity can be expressed as enzyme unit (U) per milligram of protein.

The NADH oxidase assay kit (Fluorometric) from Abcam (Cambridge, UK) was used for measuring NOX activity in the samples as described in our previous work (chapter 3) [27]. The reaction mechanisms are related to the oxidation of NADH to $NAD^+$ by NOX and the reduction of substrate to a colored product, generating fluorescence at Ex/Em = 535/587 nm. The fluorescence generated is directly correlated with NOX activity in samples. One unit (U) of NOX activity was defined as the amount of enzyme capable of reducing 1 µmol of substrate per minute under the specified conditions and the specific enzyme activity could be expressed as µU per milligram of protein.

### 4.2.5 Production of 2,3-BD by fed-batch bioreactor culture

The most efficient DO control condition from the aforementioned trials (in 4.2.3) was chosen for fed-batch fermentation in a 2-L benchtop Biostat® B bioreactor (Sartorius, Germany), containing 1.2 L of optimized fermentation medium. The bioreactor was equipped with sensors for temperature, pH, and DO. Air was supplied at a rate of 1.0 vvm. The fermentation was carried out at 30 ºC and 180 rpm stirring rate. The pH value was maintained at 6.0 ± 0.1 with addition of 4 M NaOH or 4 M HCl. 10% (*w/v*) Antifoam B (Sigma Aldrich, UK) was used when needed. A concentrated feeding solution of sucrose (500 g/L) was fed into the bioreactor in pulses when the

sucrose concentration dropped below 10 g/L to maintain its concentration at 20 g/L. Samples were collected at regular intervals during the fermentation for the determination of cell growth, metabolites, and residual sugar concentrations.

### 4.2.6 Analytical methods

Cell growth, sugar consumption, metabolic byproducts, and 2,3-BD production were determined based on the methods reported by Suttikul et al. [27]. Briefly, the cell-free supernatant was hydrolysed with 10% (*v/v*) $H_2SO_4$ for 30 min at 100 ℃ and then syringed filtered (0.2 μm, PVDF, Cole-Parmer, Saint Neots, UK) before HPLC analysis [34]. The separation and quantification of sugars and metabolites were conducted using an Agilent 1260 Infinity HPLC system (Agilent, Didcot, UK) with diode array detector (DAD) and refractive index detector (RID). The HPLC was equipped with a Bio-Rad Aminex HPX-87H organic acid column with a cation $H^+$ guard cartridge. The mobile phase was a 5 mM $H_2SO_4$ solution. The sample injection volume was set at 20 μL with an eluent flow rate of 0.6 mL/min, and a column heating temperature at 65 °C. Sugar, organic acids, and 2,3-BD were analysed by RID, whereas acetoin was quantified by a diode array detector (DAD). All sugars and metabolites concentrations were analysed using external calibration standards.

Stereoisomeric forms of 2,3-BD and acetoin were identified by GC-FID. Samples were extracted with ethyl acetate using Fast prep 24™ instrument (MP Biomedicals, Eschwege, Germany), then nitrogen gas was used to evaporate solvent from the samples. The stereoisomers of 2,3-BD and acetoin were separated and quantified using a GC system Agilent 7890B (Agilent, Denver, CO, USA) with a flame ionization detector. The GC system equipped with a chiral column (CP-Chirasil-Dex

DB 25 m length, and 0.25 mm inner diameter, Agilent Technologies, Denver, CO, USA). The operating conditions were based on a previous protocol of Caligiani et al. [35], which were as follows: helium with a flow rate of 1.2 mL/min was employed as a carrier gas; the injector port and detector temperature were set at 250 ℃. The column temperature was programmed at 60 ℃ and held for 1 min, then increased to 120 ℃ at a rate of 10 ℃/min followed by 40 ℃/min ramp to 200 ℃. The concentrations of acetoin and 2,3-BD stereoisomers were quantified using external standard curves of those chemicals (Sigma-Aldrich, Gillingham, UK).

## 4.3 Results and Discussion

### 4.3.1 Impact of alternative low-cost nitrogen sources on 2,3-BD production

Studies on the biotechnological production of 2,3-BD, have been mainly focused on the evaluation of renewable resources as growth substrates. One of the crucial factors to be considered in the commercialization of 2,3-BD is the cost of fermentation media. Apart from the carbon source which is the major component in culture media, cost-effective nitrogen sources still remain a challenge, especially when considering the cost of commercial nitrogen sources such as yeast extract, peptone, amino acids, which may be not economically favourable for platform chemicals production at industrial scale. This work evaluated the potential of low-cost nitrogen source to replace costly nitrogen source, such as yeast extract, peptone, and beef extract in commercial (MRS) medium for 2,3-BD production.

Corn steep liquor (CSL), soybean meal (SBM), and ammonium sulphate (AMS) with and without vitamin addition, were evaluated separately as substitutes of more expensive nitrogen sources. As shown in Table 4.1, higher bacterial growth (2.68 g/L of

biomass) was observed at 30 h in cultures with unmodified MRS medium (control), compared to other sources of nitrogen. In terms of 2,3-BD production, CSL was the best nitrogen source, as it exhibited the highest 2,3-BD production for *S. plymuthica*, reaching 5.85 g/L at 30 h. It was also noted that less acetoin was produced in CSL media (1.15 g/L), compared to the control (3.28 g/L).

**Table 4.1** Impact of different nitrogen sources on biomass and 2,3-BD production by *S. plymuthica* at 30 h cultivated under aerobic conditions in MMRS medium

| N-sources [a] | DCW (g/L) | pH | 2,3-BD (g/L) | Acetoin (g/L) | 2,3-BD production yield (g/g) |
|---|---|---|---|---|---|
| Control | 2.68 ± 0.19 | 6.26 ± 0.01 | 4.97 ± 0.03 | 3.28 ± 0.04 | 0.26 ± 0.01 |
| CSL | 1.80 ± 0.17 | 6.15 ± 0.01 | 5.85 ± 0.03 | 1.15 ± 0.01 | 0.37 ± 0.02 |
| SBM | 1.90 ± 0.17 | 6.25 ± 0.01 | 2.67 ± 0.03 | 0.51 ± 0.02 | 0.18 ± 0.02 |
| AMS 20 g/L | 1.83 ± 0.01 | 5.77 ± 0.01 | 1.77 ± 0.05 | 0.42 ± 0.05 | 0.12 ± 0.01 |
| AMS 10 g/L | 1.81 ± 0.04 | 5.71 ± 0.01 | 1.62 ± 0.05 | 0.36 ± 0.06 | 0.13 ± 0.01 |
| AMS 10 g/L +Vitamins | 2.06 ± 0.01 | 5.82 ± 0.02 | 2.52 ± 0.08 | 0.69 ± 0.01 | 0.17 ± 0.01 |

Culture conditions: T = 30◦C; initial pH = 7.0; agitation rate = 180 rpm. [a] Control: Peptone + Yeast extract + Beef extract, CSL: Corn steep liquor, SBM: Soybean meal, AMS: Ammonium sulphate. Data shown are means ± SD of duplicate independent fermentations

CSL is a by-product of the wet-milling process of corn starch manufacturing [36]. This corn soak water is often disposed of directly into the environment, [37]. Thus, utilisation of CSL as a nitrogen source for platform chemicals could not only reduce non-environmentally friendly waste disposal practices, but also add value to by-products of the food industry. CSL comprises mostly of 40% crude proteins, 7.18% nitrogen, vitamins, amino acids, reducing sugars, minerals, and organic acid [38, 39]. It is a cheap

nutrient-rich source that has been employed as a supplement in various bio-industries [40, 41]. Yang et al. [42] claimed that CSL has beneficial impact on the production of 2,3-BD over other N-sources, such as beef extract and soy peptone. They also reported that organic sources of nitrogen could promote the conversion of acetoin to 2,3-BD. Soybean meal comprises of 44-48% crude protein and 8.39% of nitrogen, which has been reported as an effective nitrogen source to replace yeast extract for 2,3-BD and acetoin production [43]. Soy protein is a globulin protein, with glycinin and conglycinin as major components. Even though Soy bean mean (SBM) has high nutritional content, it was not comparable to CSL for the production of 2,3-BD in this study. The reason might be due to CSL being more easily assimilable than SBM by this strain. In addition, it is possible that the proteolytic enzymes produced by *S. plymuthica* might not be specific and effective in hydrolysing SBM protein. SBM proteins may need to be further hydrolysed into small molecules of peptides and amino acids prior to being utilised by microorganisms [44]. This suggests that the use of SBM might require pretreatment, which contributes to additional cost. With regards to inorganic nitrogen sources, Maina et al. [45] reported that the use of inorganic nitrogen source (ammonium sulphate) could enhance 2,3-BD and acetoin production by *Enterobacter ludwigii*; however this was not the case for *S. plymuthica* in this study. 2,3-BD is important primary metabolites like ethanol, lactate, and acetate in a variety of bacteria and the excretion of 2,3-BD has vital physiological implications [46]. As 2,3-BD production is typically claimed to be a growth-associated phenomenon [47], in which the fermentation conditions for the maximum product formation are approximately the same as those for the maximum biomass yield [14]. As observed in Table 4.1, the results showed that 2,3-BD production by *S. plymuthica* in this study, is a non-growth associated metabolite. Although the biomass (DCW) concentrations obtained from CSL, SBM, and AMS were relatively

similar (1.8 – 2.1 g/L), however 2,3-BD concentration from CSL was obviously higher than those from other nitrogen sources. The higher concentration of 2,3-BD in case of CSL because higher amount of sucrose was consumed and used for supporting product formation rather than cell growth. Based on these initial trials, all subsequent fermentations were carried out using corn steep liquor (CSL) as nitrogen source.

### 4.3.2 Impact of carbon to nitrogen (C/N) molar ratio on 2,3-BD production

Carbon is required for the provision of energy and carbon and nitrogen are necessary for cell biosynthesis in microbial metabolism. To investigate the effect of the C/N ratio of substrate on microbial growth and 2,3-BD production, media with varying carbon to nitrogen molar ratio were employed.

As seen in Table 4.2, the lowest dry cell mass (1.32 g/L) and specific growth rate (0.03 $h^{-1}$) were obtained in cultures with a C/N ratio of 6. Moreover, trials with C/N ratio of 22 provided the lowest 2,3-BD production. This might indicate an imbalanced C/N ratio for *S. plymuthica*, and specifically either an inadequate (C/N 22) or overabundant (C/N 6) supply of nitrogen. Maximum cell mass (2.31 g/L), specific growth rate (0.08 $h^{-1}$) and 2,3-BD production yield (0.38 g/g) were achieved at 30 h in media with a C/N ratio of 17. Considering those parameters, a C/N ratio of 17, provided a satisfactory balance between carbon and nitrogen supply during batch cultivation, and was therefore chosen for further studies. It has been reported that the C/N ratio plays a significant role on microbial growth and the biosynthesis of metabolites for a diversity of microorganisms [40,41]. Yang et al. [42] claimed that the initial concentration of CSL affected the production of 2,3-BD and acetoin as well as the ratio of 2,3-BD to acetoin. They reported that increasing in CSL concentration, bacterial growth was enhanced,

acetoin reductase (AR) was activated, 2,3-BD concentration increased by 78.6%, while acetoin proportion decreased by 61.9%, and the ratio of 2,3-BD to acetoin improved by 3.69-fold. In addition, C/N ratio was found to be an important parameter affecting the conversion between 2,3-BD and acetoin in some bacteria such as *B. subtilis* [48].

**Table 4.2** Impact of different carbon to nitrogen (C/N) molar ratio on bacterial growth and 2,3- BD production by *S. plymuthica* at 30 h cultivated under aerobic conditions at 30°C in MMRS medium.

| C/N ratio | DCW (g/L) | Specific growth rate ($h^{-1}$) | 2,3-BD (g/L) | 2,3-BD production yield (g/g) |
|---|---|---|---|---|
| 6 | 1.32 ± 0.14 | 0.03± 0.002 | 5.03 ± 0.16 | 0.29 ± 0.02 |
| 11 | 2.05 ± 0.12 | 0.07 ± 0.006 | 6.14 ± 0.02 | 0.35 ± 0.01 |
| 17 | 2.31 ± 0.02 | 0.08 ± 0.002 | 6.66 ± 0.16 | 0.38 ± 0.02 |
| 22 | 2.22 ± 0.02 | 0.08 ± 0.001 | 3.71 ± 0.03 | 0.22 ± 0.01 |

Data shown are means ± SD of duplicate independent experiments.

### 4.3.3 Influence of dissolved oxygen (DO) concentration on cell growth and 2,3-BD production in 2-L batch bioreactor

The influence of three dissolved oxygen DO control strategies on *S. plymuthica* growth and 2,3-BD production were explored in 2-L benchtop bioreactor under batch fermentations. Unless stated otherwise, fermentations were conducted at 30°C, at pH of 6.0 and aeration rate of 1.0 vvm throughout the cultivation. The growth curves of *S. plymuthica* during three different DO control regimes are shown in Figure 4.1. Lag phase was observed from 0 to 12 h in all three DO control conditions. Higher growth levels were obtained when DO concentration was maintained at 5 %, with cascade agitation throughout the cultivation compared to cultures with uncontrolled DO. This

indicated that higher stirring rate provided better mixing between culture medium and bacteria in the bioreactor and generated higher oxygen saturation, thus stimulating bacterial growth. It is noted that *S. plymuthica* entered log phase after 12 h and the maximum DCW (5 g/L) was achieved within 48 h (5% DO control conditions). A slower growth was observed in trials with uncontrolled DO, where maximum DCW (3.1 g/L) was reached within 48 h of cultivation. Then the strain entered stationary phase of growth, presenting stable growth until the end of fermentation. In the case of two-stage DO control regime, maximum DCW of 2.8 g/L was observed during cascade agitation at 5% until 30 h. During anaerobic conditions (after 30 h), cell growth effectively ceased (Figure 4.1). This is possibly due to a lack of dissolved oxygen in the culture medium. In terms of maximum specific growth rate ($\mu_{max}$), the highest $\mu_{max}$ of 0.12 $h^{-1}$ was obtained in trials with controlling DO at 5% throughout cultivation, followed by a $\mu_{max}$ of 0.10 $h^{-1}$ and 0.08 $h^{-1}$ during two-stage DO control and uncontrolled DO regimes, respectively.

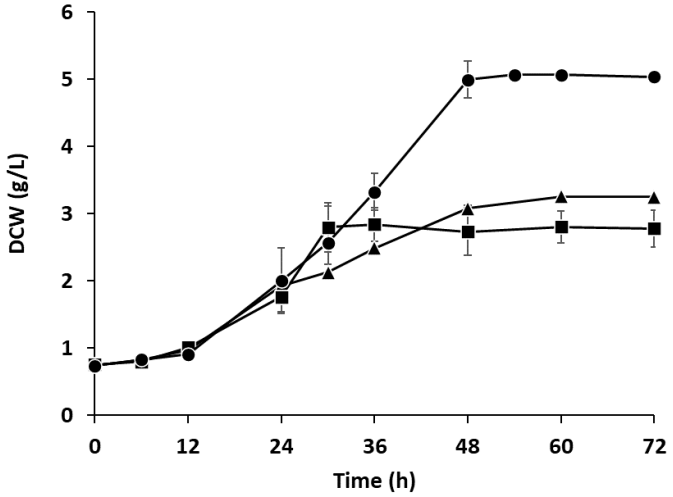


**Figure 4.1** Cell growth of *S. plymuthica* at 30°C, pH 6 in 2-L bioreactor under different DO control regimes: (▲): uncontrolled DO at a fixed stirring rate of 180 rpm, (■): controlling DO with cascade agitation at 5% for 30 h, followed by anaerobic conditions, and (●): controlling DO with cascade agitation at 5% throughout cultivation. Data shown are means ± SD of duplicate independent experiments.

In addition, the influence of different DO control strategies on metabolites production were assessed. Uncontrolled DO at a fixed agitation rate of 180 rpm (Figure 4.2a), led to dissolved oxygen reduction at 0% within 24 h, and favoured 2,3-BD production, resulting in a maximum 7.41 g/L of 2,3-BD, with a production yield of 0.38 g/g sucrose, and productivity of 0.18 g/L/h after 48 h of fermentation. It has been reported that the enzymes involved in the 2,3-BD production pathway (α-acetolactate synthase, α-acetolactate decarboxylase, and acetoin reductase) are typically activated under oxygen limited conditions, while can be inactivated under fully aerobic conditions. Thus, it has been suggested that microbial 2,3-BD production could be improved by manipulating an appropriate oxygen saturation in the bioreactor to limit cellular respiration [49]. As observed in Figure 4.2a, notable concentrations of 2,3-BD were achieved during growth phase (12-48 h), denoting that 2,3-BD is a growth associated metabolite for this specific strain. Subsequently, after the sugar was depleted, 2,3-BD continuously declined and was converted back into acetoin, providing novel evidence on the reverse activity of acetoin reductase in *S. plymuthica*. Several bacterial strains such as *K. oxytoca*, *B. subtilis*, *B. licheniformis* and *Gluconobacter oxydans* have been reported that 2,3-BD and acetoin could be transformed into each other directly [5, 48, 50, 51]. Primarily, the produced acetoin is converted into 2,3-BD by the action of acetoin reductase (AR), subsequently, 2,3-BD is reversibly converted to acetoin by butanediol dehydrogenase (BDH) [52]. In the reversed reaction, a molecule of NADH is generated through the BDH activity in order to maintain microbial growth [48]. The reverse activity of acetoin reductase could be illustrated by microbial cell metabolism. When the sugar is depleted, the NADH required for cell metabolism is produced through the conversion of 2,3-BD to acetoin for the maintenance of redox balance. This particular phenomenon has been reported in several strains i.e. *B. subtilis*,

*B. amyloliquefaciens*, *B. licheniformis*, and *S. marcescens* that used sugars as substrate for 2,3-BD production [48, 53-55].

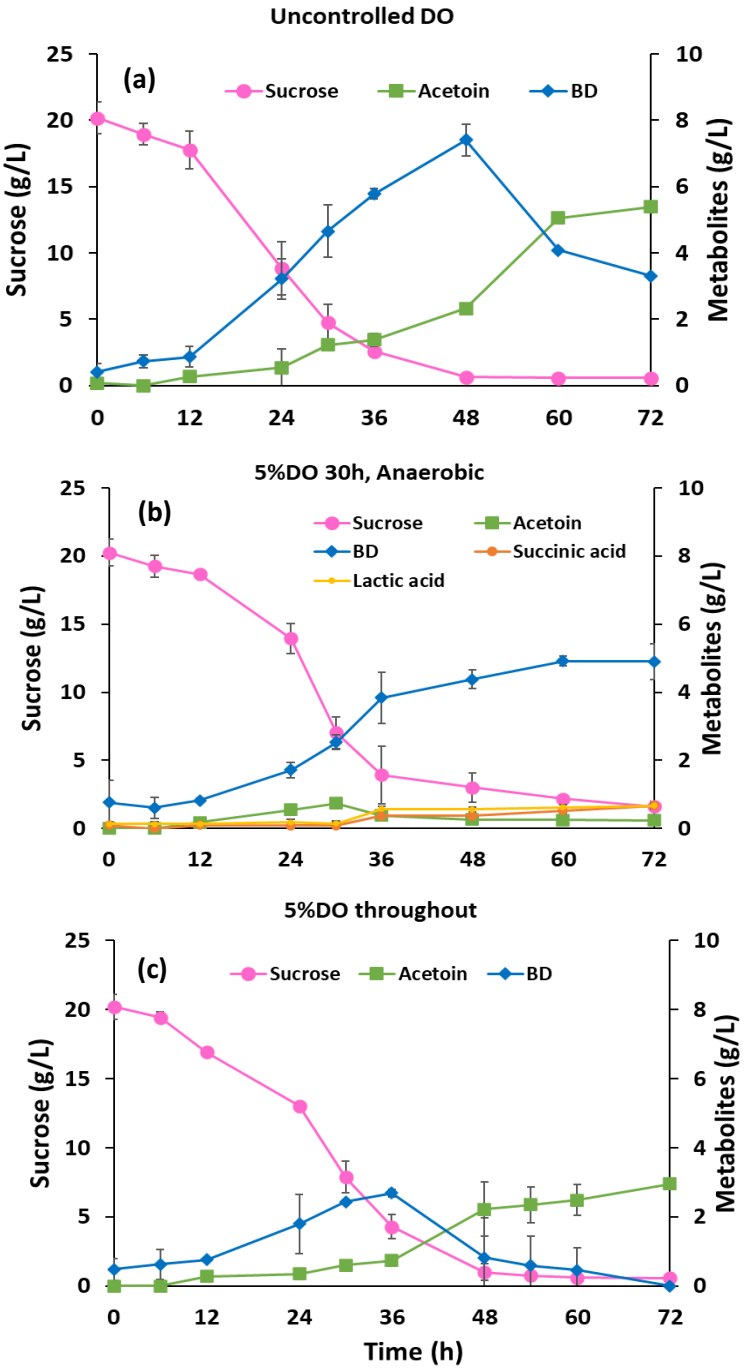


**Figure 4.2** Time course of sucrose consumption and production of metabolites by *S. plymuthica* in 2-L bioreactor at 30°C, pH 6, 1.0 vvm aeration, under (a) uncontrolled DO at fixed stirring speed at 180 rpm throughout fermentation, (b) cascade agitation at 5% during the first 30 h, followed by anaerobic conditions, and (c) cascade agitation at 5% throughout fermentation.

The two-stage DO control strategy was investigated to verify the impact of oxygen on metabolite production (Figure 4.2b). The DO was controlled at 5% with cascade agitation for 30 h, followed by anaerobic conditions. Under these conditions, the bacteria consumed less sugar and at a slower rate, particularly during anaerobic conditions as ~3 g/L of sucrose remained unconsumed at 48 h of fermentation, compared with the other DO control regimes. Interestingly, a small amount of acetoin (less than 1 g/L) was produced along with 2,3-BD (2.5 g/L) during the first 30 h of fermentation, while during anaerobic conditions, the pathway for conversion from acetoin to 2,3-BD was more active. Consequently, 2,3-BD concentrations increased to 4.4 g/L, with a yield of 0.25 g/g after 48 h of cultivation. Moreover, in this phase, by-products such as lactic and succinic acid (less than 1 g/L each) were also produced, whereas acetoin decreased to nearly zero towards the end of cultivation. Specifically, limited oxygen availability during anaerobic fermentation promoted the AR activity and the reductive reaction, in which utilising acetoin and NADH for conversion into 2,3-BD was activated. This led to no observation of acetoin accumulation. Interestingly, the oxidative pathway, whereby 2,3-BD converted back into acetoin was not active. Moreover, it is likely that there is no evidence that the strain consumed 2,3-BD as carbon source when sugar was exhausted during anaerobic fermentation. However, eliminating all oxygen supply (anaerobic fermentation) could force the bacteria to produce organic acids, thus lactic acid and succinic acid were generated even in traces as by-products. As only one mole of NADH from the two NADH formed in glycolysis, is used for R-acetoin production under anaerobic condition (limited presence of oxygen). Thus, the NADH excess remains to be used by the other fermentative pathways of NADH-consuming metabolites such as lactic acid, succinic acid and ethanol [21, 56].

In experiments under DO at 5% throughout the cultivation (Figure 4.2c), *S. plymuthica* produced 2.7 g/L 2,3-BD at 36 h, and when sucrose was depleted, 2,3-BD was converted to acetoin (2.2 g/L) after 48 h of fermentation. The conversion of 2,3-BD into acetoin when sugar was exhausted could be explained by the microbial metabolism. When sugar is completely consumed, the NADH required for cell metabolism is generated through conversion of 2,3-BD to acetoin in order to maintain a balance ratio of intracellular NADH/NAD+ [53, 55]. Fu et.al. [57] also reported that when the glucose was almost consumed during late stationary phase of growth, 2,3-BD concentration declined gradually to almost reached zero, whereas acetoin concentration rose significantly, which mainly caused by the reversible transformation from 2,3-BD to acetoin. Moreover, controlling DO at 5% contributed to high oxygen saturation and provided better mixing between bacteria and medium. As a result, the strain preferably oxidized sucrose towards cell growth rather than metabolite production.

### 4.3.4 Investigation of metabolic pathways in *S. plymuthica* through enzyme activity assays

As a pair of cellular cofactors, $NAD^+$ and NADH play a significant role in a number of biochemical reactions [58, 59], which support the bacterium to maintain a constant redox state for cellular metabolism [60]. The reduction and oxidation reactions for the transformation between acetoin and 2,3-BD participate in the regulation of NADH/NAD+ ratios in microbial cells was illustrated in our previous published work [27]. To assess the activities of enzymes (AR, BDH, and NOX) involved in acetoin reduction and 2,3-BD oxidation, crude protein extracts of *S. plymuthica* obtained from cultures with different DO control patterns, were determined. As shown in Table 4.3, it

was noted that high AR activities of 10.68 U/mg correlated with the highest 7.4 g/L of 2,3-BD achieved from uncontrolled DO regime at 48 h, (Figure 4.2a). Similarly, high AR activity of 9.08 U/mg obtained at 48 h of the two-stage DO control regime was verified by a notable 2,3-BD concentrations of ~5g/L was generated during anaerobic fermentation (Figure 4.2b).

In contrast, very low concentration of 2,3-BD (0.8 g/L) was produced at 48 h in the case of controlling 5%DO throughout the cultivation (Figure 4.2c), in which only 0.59 U/mg AR activity was detected. Under anaerobic fermentation or oxygen limiting conditions, AR in *S. plymuthica* would preferentially function as a reduction rather than oxidation. There have been reported in many studies that several ARs from diverse microbial strains exhibited 2- to 3-fold higher AR reduction activity in comparison with 2,3-BD oxidation activity [61, 62]. Based on these results, it could be verified that oxygen limiting conditions could reduce acetoin accumulation while enhance 2,3-BD production.

Considering the specific BDH activities (Table 4.3), the highest BDH activity of 4.35 U/mg was obtained in uncontrolled DO regime and was 2.5-fold lower than the specific AR activity (10.68 U/mg) at 48 h of fermentation, indicating that the reversible activity of BDH was also activated during this time; however, the reductive reaction was more favourable than the oxidative one. Therefore, the strain produced relatively high concentrations (7.4 g/L) of 2,3-BD, with lower concentrations of ~2 g/L acetoin (Figure 4.2a). These results were similar to those reported by Cho et al. [63], in which the AR activity in *Klebsiella oxytoca* M1 was higher than BDH activity, resulting in higher concentrations of 2,3-BD compared to acetoin. This denotes that AR is key enzyme in native microorganisms for conversion from acetoin to 2,3-BD [31, 64]. Moreover, the reversible reaction of BDHs was also active after 48 h (5.30 U/mg) in the case of

controlled DO at 5% throughout the cultivation (Figure 4.2c), at a time point where sucrose was depleted and 2,3-BD declined to zero, while acetoin concentrations increased to 2.8 g/L. The rationale could be that after carbon depletion, the NADH required for cell metabolism is generated through the reverse conversion from 2,3-BD to acetoin by BDH. For the two-stage DO control, particularly during anaerobic conditions (48 h), the AR enzyme was much more active (9.08 U/mg) than BDH (1.23 U/mg). Thus, ~5 g/L of 2,3-BD was produced, whilst acetoin decreased to nearly zero (Figure 4.2b). These suggested that AR is stimulated under limited presence of oxygen, exhibiting a much higher acetoin reduction activity compared to that of 2,3-BD oxidation, and acetoin accumulation is avoided. On the other hand, the presence of oxygen promoted BDH in *S. plymuthica* and thus stimulated acetoin formation.

For NADH oxidase (NOX), it is worth mentioning that both AR and NOX are NADH-dependent enzymes; AR is activated under low oxygen availability, whilst NOX is activated under high oxygen levels. In this study, under oxygen deprived conditions, considerably lower levels of NOX activity were detected (0.44 mU/mg for uncontrolled DO (at 48 h) and 0.38 mU/mg during anaerobic conditions of the two-stage DO control regime), resulting in less competition for intracellular NADH and diversion of the metabolic carbon flux towards 2,3-BD production [10]. A possible explanation could be that NOX is not generally active under limited oxygen conditions [10]. Higher NOX activity (6.77 mU/mg) was obtained in trials with controlling DO at 5% at 48 h. This could imply that NADH in the cell was utilised with oxygen, and that $NAD^+$ was continuously regenerated by NOX activity. Thus, the intracellular $NAD^+$ pools in this strain increased. Meanwhile, NADH was regenerated by BDHs for maintaining a constant redox state, thus kept a persisting on acetoin production to 3 g/L (Figure 4.2c). This result is in consistent with that of Bao et al. [65] who revealed that $NAD^+$ was

regenerated by over-expression of NOX, resulting in an enhancement of acetoin production. It has been pointed out that not only enzymes-specificities and metabolic pathways but also the balance of NADH/NAD+ is crucial for the production of specific 2,3-BD stereoisomers. The strategy for improving the pool of NADH availability could be accomplished by controlling DO saturation. Fu et al. [57] claimed that high levels of NADH availability contributed more on the production of chiral pure (R,R)-2,3-BD than AR activity in *B. subtilis*.

**Table 4.3** Specific activity of acetoin reductase (AR), butanediol dehydrogenase (BDH), and NADH oxidase (NOX) activities obtained in *S. plymuthica* during batch fermentations with different DO control strategies.

| Cultivation conditions | Fermentation time | | | | | |
|---|---|---|---|---|---|---|
| | 36 h | | | 48 h | | |
| | Specific enzyme activity | | | Specific enzyme activity | | |
| | AR (U/mg) | BDH (U/mg) | NOX (mU/mg) | AR (U/mg) | BDH (U/mg) | NOX (mU/mg) |
| Uncontrolled DO | 5.85±0.92 [a] | n.d | 0.24±0.01[a] | 10.68±0.12 [a] | 4.35±0.27 [a] | 0.44±0.01[a] |
| 5% DO (until 30 h), anaerobic afterwards | 5.23±0.14 [a] | 2.68±0.40 [b] | 0.11±0.01[a] | 9.08±0.18 [b] | 1.23±0.29 [b] | 0.38±0.03 [a] |
| 5% DO throughout | 1.10±0.08 [b] | 2.15±0.23 [b] | 3.05±0.13 [b] | 0.59±0.15 [c] | 5.30±0.36 [a] | 6.77±0.21 [b] |

Values are reported as means ± standard deviation of duplicate measurements. Values with different superscript letters (within same column) are significantly different ($p < 0.05$). n.d. is not detected. All culture conditions were conducted in 2-L bioreactors at 30 ℃, pH 6.0 using MMRS with sucrose 20 g/L and corn steep liquor 10 g/L.

### 4.3.5 Stereoisomers of 2,3-BD and acetoin produced by *S. plymuthica*

Generally, native 2,3-BD producing strains could produce mixtures of two or three stereoisomeric forms of 2,3-BD. However, the ratios of the produced 2,3-BD stereoisomers could vary greatly depending on the microbial strains and cultivation conditions [13, 19, 66]. To further understand the mechanisms of the key enzymes (AR/BDH) involved in the metabolic pathway of 2,3-BD production, the produced 2,3-BD and acetoin stereoisomeric forms were identified. According to the results of 2,3-BD production in three DO control regimes, the highest concentration of 7.4 g/L 2,3-BD was obtained in uncontrolled DO regime, thus these conditions were chosen to further investigate 2,3-stereoisomeric forms in *S. plymuthica*.

In the metabolic pathway (Figure 4.3), R-acetoin is the product of anaerobic conditions from α-acetolactate, whereas S-acetoin is generated under aerobic fermentations from diacetyl. Various AR/BDHs existed in natural 2,3-BD producers, lead to the formation of mixed 2,3-BD stereoisomers [67].

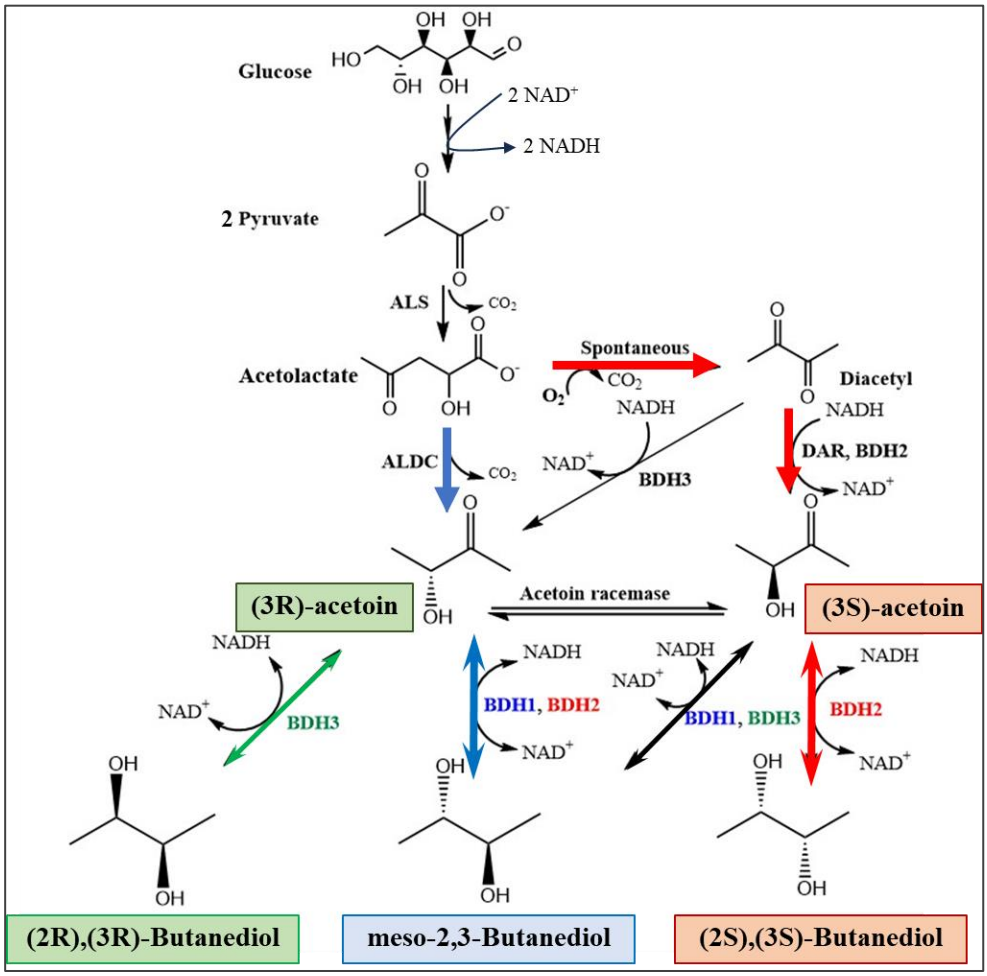


**Figure 4.3** Metabolic pathway for the production of three isomeric forms of 2,3-BD with mixed acid pathway: ALS: acetolactate synthase; ALDC: acetolactate decarboxylase; AR/BDH1: meso-2,3- BD dehydrogenase; AR/BDH2: (2S,3S)-2,3-BD dehydrogenase; AR/BDH3: (2R,3R)-2,3-BD dehydrogenase; DAR: diacetyl reductase (Modified, based on Zhang et al., 2016; Song et al., 2019 [13, 16]).

As observed in Figure 4.4, *S. plymuthica* produced high chiral of meso-2,3-BD (95%) and R-acetoin during growth phase. Interestingly, (S,S)-2,3-BD (5%) and S-acetoin were started to produce after sugar was exhausted after 36 h of cultivation. In addition, it is noted that the strain produced high proportion of R-acetoin with less fraction of S-acetoin.

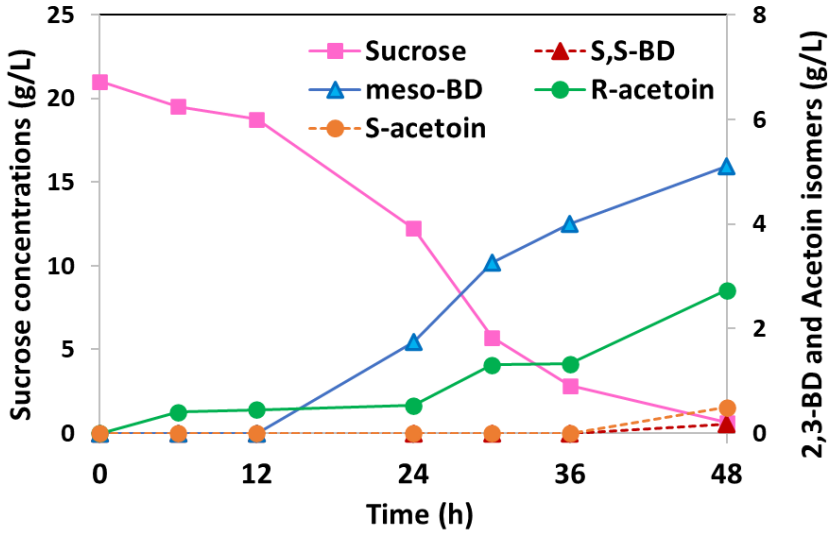


**Figure 4.4** Time course of 2,3-BD and acetoin isomers produced by *S. plymuthica* from sucrose-based medium in 2-L bioreactor under uncontrolled DO regime at 30°C, pH 6, 1.0 vvm aeration rate.

This could be explained by the metabolic pathway for 2,3-BD biosynthesis in the Figure 4.3, the pathway was initiated from R-acetoin to produce meso-2,3-BD by the enzyme (S,S)-acetoin reductase/butanediol dehydrogenase (BDH2) (The blue route). Subsequently, after sugar was completed consumed after 36 h of fermentation, it is likely that S-acetoin was generated from diacetyl, which generally occurs under the oxic conditions. Another possible route is that R-acetoin could be reversibly transformed to S-acetoin by acetoin racemase. Finally, S-acetoin transformed into S,S-2,3-BD by the enzyme (S,S)-acetoin reductase/butanediol dehydrogenase (BDH2) (The red route).

There were several possible explanations for the mixed formation of 2,3-BD stereoisomers, including non-stereospecific dehydrogenases, aeration conditions (redox balance), multiple pathways and multiple stereospecific dehydrogenase [68]. The result in this study is similar to several studies that have been reported the production of meso-2,3-BD with the purity between 96 to 99% by *Serratia* sp. For example, *S. marcescens* has been reported to be able to produce meso-2,3-BD as the major product [2, 69]. To our knowledge, this is the first study to identify stereoisomeric forms of the produced 2,3-BD and acetoin in the strain of *S. plymuthica*.

### 4.3.6 Production of 2,3-BD in fed-batch fermentation

As observed in batch cultivations, sugar depletion played a critical role in influencing the reverse transformation of 2,3-BD into acetoin. A fed-batch fermentation was therefore performed to further improve the production of 2,3-BD and reduce the formation of other by-products, particularly, acetoin. Maximum 2,3-BD concentration of 26.23 g/L was achieved at 96 h of fermentation (Figure 4.5), with a yield of 0.34 g/g and a productivity of 0.3 g/L/h.

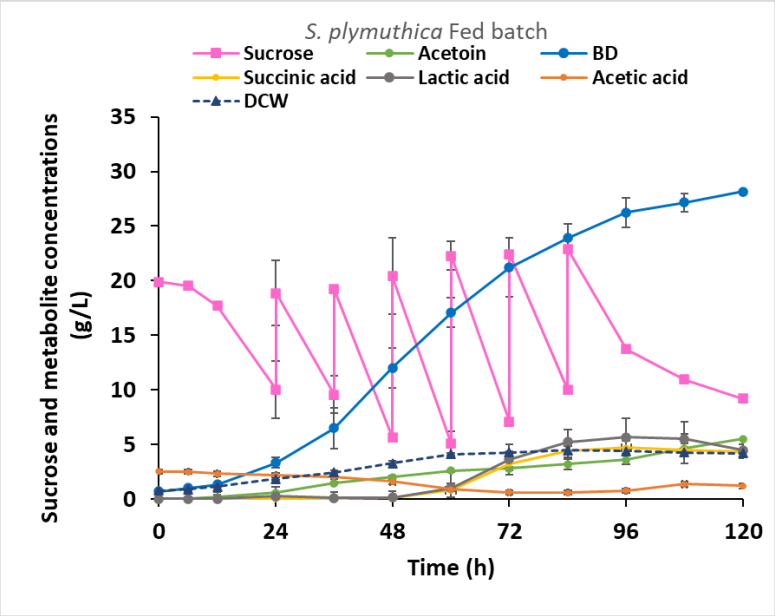


**Figure 4.5** Fed-batch fermentation by *S. plymuthica* in MMRS medium in 2-L bioreactor with uncontrolled DO and a constant residual sugar feeding strategy, at 30°C, pH 6.0, agitation rate of 180 rpm, aeration rate of 1.0 vvm.

Interestingly, although acetoin was still produced by the strain and seemed to increase gradually towards the final period of fermentation, the ratio of acetoin to 2,3-BD (0.14) in the fed-batch process appeared to noticeably decrease (~55%) compared with its ratio (0.31) in the uncontrolled DO regime of the batch fermentations (Figure 4.2a). It is possible that constant sugar provision was sufficient to support NADH demand for bacterial cell metabolism. Thus, the reverse conversion of 2,3-BD to acetoin was no longer necessary for NADH regeneration [70]. Apart from acetoin, however, lactic (5 g/L) and succinic acid (4 g/L) were also detected as by-products in the culture supernatant (Figure 4.5). *Serratia* sp., like other members of Enterobacteriaceae, generally utilise sugar to produce organic acid products, mainly lactate, acetate, succinate, and formate, leading to excessive acidification and growth inhibition during the cultivation. Nevertheless, *Serratia* sp. has the ability to switch its metabolism to the

production of neutral compounds such as 2,3-BD, acetoin, butanol, alcohol [71, 72]. The results of the present study were in agreement with the hypothesis that the production of neutral compounds by *Serratia* sp. is impacted by the reduction of the pH due to acid products formation. Thus, it allows the bacteria produce 2,3-BD and acetoin to prevent lethal acidification caused by the production of organic acids such as succinic and lactic acids. In recent studies, it has been reported that 2,3-BD and acetoin production in *S. plymuthica* RVH1 could be regulated by signals of quorum sensing and acidification [73, 74]. However, the studies did not investigate the impact of DO control on 2,3-BD production in *S. plymuthica*.

In several cases, the Enterobacteriaceae family members consume sugar to produce mixed acids, including acetate, succinate and lactate, resulting in excessive acidification and growth inhibition during the fermentation process. However, some of that family members, such as *Serratia* sp. and *Klebsiella* sp. possess the ability of switching their metabolism to produce neutral compounds such as 2,3-BD, acetoin, alcohols, or 1,3-PD [71, 72, 75]. *S. marcescens* MG1 could produce a large quantity of neutral products, such as 2,3-BD and acetoin, instead of organic acids, during the fermentation from carbohydrate carbon sources. In general, *Serratia* sp. produces acid products during growth phase, leading to a decrease in the pH of fermentation medium. Thus, the strain uses defensive abilities to prevent excessive acidification of internal environment by switching from acidogenesis to solventogenesis. Van Houdt et al. [76] reported that AHL (acylated homoserine lactones)-dependent quorum sensing systems regulated 2,3-BD fermentation in *S. plymuthica* RVH1. In both cases of *S. plymuthica* RVH1 and *S. marcescens* MG1, inactivation of the AHL synthase encoding gene led to a reduction of 2,3-BD production and to a continuous production of acid products at the

end of the exponential and throughout the stationary growth phase, which contributing to early growth inhibit in the presence of fermentable sugars.

Table 4.4 shows the comparison of 2,3-BD production with pros and cons by the genus of *Serratia* of this study and other literature reports.

**Table 4.4** Comparison of 2,3-BD production by *Serratia* sp.

| Bacterial strains | Substrate/ fermentation process | 2,3-BD concentration (g/L) | 2,3-BD production yield (g/g) | 2,3-BD productivity (g/L/h) | Advantages | Disadvantages | Ref. |
|---|---|---|---|---|---|---|---|
| *S. marcescens** | Sucrose/ fed-batch | 139.9 | 0.47 | 3.49 | - High product concentration and productivity | - Pathogenic and engineered/ mutant strain<br>- Pure sugar substrate<br>- High-cost N source (yeast extract) | [19] |
| *S. marcescens** | Sucrose/ fed-batch | 152 | 0.46 | 2.67 | - High product concentration and productivity | - Pathogenic and engineered/ mutant strain<br>- Pure sugar substrate<br>- high-cost N source (yeast extract) | [77] |
| *S. marcescens* | SSJ/ fed-batch | 109.44 | 0.42 | 1.40 | - Wild-type strain<br>- Alternative sugar feedstock<br>- High product concentration | - Pathogenic strain<br>- High-cost N source (yeast extract) | [78] |
| *S. plymuthica* | Sucrose/ fed-batch | 26.23 | 0.34 | 0.27 | - Non-pathogenic and wild-type strain<br>- Low-cost N-source<br>- High chiral of meso-2,3-BD | - Low product concentration<br>- Pure sugar feedstock | This study |

* : Engineered or mutant strain

## 4.4 Conclusions

As a promising platform chemical, 2,3-butanediol has been granted credit for having extensive application in diverse fields such as chemical industry, fuel, food industry, and pharmaceutical industry. This study demonstrated that corn steep liquor could be applied as an alternative low-cost nitrogen source for production 2,3-BD production by wild-type *S. plymuthica.* This could lead to the reduction of manufacturing costs when considering large scale production. Additionally, the assessment of dissolved oxygen saturation showed that limited presence of oxygen favoured 2,3-BD production, whereas the presence of oxygen promoted microbial growth and other by-products such as acetoin production. Therefore, batch fermentation with uncontrolled DO has potential for developing an effective and economically viable process for the production of high chiral meso-2,3-BD (purity 95%).

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# Chapter 5

# Integration of Activated Charcoal Treatment and Ionic Liquids for Bio-based 2,3-Butanediol Extraction


**Abstract**

The recovery of microbially produced 2,3-butanediol (2,3-BD) is challenging due to its high hydrophilicity, high boiling point, and the presence of various metabolites or nutrient residues in the fermentation broth. This work demonstrated the development of separation processes for the downstream recovery of 2,3-BD from fermentation broth. Various concentrations of activated charcoal were investigated for colour reduction and macromolecules removal from two different fermentation broths. Subsequently, the ionic liquid (IL) 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([$C_2$mim][$CF_3SO_3$]), was first investigated in an aqueous two phase system with inorganic salts, aiming at evaluating its ability towards the recovery of 2,3-BD from *S. plymuthica* culture broth. High recovery yield of 2,3-BD (95.63%) was achieved with the system composed of 30% [$C_2$mim][$CF_3SO_3$] - 35% $K_2HPO_4$ from *S. plymuthica* culture broth, with a partition coefficient of 2,3-BD equal to 24.55. In addition, 35% [$C_2$mim][$CF_3SO_3$] - 30% $K_2HPO_4$ provided a high yield for recovering 2,3-BD (97.31%) from *B. subtilis* fermentation broth, thus verified the effectiveness of this separation method. IL was also effectively recovered (90% to 98%). These findings highlight the potential of activated charcoal treatment and IL-based salting out system as green separation techniques for bioprocessing industries, and more specifically for developing a cleaner production of 2,3-BD.

## 5.1 Introduction

2,3-butanediol (2,3-BD) is an important platform chemical with extensive industrial applications. It can be efficiently used in a variety of industries such as fuel additive for aviation transportation, in the production of methyl ethyl ketone and 1,3-butadiene for industrial solvents, as a precursor for synthetic rubber production, and in pharmaceutical industries [1]. Even though most of 2,3-BD is chemically produced, bio-based 2,3-BD has attracted a great attention in various industries due to high cost and environmentally unfriendly processes associated with petroleum-based 2,3-BD production. Apart from the considerable progress in microbial fermentation, the separation of 2,3-BD from the fermentation broth is still challenging due to its high boiling point (177-182ºC) and high polarity, as well as the usually low concentrations of 2,3-BD in complex fermentation broth [2]. The development of low-cost and highly efficient separation process is relatively slow [3], which restricts scaling-up of bio-based 2,3-BD [4]. Thus, it is important to develop cost effective and highly efficient separation technologies to support commercial 2,3-BD production process. Moreover, the separation and purification of 2,3-BD from the fermentation broth is a critical bottleneck of the entire process, representing 60 to 70% of the total production costs [5-8]. Several methods have been suggested for the separation and purification 2,3-BD from the broth, however most of them are accompanied by drawbacks and/ or limitations [7]. Distillation consumes too much energy and is cost intensive due to the high boiling point of 2,3-BD [8]. Consequently, other alternative separation processes, including adsorption, solvent extraction, reactive extraction [9], membrane separation have been developed. So far, most of the liquid-liquid extraction research targeted towards diols has been conducted based on conventional organic solvents. However, most organic

solvents contribute to air pollution because of their high volatility. Also, most of them are toxic and flammable as well as generating waste water. Reactive extraction using aldehydes as extractants is a costly method and the remaining aldehydes are likely to provide adverse effect to the applications of 2,3-BD as they prohibit subsequent polymerization. Due to environmental issues and toxicity concerns associated with common volatile organic solvents, ionic liquids (ILs), are being currently investigated for extraction purposes as a novel and greener class of non-volatile alternative solvents. ILs possess several obvious advantages due to their outstanding unique properties. They have extremely low volatility under atmospheric conditions, and typically exhibit good thermal and chemical stability, solvation ability and are of non-flammable nature, all of which contribute to their extensive recognition as ambient-friendly compounds [10]. Their valuable properties endorse their applications in diverse areas ranging from catalysis, organic synthesis, extraction and separation processes [11]. Furthermore, a wide range of prospective combinations between cations and anions enable a high degree of tunability of ILs properties. However, the relatively high costs of ILs have limited their application for large scale separation. Thus, selecting a lower price of IL with reasonably high extraction efficiency and enhanced recyclability is considered key.

ILs consist of an organic cation and an organic or inorganic anion [12]. The separation of higher polarity alcohols, particularly, diols, are more challenging because their properties are relatively close to those of water. There are two possible types of ILs applications in aqueous-based extraction systems, hydrophilic ILs that can be used in aqueous two-phase systems [13] or hydrophobic ILs, which can be applied as a second immiscible phase [14-19]. Generally, ILs can be divided into two distinct families: aprotic ILs (AILs), which possess excellent extraction ability, however, they have unfavourable physical properties, i.e, high viscosity. Another family is protic ILs (PILs),

which are easier to produce through a neutralization reaction between Brønsted acid and Brønsted base at relatively low price. Aqueous two-phase system (ATPS), comprises of two liquid phases with high contents of water and is commonly employed for the extraction of biomolecules. In the ATPS, phases are obtained by the addition of a sufficient amount of two different phase forming components (PFC) to water or fermentation broth. PFCs are commonly water-soluble solutions and mutually incompatible, allowing phase separation to occur. General combinations of PFCs are polymer/polymer (e.g., dextran/polyethylene glycol (PEG)) and polymer/salt (PEG/inorganic salt). For IL-based ATPSs, the phase formation can be obtained by adding an IL and inorganic salt. IL-based ATPSs are widely investigated with focusing on the impact of various inorganic salts and ILs on the phase formation [20-22] and the distribution coefficient of different components. Alkylimidazolium structure is considered as common cation with reasonable costs and flexibility [23], thus imidazolium ILs have been extensive studied in many biopprocessing applications [24-28]. Among various alkylimidazolium-based ILs systems, 1-ethyl-3-methylimidazolium trifluoromethanesulfonate [$C_2$mim][$CF_3SO_3$] could enable the substitution of 1-n-butyl-3-methylimidazolium chloride [$C_4$mim][Cl] for cheaper, less toxic, and more biodegradable alternatives.

2,3-BD requires a polar extractant, thus hydrophilic ILs-based salting out extraction systems were investigated in this work. This work aims to evaluate the influence of activated charcoal treatment and IL-based salting out extraction in the recovery of 2,3-BD from fermentation broths. Firstly, different amounts of activated charcoal were applied for treatment of the two different fermentation broths. Further, the abilities of different types of salt to form ATPSs with [$C_2$mim][$CF_3SO_3$] were evaluated by determining phase volume ratio, partition coefficient, and recovery of 2,3-BD.

Subsequently, different amounts of IL and salt were explored to determine the most effective composition for recovering 2,3-BD from two different compositions of fermentation broths (i.e, *Serratia plymuthica* and *Bacillus subtilis* fermentative broths). This study provides further insights into extraction ability for design of appropriate composition of IL-based salting out system.

## 5.2 Materials and Methods

### 5.2.1 Materials

All chemicals used in this study were purchase from Sigma-Aldrich, Gillingham, UK (unless otherwise stated): 2,3-BD, acetoin, ethanol, glucose, fructose, and organic standards, activated charcoal. The ionic liquid used was 1-ethyl-3-methylimidazolium trifluoromethanesulfonate [$C_2$mim][$CF_3SO_3$], whose chemical structure is shown in Figure 5.1. Dipotassium phosphate ($K_2HPO_4$), monosodium phosphate ($NaH_2PO_4$), ammonium sulphate ($(NH_4)_2SO_4$), tripotassium phosphate ($K_3PO_4$), and potassium carbonate ($K_2CO_3$) were used as inorganic salts, and Bradford reagent was used for total soluble protein quantification. The glucose assay kit (K-gluc assay kit) was purchase from Megazyme, USA.

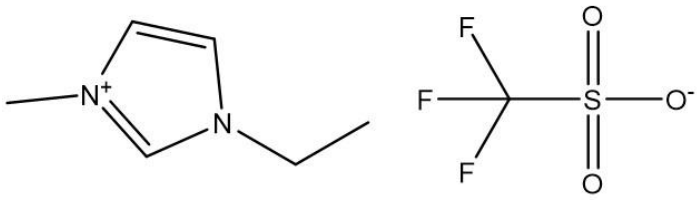


**Figure 5.1** Chemical structure of 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([$C_2$mim][$CF_3SO_3$]).

### 5.2.2 Fermentation broth preparation

The culture broths used for separation processes were obtained independently from fed-batch fermentations of *S. plymuthica* and *B. subtilis*, as described in Chapters 3 and 4. The culture broth from fed-batch fermentation of *S. plymuthica* comprised of sucrose 10.28 g/L, acetoin 5.46 g/L, 2,3-BD 27.57 g/L, succinic acid 4.12 g/L, lactic acid 4.39 g/L, acetic acid 1.06 g/L. The fermentation broth from fed-batch cultivation of *B. subtilis* contained sucrose 14.66 g/L, acetoin 10.80 g/L, 2,3-BD 42.77 g/L, acetic acid 0.2 g/L.

### 5.2.3 Treatment of fermentation broth by activated charcoal

The fermentation broth was centrifuged at 4ºC, (11,627 × *g*) for 10 min to separate cell biomass and supernatant. The supernatant was treated with different concentrations of activated charcoal, including 0% (control), 0.5, 1, 1.5, 2, 3, 4, 5% (w/w). The sample mixtures were shaken at 200 rpm, 30 ºC for 90 min and were subsequently centrifuged at 4ºC, (11,627 × *g*) for 10 min. The treated samples were subjected to the HPLC for the analysis of sucrose, 2,3-BD, and other metabolites such as acetoin, succinic acid, lactic acid, and acetic acid. Moreover, protein and free amino nitrogen (FAN) concentrations were determined by Bradford [29] and Lie [30] methods, respectively. In addition, the HunterLab Vista colour spectrophotometer (HunterLab, Murnau, Germany) was used for the measurements of colour indices of the activated charcoal treated samples based on the three-colour coordinates, L*, a*, and b* at D65 standard illuminant and 10º standard observer. The instrument was calibrated using the fermentation broth of each strain. The sample colour was expressed in Hunter Lab units

$L^*_2$ (lightness/ darkness; 0–100), $a^*_2$ (positive = redness/negative = greenness) and $b^*_2$ (positive = yellowness/negative = blueness). The total colour difference (TCD, $\Delta E^*_{ab}$) between any two samples was calculated according to the equation (1) [31]:

$$\Delta E^*_{ab} = \sqrt{(L^*_2 - L^*_1)^2 + (a^*_2 - a^*_1)^2 + (b^*_2 - b^*_1)^2} \quad (1)$$

where, $L^*_1$, $a^*_1$, $b^*_1$ = blank values of the control sample (fermentation broth without activated charcoal treatment).

The Gardner index, a visual scale described in ASTM D 6166, was determined. The Gardner colour scale is a number with a series of 18 color variations, ranging from nearly clear or light yellow (Gardner 1) to dark brown (Gardner 18). Gardner 0 is defined by clear distilled water [32]. The Yellowness Index was also evaluated to characterize the transition from clear to yellow in the colour of test samples as described in ASTM E313.

### 5.2.4 Screening of salt type for ILs- based salting out extraction

The best performing activated charcoal concentration was selected for treatment of the fermentation broth in this experiment. 1-ethyl-3-methylimidazolium trifluoromethanesulfonate $[C_2mim][CF_3SO_3]$ was employed in IL-based salting out system. Briefly, 2 g of five different inorganic salts including $K_2HPO_4$, $NaH_2PO_4$, $(NH_4)_2SO_4$, $K_3PO_4$, and $K_2CO_3$ were dissolved independently in 4 mL of *S. plymuthica* fermentation broth. Subsequently, 2 mL of $[C_2mim][CF_3SO_3]$ was added into the reaction mixture and mixed thoroughly for 2 min by using vortex mixer. The mixtures were left standing overnight (16-24 h) at room temperature (18-25 $^oC$) for phase

separation as shown in Figure 5.2. The volumes of the top and the bottom phases were carefully retrieved with syringe and were prepared for HPLC analysis as described in Chapter 3. The phase ratio (R), partition coefficient (K), recovery (Y), selectivity (S) were calculated as suggested by Dai [33] and Li [34] as follows:

$$R = \frac{Vt}{Vb} \tag{2}$$

$$Kj = \frac{Cjt}{Cjb} \tag{3}$$

$$Yj(\%) = \left(\frac{Cjt \times Vt}{Cjt \times Vt + Cjb \times Vb}\right) x\ 100 \tag{4}$$

$$Sj = \frac{KBD}{Kj} \tag{5}$$

Where $V_t$ and $V_b$ represent the volume of top and bottom phases, respectively; $C_{jt}$ and $C_{jb}$ denote the concentrations of chemical j in the top and bottom phases; $K_{BD}$ is the partition coefficient of 2,3-BD; $K_j$ is the partition coefficient of chemical j; Yj is recovery of chemical j

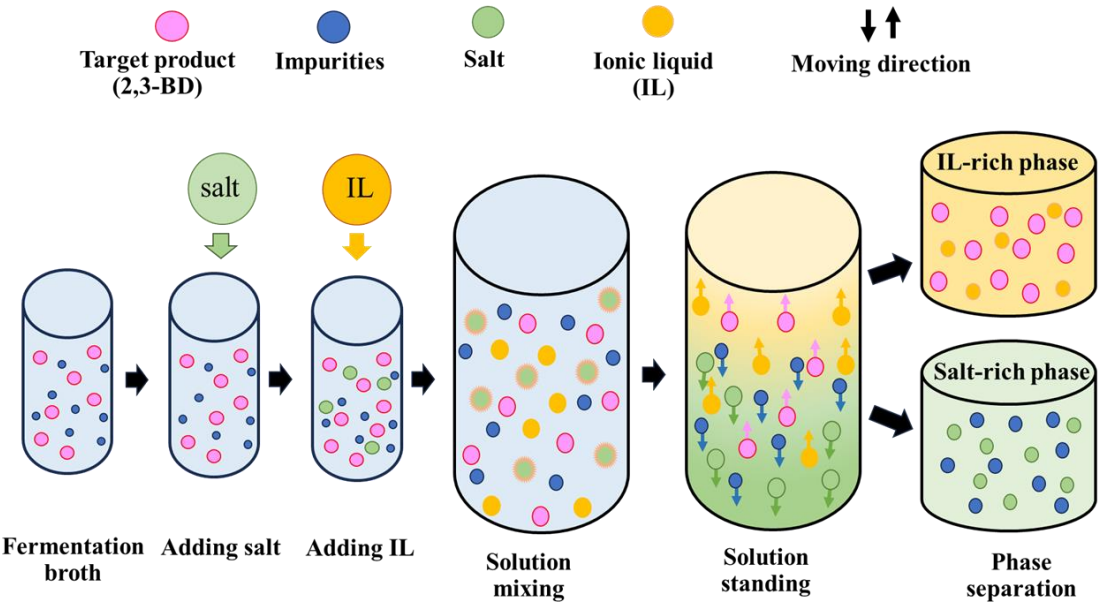


**Figure 5.2** Schematic representation of IL-based salting out extraction of 2,3-BD from fermentation broth.

### 5.2.5 Effect of different amounts of IL and salt in IL-based salting out extraction

The most effective salt in terms of partition behaviour and recovery of 2,3-BD in the previous experiment, was chosen for evaluating IL and salt on extraction efficiency for the recovery of 2,3-BD from the fermentation broth. The IL- based salting out systems were prepared gravimetrically using glass vials by varying amounts of three contents, including 15 to 35 wt% of $[C_2mim][CF_3SO_3]$, 15 to 35 wt% of salt, and fermentation broth to fulfill 100 wt% of 8 g total weight. The extraction and analysis procedures were carried out with the same as aforementioned procedure in 5.2.4.

### 5.2.6 Effect of different components of fermentation broth on partition coefficient and the recovery of 2,3-BD

The activated charcoal treated fermentation broth of *B. subtilis* was employed for evaluating an impact of various fermentation metabolites on the partition coefficient and the recovery of 2,3-BD. The three systems of $[C_2mim][CF_3SO_3]$-$K_2HPO_4$ that gave high extraction ability and recovery of 2,3-BD from the experiment described in 5.2.5, were used to compare the extraction efficiency in this study. Moreover, the untreated fermentation broths of *S. plymuthica* and *B. subtilis* were also assessed for their extraction efficiency to recover 2,3-BD. The extraction and analysis procedures were carried out with the same as aforementioned procedure in 5.2.5.

### 5.2.7 Analytical methods

Since peak of glucose overlapped with phosphate in $[C_2mim][CF_3SO_3]$ - $K_2HPO_4$, and $[C_2mim][CF_3SO_3]$ - $K_3PO_4$ systems, glucose concentration was detected by using glucose assay kit (K-gluc assay kit, Megazyme, USA). Protein concentration was determined by Bradford protocol using bovine serum albumin (BSA) as protein standard [29]. Free amino nitrogen (FAN) was measured by the method of Lie [30] with some modifications. Briefly, 0.25 mL of diluted sample was mixed with 0.125 mL of colour reagent (49.71 g of $Na_2HPO_4 \cdot 2H_2O$, 5 g of ninhydrin, 3 g of fructose and ~ 60 g of $KH_2PO_4$ dissolved in 1 L of distilled water; pH 6.6 – 6.8) in a microtube. The mixture was heated at 100 $^oC$ in a thermal block (Grant, Cambridge) for exactly 16 minutes and immediately cooled to room temperature. Then, 0.625 mL of dilution reagent (2 g potassium iodate, $KIO_3$, in 616 mL distilled water and 384 mL 96% ethanol) was added and the free amino nitrogen content was measured at 570 nm against the blank. A calibration curve was constructed using glycine as standard at several concentrations (0.25 – 2 mg/L).

### 5.2.8 Statistical Analysis

Statistical analysis was performed using the SPSS Statistics for Windows, Version 27 (IBM SPSS Statistics, Armonk, NY: IBM Corp). The significant differences in mean values between treatments were evaluated using the analysis of variance (ANOVA) method, at a confidence level of 95% ($P < 0.05$). Results are presented as mean ± standard deviation.

## 5.3 Results and Discussion

### 5.3.1 Effect of different concentrations of activated charcoal on fermentation broth

Activated charcoal is a highly porous material made from wood, bamboo, or other organic substances. It has a large surface area that can effectively adsorb biological and organic chemical substances, colour as well as odor from water and air. Fermentation broth generally contains various components such as residual sugars, target metabolites, by-products, proteins, and other residual nutrients.

### 5.3.2 Effect of activated charcoal on decolouring of the fermentation broth

Fermentation broths contained residual sugars (sucrose, glucose, fructose), organic nitrogen, free amino acids, and by-products of microbial metabolism, such as acetoin and traces of organic acids. Some of them could contribute to the yellowish brown colour of the fermentation broth. It is likely that a significant amount of the yellowish-brown colour of the culture medium was caused by the brown colour of corn steep liquor, which was used as nitrogen source for microbial fermentation. Moreover, the brown colour could also be attributed to the MRS medium. Thus, prior to extraction, the fermentation broth was initially treated with activated charcoal, aiming to remove colour and some residual molecules. For *S. plymuthica* fermentation broth (Figure 5.3a), a positive correlation was observed as the activated charcoal concentrations increased from 0.5% to 1.5% (w/v), where the colour of the fermentation broths became notably lighter. For higher concentrations of activated charcoal (2% to 5%, w/v) no significant changes were observed. Similarly, a lighter colour was observed in *B. subtilis* fermentation broth as the activated charcoal concentrations increased from 0.5% to 2%

(w/v), whereas no further significant changes were observed in samples treated with 3% to 5% (w/v) (Figure 5.3b).

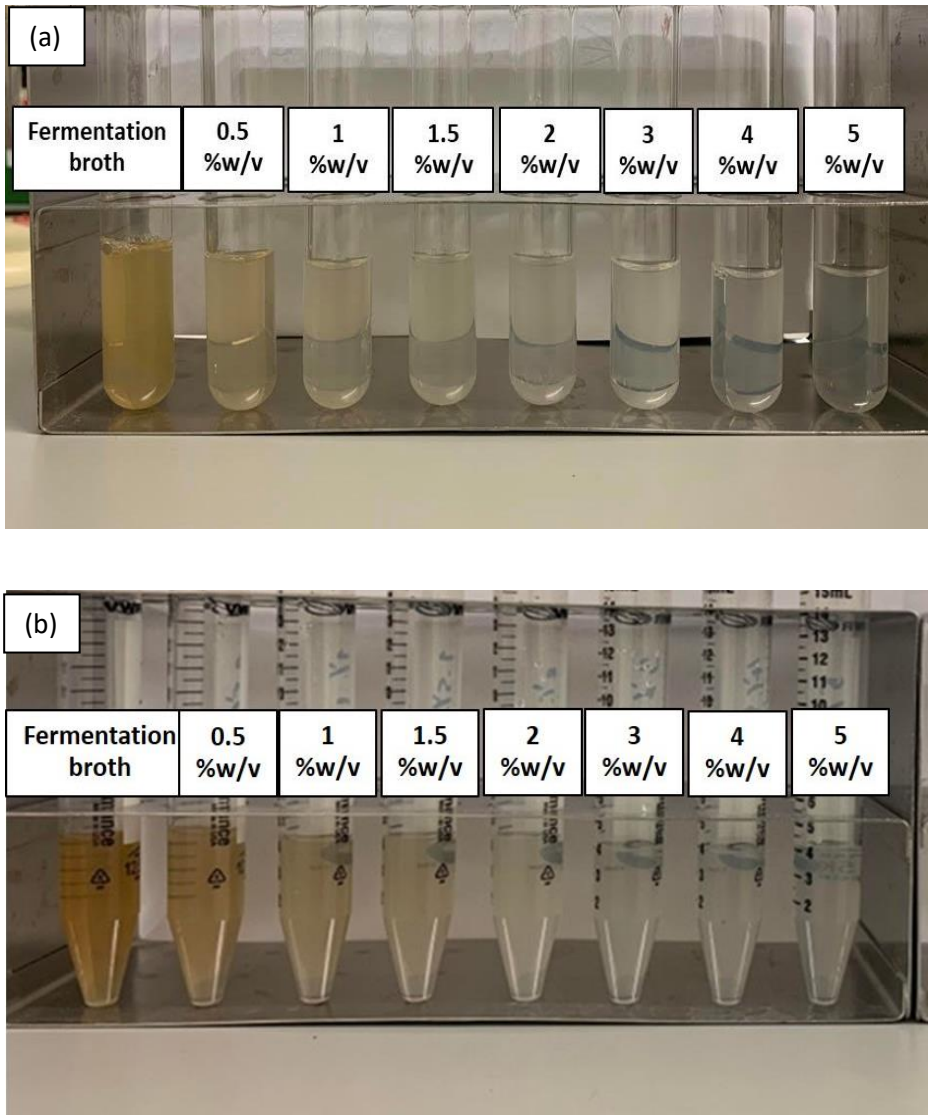


**Figure 5.3** Fermentation broth of *S. plymuthica* (a) and *B. subtilis* (b) treated with different concentrations of activated charcoal at 30 °C for 1.5 h and centrifuged at 11,627 ×g, 4ºC, 10 min.

HunterLab spectrophotometer was used to determine the colour difference and colour indices of activated charcoal treated broths. The total colour difference (TCD) value of *S. plymuthica* broth treated with different concentrations of activated charcoal

(0.5% to 5%, w/v) was measured by comparing against the control (original fermentation broth). As observed in Figure 5.4a, the TCD values in samples treated with 1% to 5% (w/v) were not different, but were higher than that of 0.5% (w/v) treated samples. In the case of *B. subtilis* broth, relatively different TCD values were obtained when 0.5% to 2% of activated charcoal was used, whereas TCD values were not different when higher than 2% w/v of activated charcoal concentrations were employed for broth decolourisation. This reflects the effectiveness of activated charcoal since its small particles provide large surface area to which large coloured molecules could be adsorbed. As observed in Figure 5.4b, the Gardner values (yellowness) decreased faster when activated charcoal concentrations increased from 0.5% to 1.5%, then gradually decreased to constant levels with increasing concentrations of activated charcoal from 2% to 5% (w/v) in the case of *S. plymuthica*. Stable levels of the Gardner index were obtained in activated charcoal concentrations of 3% to 5% (w/v) in *B. subtilis* broth. In terms of the yellowness index (Figure 5.4c), a significant decrease was observed when activated charcoal concentrations increased from 1% to 1.5% (w/v) in *S. plymuthica* broth, whereas a notable decrease in yellowness index was obtained when 1 to 2% (w/v) was applied in *B. subtilis* broth. Considering those results, the most effective and suitable concentrations of activated charcoal for the treatment of *S. plymuthica* and *B. subtilis* fermentation broths, were 1.5% and 2% (w/v), respectively. The results from this study support previous studies indicating that activated charcoal could be efficiently used for decolouring of various solutions such as syrups [35, 36].

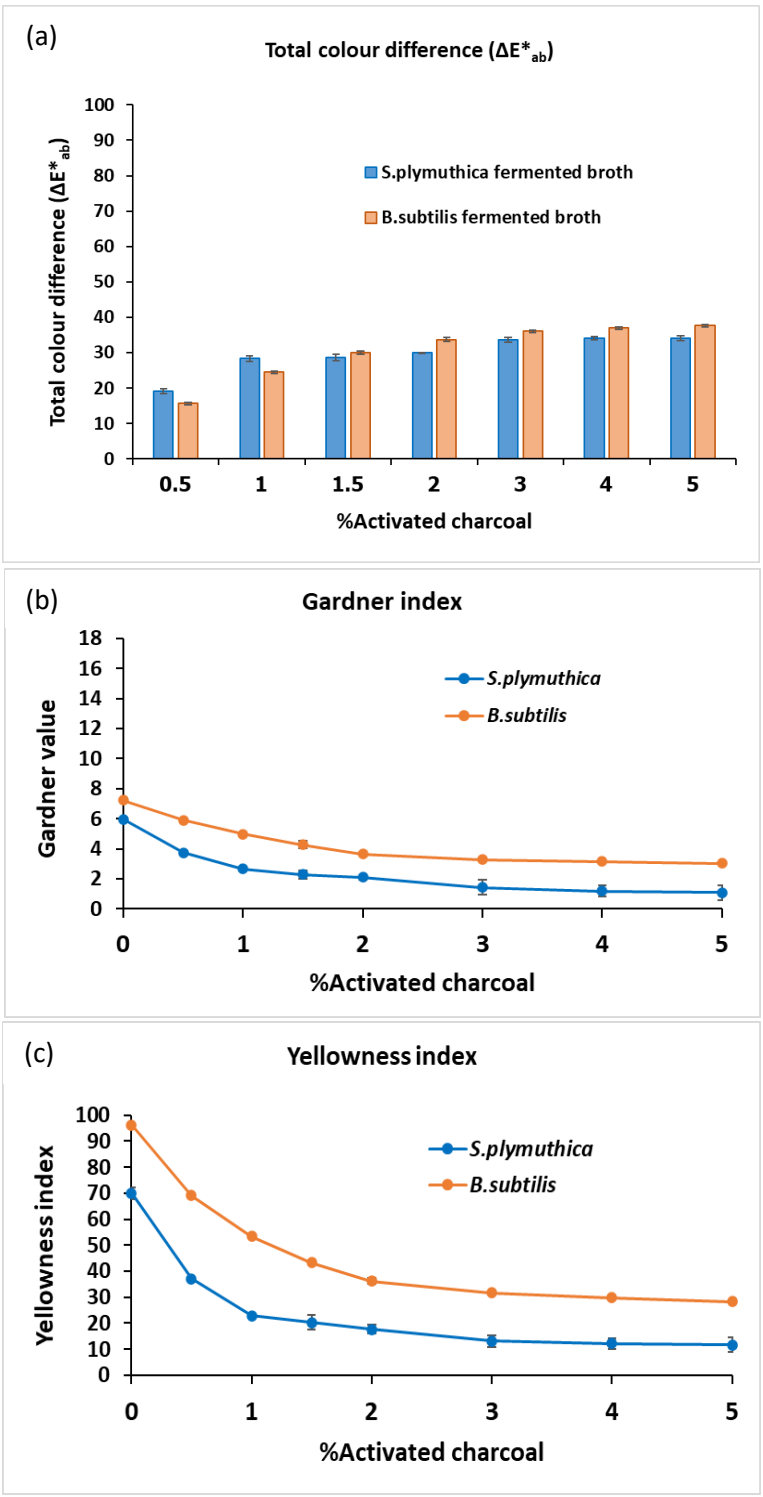


**Figure 5.4** The total colour difference (a), Gardner index (b), and yellowness index (c) of *S. plymuthica* and *B. subtilis* fermentation broth after treated with different concentrations of activated charcoal

### 5.3.3 Effect of activated charcoal on the recovery of 2,3-BD and other molecules from the fermentation broth

The effect of activated charcoal concentration on the recovery of 2,3-BD, acetoin, sucrose, proteins and free amino nitrogen from the fermentation broth of *S. plymuthica* is shown in Table 5.1. As activated charcoal can adsorb proteins, sugars, and chemicals (such as 2,3-BD, acetoin, organic acids). Thus, the first row of Table 5.1 and 5.2 represents the concentrations of all components in the original fermentation broth, whereby 100% recovery yield of each component is shown. The recovery of 2,3-BD gradually decreased as the concentration of activated charcoal increased. Similarly, gradual reduction in acetoin, sucrose and FAN concentrations were noted when the activated charcoal concentration increased. Unlike other compounds, protein was efficiently removed from the fermentation broth at increasing concentrations of activated charcoal. Kopper et al. [37] reported that activated charcoal could be applied for adsorbing peanut protein allergens. Moreover, Stone and Kozlov [38] reported that activated charcoal could adsorb low molecular weight proteins, in line with the results of this study. In addition, activated charcoal can remove organic compounds due to its physical features, like surface area, pore size distribution, pore volume, and surface functional groups [39]. Thus, activated charcoal could help to decolourise and remove some impurities from the fermentation broths. Based on the above results, 1.5% (w/v) activated charcoal was chosen as a suitable concentration to pretreat the *S. plymuthica* fermentation broth for efficient 2,3-BD recovery (aiming at higher than 80%), with simultaneous removal of other impurities, like sugars, acetoin, proteins, and some amino acids.

As higher concentrations of corn steep liquor (20 g/L) were initially employed in *B. subtilis* culture medium compared to that 10 g/L of CSL employed in *S. plymuthica* fermentation medium, according to the results of investigating a suitable C/N ratio. This contributed to higher protein concentrations and slightly darker of *B. subtilis* broth than those of *S. plymuthica* broth. Moreover, the compositions and concentrations of all components of *B. subtilis* fermentation broth are difference from those of *S. plymuthica* broth. Therefore, a slightly higher concentration of activated charcoal is required for treatment of *B. subtilis* compared to *S. plymuthica* broth. 2,3-BD recovery gradually decreased as the concentration of activated charcoal increased. As seen in Table 5.2, approximately 55% of proteins and 20% of sucrose and acetoin were removed from *B. subtilis* fermentation broth with 2% (w/v) of activated charcoal. Thus, in order to remove as many impurities as possible whilst ensure a satisfactory recovery of 2,3-BD, 2% (w/v) activated charcoal was selected as the most appropriate concentration for treatment of the *B. subtilis* fermentation broth.

**Table 5.1** Recovery of 2,3-BD, acetoin, sucrose, proteins, and free amino nitrogen from *S. plymuthica* broth after treatment with activated charcoal at different concentrations

| Activated charcoal (%) | Recovery (%)* | | | | |
|---|---|---|---|---|---|
| | **2,3-BD** | **Acetoin** | **Sucrose** | **Protein** | **FAN** |
| 0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| 0.5 | 92.3±1.8 | 92.8±0.4 | 83.8±1.9 | 78.1±2.1 | 93.1±0.1 |
| 1 | 81.7±0.5 | 80.4±0.3 | 68.5±0.9 | 65.7±1.1 | 81.8±0.2 |
| **1.5** | **81.4±1.2** | **77.1±0.3** | **67.5±0.8** | **59.8±1.0** | **80.5±0.5** |
| 2 | 79.4±1.5 | 75.5±1.2 | 64.3±3.7 | 44.8±0.2 | 78.4±2.2 |
| 3 | 71.4±1.6 | 66.4±1.1 | 55.1±0.2 | 26.7±1.6 | 71.7±1.9 |
| 4 | 66.7±0.2 | 60.3±0.3 | 52.8±0.4 | 20.6±0.4 | 70.6±0.4 |
| 5 | 66.5±0.0 | 58.2±0.1 | 50.8±2.2 | 17.3±1.0 | 70.1±0.2 |

* Initial concentrations in the fermentation broth of *S. plymuthica*: 27.57 g/L 2,3-BD, 5.46 g/L acetoin, 10.28 g/L sucrose, 0.47 g/L protein, and 116.86 mg/L FAN.

**Table 5.2** Recovery of 2,3-BD, acetoin, sucrose, proteins, and free amino nitrogen from *B. subtilis* broth after treatment with activated charcoal at different concentrations

| Activated charcoal (%) | Recovery (%)* | | | | |
|---|---|---|---|---|---|
| | **2,3-BD** | **Acetoin** | **Sucrose** | **Protein** | **FAN** |
| 0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| 0.5 | 94.1±0.9 | 94.4±0.5 | 93.5±1.0 | 76.9±0.7 | 87.6±0.9 |
| 1 | 91.2±0.1 | 90.3±3.7 | 89.4±4.9 | 63.9±0.4 | 80.8±0.6 |
| 1.5 | 88.0±1.2 | 86.8±1.4 | 85.7±1.7 | 56.6±0.4 | 76.4±1.3 |
| **2** | **86.5±0.7** | **84.5±0.6** | **83.5±0.4** | **44.0±0.3** | **72.6±0.9** |
| 3 | 79.9±0.6 | 77.9±0.4 | 76.0±0.9 | 33.6±0.7 | 70.0±0.2 |
| 4 | 77.7±0.7 | 75.3±0.9 | 72.2±0.9 | 28.6±1.2 | 65.5±1.1 |
| 5 | 75.7±0.4 | 73.5±0.5 | 69.6±0.4 | 24.3±2.1 | 64.7±0.9 |

* Initial concentrations in the fermentation broth of *B. subtilis* fermentation broth contained: 42.77 g/L 2,3-BD, 10.80 g/L acetoin, 14.66 g/L sucrose, 0.17 g/L protein, and 86.4 mg/L (FAN).

### 5.3.4 Screening of salts for $[C_2mim][CF_3SO_3]$-based extraction

In several studies, $[C_4mim][Cl]$ (an IL commonly used in the pretreatment of lignocellulosic biomass) has been used during screening of salts in extraction experiments for the recovery of 2,3-BD and phase formation [12]. Even though $[Cl]^-$ and $[BF4]^-$ anions of ILs are frequently employed in extraction and separation of bioproducts from biomass [40], $[C_4mim][BF_4]$ could not form aqueous two phases with potassium salt, such as $K_2HPO_4$ [12]. In the case of 1,3-propanediol (1,3-PD), the anion $[CF_3SO_3]^-$ could perform stable ILs and $[C_4mim][CF_3SO_3]$ exhibited good efficiency in the recovery of 1,3-PD. When IL extraction system is coupled with the same cation such as alkyl imidazolium, decreasing anion toxicity was reported as $[NTf_2]^- \geq [PF_6]^- > [CH_3SO_4]^- > [BF_4]^- > [CF_3SO_3]^- > [Br]^- \approx [Cl]^-$ [41-43]. Imidazolium cation with a shorter alkyl chain is relatively nontoxic, whilst tetra-alkyl phosphonium, $[P66614]^+$-

based ILs showed similar toxicity to imidazolium salt [44]. Apart from $[C_4mim]^+$, ILs containing $[C_2mim]^+$ or $[C_2OHmim]^+$ have been also investigated as they presented higher hydrophilicity and lower toxicity compared to $[C_4mim]^+$ [45]. However, $[C_2OHmim]^+$ could not form aqueous two phases with potassium salt. The obvious disadvantage of ionic liquids is that they are relatively expensive compared to traditional organic solvents. However, costs could be minimised by selecting less expensive IL and by evaluating IL recyclability potential.

Based on the above, $[C_2mim][CF_3SO_3]$ was investigated for its ability to recover 2,3-BD from the fermentation broth in the present study. The phase forming ability correlates with the ability to create water-ion hydration complexes, which depend upon the hydrogen bond accepting ability (β) of ILs anions. A hydrogen bond acceptor is an electronegative atom of an ion or molecule, which contains a non-bonding electron pair that can accept hydrogen from other atom, and is capable of establishing a hydrogen bond. With increasing capacity of anions as hydrogen bond acceptors, the phase forming ability of ILs with the same cation decreases [46]. On the other hand, lower hydrogen bond accepting ability (β) of an anion demonstrates limited ability to form coordinated bonding between IL and water, leading to easy salting out by $K_2HPO_4$ [47]. It seems that lower β and higher α values in ILs lead to an induction of phase separation and improvement of product recovery yield. Kurnia et al.[48] revealed that the hydrogen bond donating ability (α) of $[C_2mim]^+$ was higher than that of $[C_4mim]^+$, leading to a greater recovery yield of 2,3-BD in the system of $[C_2mim][CF_3SO_3]$ compared to that of $[C_4mim][CF_3SO_3]$.

The pH of sample solution is one parameter affecting the efficiency of extraction. In particular, the pH of solution could affect salting out ability during separation by changing the degree of dissociation and solubility of inorganic salt, thus impacting on

extraction ability [49]. However, several studies reported no significant change in the recovery of 2,3-BD in the presence of varying pH values [50].

In this study, five different salts, namely $K_2HPO_4$, $(NH_4)_2SO_4$, $NaH_2PO_4$, $K_3PO_4$, and $K_2CO_3$, were investigated with $[C_2mim][CF_3SO_3]$ for phase separation and extraction of 2,3-BD from *S. plymuthica* fermentation broth. In general, aqueous two phases were obtained in ILs- based salting out system, with the IL-rich and the salt-rich phases represented as the top and the bottom phases, respectively. As observed in Figure 5.5, aqueous two phases were formed when the system contained $K_2HPO_4$, $(NH_4)_2SO_4$, $NaH_2PO_4$, $K_3PO_4$, and $K_2CO_3$, and among aforementioned types of salt, most of 2,3-BD and IL were recovered in the top phase of the system. Even though $(NH_4)_2SO_4$ could also provide phase separation, most of 2,3-BD and IL contents were recovered in the bottom phase. The reason for this is that the density of salt-rich phase (top phase) was lower than that of the IL-rich phase (bottom phase). Moreover, it should be noted that salt precipitation occurred when $K_2CO_3$ was used with $[C_2mim][CF_3SO_3]$. This could be caused by the IL binding strongly to water molecules, which led to IL-rich phase containing large amount of water. At the same time, the salt-rich phase became saturated as its water content decreased, contributing to $K_2CO_3$ (salt) precipitating. However, it has been reported in the study of Dai [33] that solid-liquid two phases could be observed in $[C_4mim][Cl]$ - $(NH_4)_2SO_4$ extraction systems during 2,3-BD recovery.

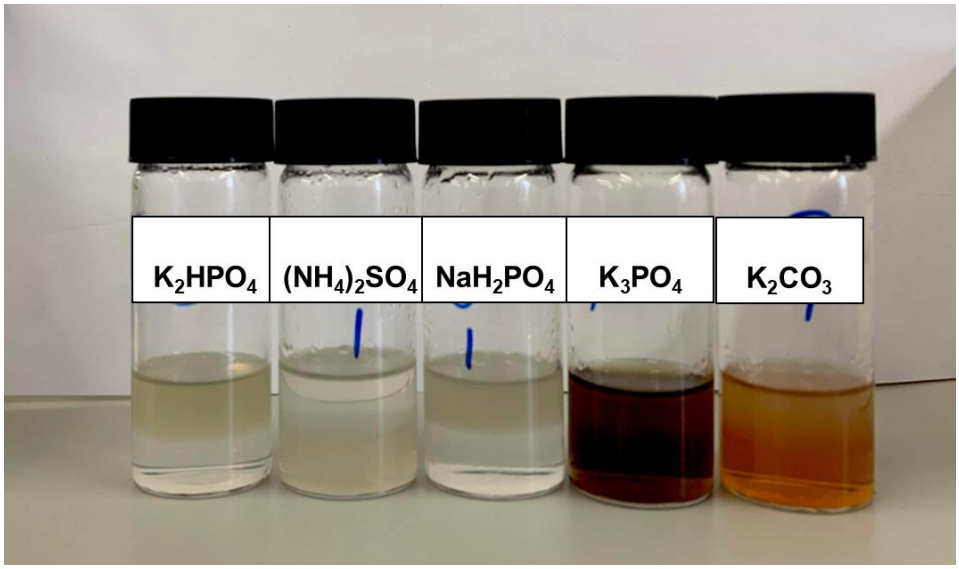


**Figure 5.5** Phase separation obtained from [$C_2$mim][$CF_3SO_3$] extraction with different salts and allowed to stand for 18-24 h for the recovery of 2,3-BD from *S.plymuthica* fermentation broth

Table 5.3 shows the results of the recovery of 2,3-BD ($Y_{BD}$), partition coefficient ($K_{BD}$), and phase ratio (R) obtained from [$C_2$mim][$CF_3SO_3$]-based salting out extraction with various types of salts. The distribution of 2,3-BD was impacted by the hydrogen bond forming ability of anion ($[CF_3SO_3]^-$) of IL system. The $K_{BD}$ values obtained from [$C_2$mim][$CF_3SO_3$]- based salting out extraction with five tested salts including $K_2HPO_4$, $(NH_4)_2SO_4$, $NaH_2PO_4$, $K_3PO_4$, and $K_2CO_3$ were 12.55, 0.48, 3.67, 12.51, and 3.05, respectively, while the 2,3-BD recovery values were 94.55, 28.69, 79.21, 93.62, and 80.84%, respectively. Moreover, R values of 1.38, 0.84, 1.05, 1.18, and 1.45 were obtained, respectively.

**Table 5.3** Extraction ability parameters obtained from [$C_2$mim][$CF_3SO_3$] system with different salts for extraction of 2,3-BD from *S. plymuthica* fermentation broth

| Type of Salt | Recovery ($Y_{BD}$) | Partition coefficient ($K_{BD}$) | Phase volume ratio (R) |
|---|---|---|---|
| $K_2HPO_4$ | 94.55±0.08 [a] | 12.55±0.32 [a] | 1.38±0.09 [c] |
| $(NH_4)_2SO_4$ | 28.69±4.71 [c] | 0.48±0.03 [c] | 0.84±0.12 [a] |
| $NaH_2PO_4$ | 79.21±2.35 [b] | 3.67±0.03 [b] | 1.05±0.17 [ab] |
| $K_3PO_4$ | 93.62±0.39 [a] | 12.51±0.31 [a] | 1.18±0.13 [bc] |
| $K_2CO_3$ | 80.84±2.54 [b] | 3.05±0.16 [b] | 1.45±0.01 [d] |

The IL extraction systems comprised of 2mL ionic liquid, 2 g of salt, and 4 mL of the *S. plymuthica* fermentation broth after treated with 1.5% activated charcoal. Indicated values are reported as means ± standard deviation. Values with the different superscript letters (within same column) are significantly different ($p < 0.05$).

The $K_{BD}$ values obtained from this study, were relatively similar to $K_{BD}$ values from imidazolium-based salting out extraction of 1,3-PD reported in the study of Müller et al. [26] (between 3 and 16.5). The greater values of $K_{BD}$ obtained in IL-based $K_2HPO_4$ and $K_3PO_4$ systems represented a higher solubility of 2,3-BD in IL-rich phase than the salt-rich phase. Higher recovery and partition coefficient of 2,3-BD were achieved when $K_2HPO_4$ and $K_3PO_4$ were employed as salt in the salting-out extraction system. As seen in Table 5.3, no significant differences in $K_{BD}$ and $Y_{BD}$ values obtained in IL-based $K_2HPO_4$ and $K_3PO_4$ systems were observed ($p>0.05$). The system of [$C_2$mim][$CF_3SO_3$] - $K_3PO_4$ provided relatively high $K_{BD}$ and $Y_{BD}$ values, which were close to those of [$C_2$mim][$CF_3SO_3$] - $K_2HPO_4$. The reason might be that the volume of the top phase in [$C_2$mim][$CF_3SO_3$] - $K_2HPO_4$ was higher than that in [$C_2$mim][$CF_3SO_3$] - $K_3PO_4$ because of a higher hydration ability of $K_3PO_4$, resulted in a lower of its 2,3-BD recovery yield.

Although $[C_2mim][CF_3SO_3]$ - $K_3PO_4$ system gave a smaller R value compared to $[C_2mim][CF_3SO_3]$ - $K_2HPO_4$, and that would be good for the further purification process. However, a dark brown colour was generated from the mixture of $[C_2mim][CF_3SO_3]$ - $K_3PO_4$ extraction as shown in the Figure 5.5. The strong alkaline properties of $K_3PO_4$ could affect fermentation broth stability and generated an exothermic reaction when the fermentation broth was mixed with $K_3PO_4$, which resulted in colour change (dark brown). Even though some inorganic salts such as $K_2CO_3$, $K_3PO_4$, $K_4P_2O_7$ have been reported their effectiveness towards enrichment of tertiary butanol and were used for separation of 1,3-propanediol and 2,3-BD aqueous solutions [51-54]. Nevertheless, the use of $K_2CO_3$ as salt in this study provided low partition coefficient (3.05) and generated an orange colour (Figure 5.5), which might be caused by the reaction of IL and strong alkaline properties of $K_2CO_3$. Taking into account recovery values, partition coefficient, and convenience for operation, $K_2HPO_4$ was chosen together with $[C_2mim][CF_3SO_3]$ for further studies of recovering 2,3-BD from the fermentation broth.

### 5.3.5 Impact of the concentration of $[C_2mim][CF_3SO_3]$ and $K_2HPO_4$ on the phase ratio (R)

The phase ratio (R) is an important parameter for practical applications. In the aqueous two phases system (ATPS), a large R value usually indicates significant amounts of water in the top phase compared to the bottom phase, which would require more energy input for water removal in the subsequent process of product purification. The R value for IL system should be less than 1 as it would be beneficial for water removal of the top phase. As seen in Figure 5.6, the phase volume ratio (R) of the top to

bottom phase decreased with increasing amount of $K_2HPO_4$, whilst increased when [$C_2$mim][$CF_3SO_3$] concentrations increased. However, at 30% and 35% of IL, relatively close values of R were obtained at same concentrations of salt from 25% to 35%. Moreover, most conditions in this study yielded R values close to or less than 1. Wan et al. [50] found that higher salt concentration was beneficial for phase formation and led to shorter phase equilibrium time. Additionally, it was reported that in [$C_2$mim][$CF_3SO_3$] - $K_2HPO_4$ systems that a third phase or interphase could be formed by the accumulation of precipitated substances at the interface between the top and bottom phases. It should be noted that protein precipitation was observed between the top and bottom phase in this IL-sating out system which was in agreement of the work of Müller [26]. However, in this study the fermentation broth was pretreated with activated charcoal, thus very small amount of protein was present.

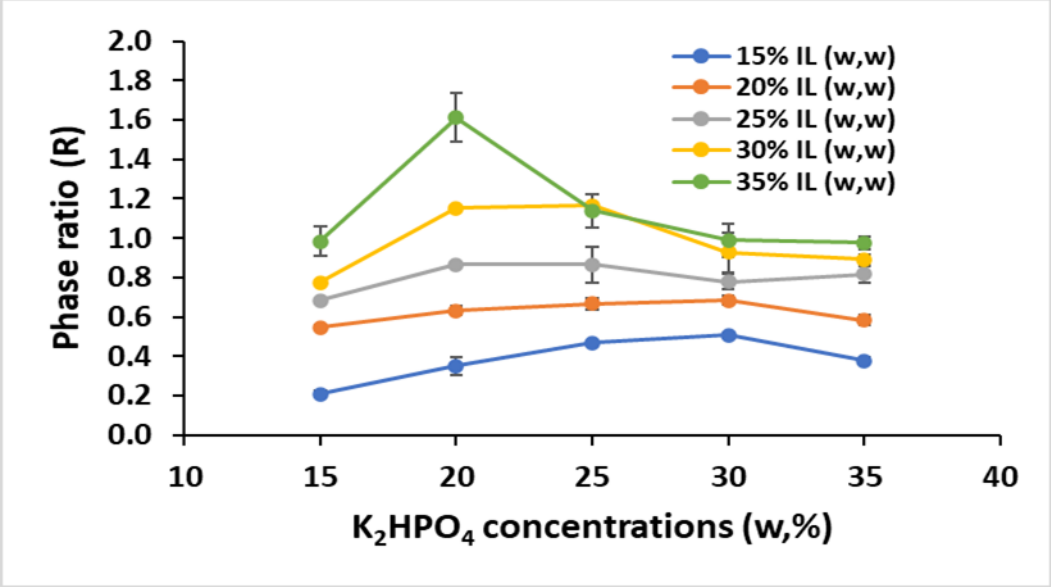


**Figure 5.6** Phase ratio from extraction of 2,3-BD from *S. plymuthica* fermentation broth with different concentrations of [$C_2$mim][$CF_3SO_3$] and $K_2HPO_4$.

### 5.3.6 Effect of the concentration of $[C_2mim][CF_3SO_3]$ and $K_2HPO_4$ on partition coefficient of 2,3-BD

The partition behavior of 2,3-BD in $[C_2mim][CF_3SO_3]$ - $K_2HPO_4$ was explored using *S. plymuthica* fermentation broth. As observed in Figure 5.7, when the concentrations of $[C_2mim][CF_3SO_3]$ and $K_2HPO_4$ increased, the partition coefficient of 2,3-BD ($K_{BD}$) values were increased due to an increase of salting out effect. 2,3-BD is considered a hydrophilic biomolecule. The results indicated that the partitioning behavior increased with IL hydrophilic nature. As seen in Figure 5.7, an increasing trend in $K_{BD}$ values occurred with increasing concentrations of $K_2HPO_4$ and $[C_2mim][CF_3SO_3]$. $K_{BD}$ values obtained with varied IL and salt concentrations ranged from 3 to 25. More specifically, at the same concentration of IL, the $K_{BD}$ values were notably increased when $K_2HPO_4$ concentrations of 25% to 35% were employed in the extraction system. This result was in consistent with studies carried out in organic solvent-based salting out extraction, in which $K_{BD}$ values showed increasing trend when increasing salt concentrations [55, 56]. Moreover, increased $K_2HPO_4$ concentrations led to increased salting out effect, contributing to increased partitioning of 2,3-BD into the top phase. This result was similar to the extraction characteristic of 1,3-propanediol by using ionic liquid based aqueous biphasic system [57]. The product partitioning is commonly regulated by solute-solvent interactions, including hydrogen-bonding, electrostatic forces and van der waals, and steric and conformational effects. Differences in targeted product distribution in the two phases depend upon the properties of the two aqueous phases and those of the solute. With the same salt, the properties of an aqueous phase in IL-based salting out system are impacted by the IL structure, and in particular, the hydrogen bond forming ability. It could be implied that hydrogen bond donating

ability (α) of [$C_2$mim][$CF_3SO_3$] was much stronger than the hydrogen bond accepting ability (β). Moreover, hydrophilicity ILs could provide better solubility and distribution ability than the hydrophobicity ones. The $K_{BD}$ of 24.5 from this study is higher than the $K_{BD}$ of 1.06 that has been reported by Garcia-Chavez et. al [58] who use Tetraoctyl ammonium 2-methyl-1-naphthoate [TOA MNaph], which is hydrophobic IL system for extracting 2,3-BD from the fermentation broth. However, they claimed that extraction of diols has been less investigated and was found to be more difficult compared to the extraction of alcohols such as 1-butanol. Also, large amounts of extractant were required to obtain high recovery yield. This reflects the better extraction ability of hydrophilic ILs than hydrophobic ILs system. Thus, the IL used in this study reaches the desirable properties of extractant, which offers high extraction efficiency and selectivity, whilst less cost and toxicity.

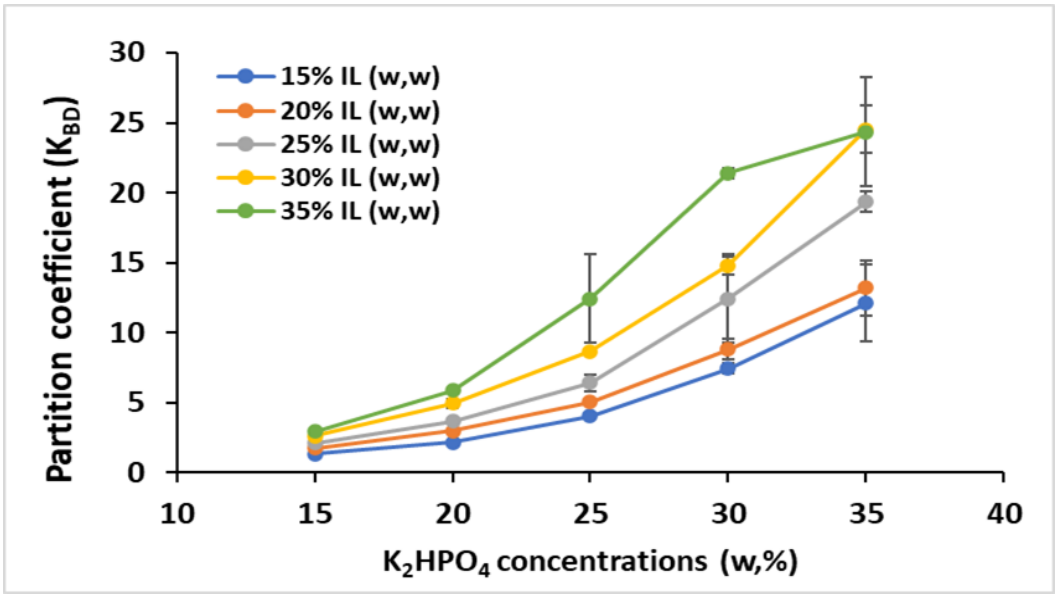


**Figure 5.7** Partition coefficient from extraction of 2,3-BD from *S. plymuthica* fermentation broth with different concentrations of [$C_2$mim][$CF_3SO_3$] and $K_2HPO_4$.

### 5.3.7 Effect of the concentration of $[C_2mim][CF_3SO_3]$ and $K_2HPO_4$ on the recovery of 2,3-BD

The recovery of 2,3-BD was found to be roughly correlated with the amount of IL in the top phase. Thus, variation of IL and salt concentrations in IL-based salting out extraction were evaluated aiming to find the smallest IL and salt concentrations that exhibited high extraction efficiency for recovering 2,3-BD from the fermentation broth. As observed in Figure 5.8, the gradual increase in the recovery of 2,3-BD seemed to be influenced by increasing $K_2HPO_4$ concentrations rather than increasing $[C_2mim][CF_3SO_3]$. The extractability or the recovery yield of 2,3-BD increased with an increase in the salt concentration at the same amount of IL. The highest recovery yield ($Y_{BD}$) was achieved with 30% (w/w) of $[C_2mim][CF_3SO_3]$ and 35% (w/w) of $K_2HPO_4$. Furthermore, over 95% of 2,3-BD was extracted from fermentation broth when the concentrations of $[C_2mim][CF_3SO_3]$ and $K_2HPO_4$ were higher than 30% (w/w). In $[C_2mim][CF_3SO_3]$ system, the recovery of 2,3-BD ($Y_{BD}$) was gradually increased as concave curves, similar to those obtained in organic solvent-based salting out system [55, 59]. This can be explained by the fact that, as the salt concentrations increase, the compatibility between top and bottom phases decrease. However, when increasing the concentrations of IL and salt from 30% to 35%, no significant increase in extraction efficiency was observed (the recovery and partition coefficient values). Thus, 30% (w/w) of $[C_2mim][CF_3SO_3]$ - 35% (w/w) of $K_2HPO_4$. was chosen as the best composition for extraction of 2,3-BD from the *S. plymuthica* broth.

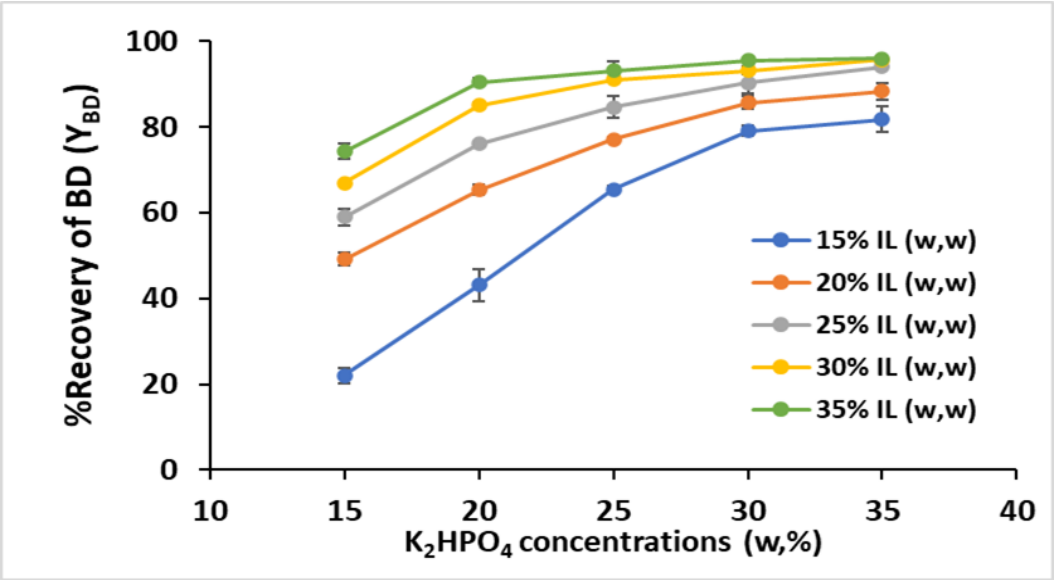


**Figure 5.8** The recovery of 2,3-BD from *S. plymuthica* fermentation broth with different concentrations of $[C_2mim][CF_3SO_3]$ and $K_2HPO_4$.

In order to obtain an appropriate composition of $[C_2mim][CF_3SO_3]$ and $K_2HPO_4$ that provides the most effective extraction ability of 2,3-BD from the fermentation broth with the smallest IL amount, the nine systems that yielded a 2,3-BD recovery of over 90% were compared. As seen in the Table 5.4, no significant differences between recovery yield (>94%) were seen in 35% IL - 35% salt, 30% IL - 35% salt, 35% IL - 30% salt, and 25% IL - 35% salt systems. While 35% IL - 35% salt and 30% IL - 35% salt systems offered highest partition coefficient value (>24), they provided reasonably good phase ratio (less than 1). Considering the aforementioned criteria, the system composed of 30% $[C_2mim][CF_3SO_3]$ and 35% $K_2HPO_4$ offers notably high recovery yield (95.63%) and partition coefficient (24.55) of 2,3-BD, and reasonable phase volume ratio (0.89) and was chosen as the best composition for the extraction of 2,3-BD from fermentation broth of *S. plymuthica*.

**Table 5.4** Comparison of the nine systems of $[C_2mim][CF_3SO_3]$ and $K_2HPO_4$ that provide high recovery and extraction ability of 2,3-BD from *S. plymuthica* fermentation broth

| ILs-salt system $[C_2mim][CF_3SO_3]$, $K_2HPO_4$ | Recovery (%) ($Y_{BD}$) | Partition coefficient ($K_{BD}$) | Phase volume ratio (R) |
|---|---|---|---|
| 35%, 35% | 95.92±0.49 [a] | 24.36±3.86 [a] | 0.98±0.03 [bc] |
| **30%, 35%** | **95.63±0.18 [a]** | **24.55±1.73 [a]** | **0.89±0.02 [ab]** |
| 35%, 30% | 95.47±0.30 [a] | 21.39±0.33 [ab] | 0.99±0.08 [bc] |
| 25%, 35% | 94.04±0.09 [a] | 19.35±0.72 [bc] | 0.82±0.04 [ab] |
| 30%, 30% | 93.17±0.96 [ab] | 14.81±0.63 [cd] | 0.93±0.10 [ab] |
| 35%, 25% | 93.14±2.10 [ab] | 12.42±3.15 [de] | 1.14±0.09 [cd] |
| 30%, 25% | 91.02±0.08 [b] | 8.69±0.08 [ef] | 1.17±0.00 [d] |
| 35%, 20% | 90.41±0.91 [b] | 5.87±0.16 [f] | 1.61±0.12 [e] |
| 25%, 30% | 90.34±2.65 [b] | 12.45±3.14 [de] | 0.78±0.04 [a] |

The IL extraction systems comprised of ionic liquid, salt, and the *S. plymuthica* fermentation broth after treated with 1.5%activated charcoal. Indicated values are reported as means ± standard deviation. Values with the different superscript letters (within same column) are significantly different ($p < 0.05$).

### 5.3.8 Selectivity of 2,3-BD over other fermentation compounds

Apart from the target product (2,3-BD), the fermentation broth also contains various impurities. The selectivity evaluation offers a comparison of the extraction behaviour of 2,3-BD over other compounds. If the selectivity value is equal to one, the distribution coefficient of 2,3-BD is same as that of other components. On the other hand, if the selectivity value higher than one, it denotes that the $K_{BD}$ of 2,3-BD is greater than those components. As seen in Figure 5.9a, acetoin was extracted with relatively equal degree to 2,3-BD, in which the selectivity was varied from 0.3 to 2 (close to 1) for

all conditions (varied IL and salt concentrations). Specifically, at [30% IL and 35% $K_2HPO_4$], [35% IL and 30% $K_2HPO_4$], and 35% IL and 35% $K_2HPO_4$, the selectivity of acetoin was higher than 1, meaning that acetoin was extracted in a less selective manner than 2,3-BD. Most of the selectivity values for acetoin were less than 2.5, indicating that acetoin was extracted into the top phase. This reflects a difficulty in separating 2,3-BD and acetoin.

In contrast to acetoin, other components (sucrose, succinic acid, and acetic acid) showed increasing trends of selectivity in higher %IL and $K_2HPO_4$ concentrations. Specifically, it was observed (Figure 5.9b) that sucrose is extracted to a far lesser degree than 2,3-BD when %IL increased at the same amount of $K_2HPO_4$. More specifically, in $K_2HPO_4$ above 20%, the selectivity of 2,3-BD over sucrose was much greater than one (5 to 80), which means that 2,3-BD was more partitioned to the top phase (IL-rich phase) than sucrose under these conditions.

In case of succinic acid (Figure 5.9c), significant increase of selectivity (10 to 175) was seen in 25 to 35% of both IL and $K_2HPO_4$ systems. This suggested that 2,3-BD is much more selectively extracted over succinic acid to the top phase. This result was in consistent with the study of Dai et al. [33] who revealed that when $C_2$mim][$CF_3SO_3$] - $K_2HPO_4$ was employed, most of succinic acid was extracted to the bottom phase (salt-rich phase).

For acetic acid (Figure 5.9d), increasing trends were observed, as acetic acids were extracted to lesser degree than 2,3-BD in all IL and salt compositions. This leads to selectivity of 2,3-BD over acetic acid in all [$C_2$mim][$CF_3SO_3$]- $K_2HPO_4$ compositions.

However, in the present study, the partition coefficient of lactic acid was very low and difficult to quantify ($K_{LA} \approx 0$). The possible reason might be that lactic acid was

mostly extracted to the bottom phase rather than the top phase and became too diluted to be detected via HPLC.

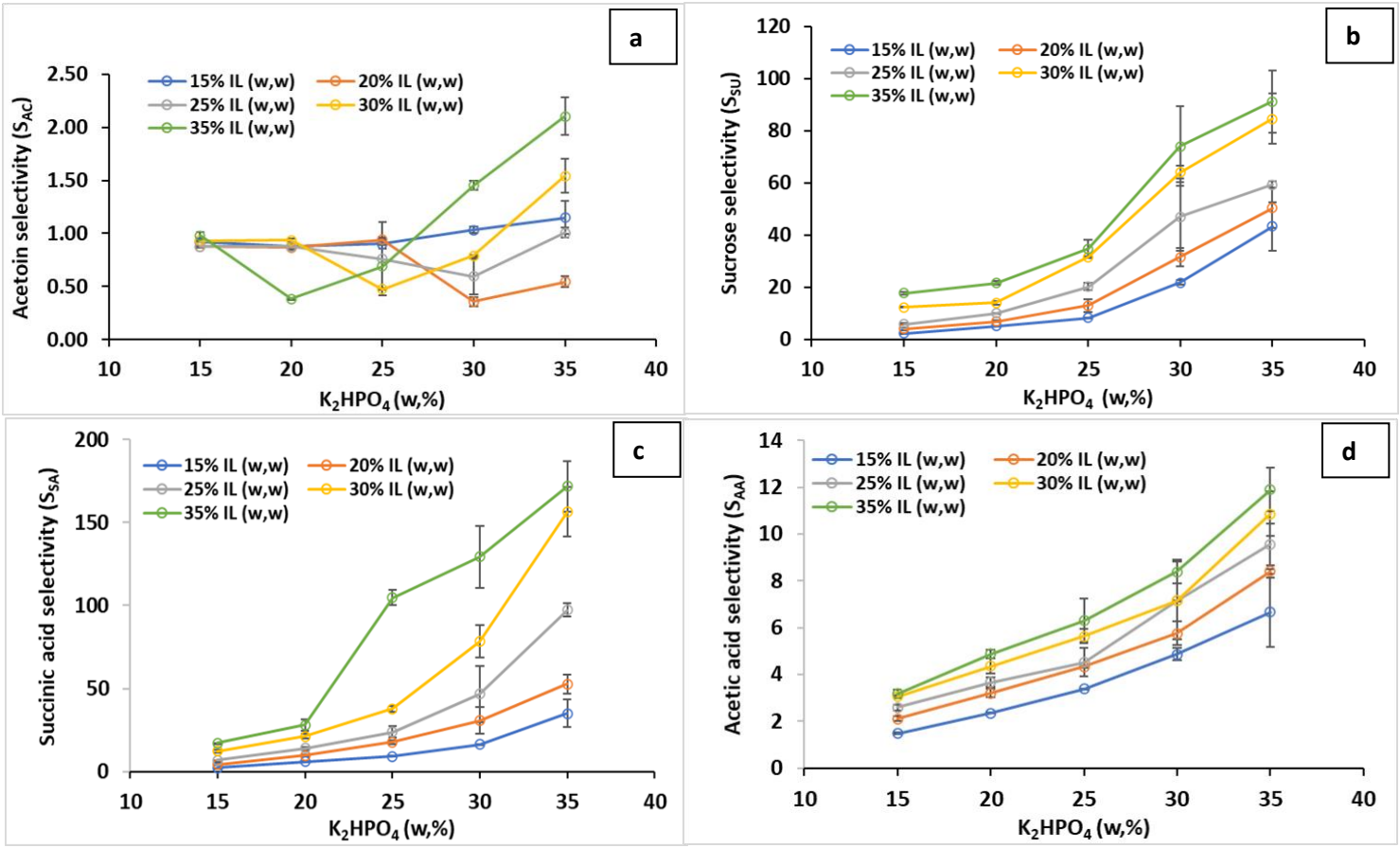


**Figure 5.9** Selectivity of 2,3-BD over (a) acetoin, (b) sucrose, (c) succinic acid, and (d) acetic acid using $[C_2mim][CF_3SO_3]$ - $K_2HPO_4$ as IL extraction system.

### 5.3.9 Impact of fermentation broth composition on extraction efficiency

In order to verify the effectiveness of the purposed system towards 2,3-BD recovery, the impact of fermentation broth composition was evaluated in this study. It was hypothesised that compositional variation of the fermentation broth would also denote polarity differences, thus ILs separation efficiency could vary [60]. As such, the three most efficient systems of $[C_2mim][CF_3SO_3]$ - $K_2HPO_4$ were used to evaluate their

effectiveness towards 2,3-BD recovery from *B. subtilis* fermentation broth. Moreover, in order to minimise the step of separation and purification process, based on the most effective system obtained from *S. plymuthica* study, 30% $[C_2mim][CF_3SO_3]$ - 35% $K_2HPO_4$ system was employed for extraction in both of original fermentation broths (*S. plymuthica* and *B. subtilis*) without the treatment with activated charcoal.

As seen in Table 5.5, the 2,3-BD recovery yield (95%) from *B. subtilis* fermentation broth was similar to *S. plymuthica* one, under the same composition of 30% $[C_2mim][CF_3SO_3]$ - 35% $K_2HPO_4$ extraction system. However, the $K_{BD}$ value from *S. plymuthica* (24.55) fermentation broth was greater than that of *B. subtilis* (14.16). The possible reason could be that higher concentration of organic acids were presented in *S. plymuthica* broth. It has been reported that the presence of organic acids in the fermentation broth can lead to an increase in distribution coefficient (K) of 1,3-PD [26]. As molecules of organic acids contain charges (ion), i.e. proton ($H^+$), thus that could increase its ability to form hydration complex with water and improve phase forming ability, leading to an increase of partition coefficient value. This result could also imply that higher 2,3-BD concentrations require higher dosages of IL and salt for extraction. As expected, when IL concentration was increased from 30% to 35%, the 2,3-BD recovery in *B. subtilis* broth increased to 97.3%, whilst the recovery yield of 2,3-BD in *S. plymuthica* was not improved. This result was in agreement with Wan et al. [50] who reported that the extraction efficiency and distribution coefficient of 2,3-BD improved with increased solute concentrations. As solute (2,3-BD) concentration increased, more 2,3-BD molecules were consequently moved from water-rich phase (bottom phase) to IL-rich phase (top phase). Indeed, the phase volume ratio increased from 0.9 (in *S. plymuthica* broth) to 1.5 (*B. subtilis* broth).

The extraction behavior as denoted by the phase ratio (R), partition coefficient of 2,3-BD ($K_{BD}$), recovery of 2,3-BD ($Y_{BD}$) parameters, was also investigated in untreated and treated fermentation broths. The results show that extraction efficiency of untreated broth was slightly lower (93% to 94%) than that of activated charcoal treated samples, indicating that biomacromolecules in the fermentation broth might possess adverse effect on the extraction behaviour. Thus, $[C_2mim][CF_3SO_3]$ - $K_2HPO_4$ was highly selective towards 2,3-BD over other compounds in the fermentation broth since most of those organic acids were extracted to the bottom phase, biomolecules were partitioned into the interphase.

Furthermore, the recovery of IL was also investigated in this study. As it can be seen in Table 5.5, in case of *S. plymuthica* broth extraction with 30% $[C_2mim][CF_3SO_3]$ and 35% $[C_2mim][CF_3SO_3]$, the recovery of IL was 96% to 98%. For *B. subtilis* broth extraction, 90% of 35% $[C_2mim][CF_3SO_3]$ could be recovered. This indicated that $[C_2mim][CF_3SO_3]$ could be recovered successfully from the system and possibly re-used for the separation of 2,3-BD. This result was similar to those from the study of Müller et al. [26] who claimed that $[C_4mim][CF_3SO_3]$ could be recovered and re-utilised for the separation process of 1,3-PD.

**Table 5.5** Comparison of different systems of $[C_2mim][CF_3SO_3]$ and $K_2HPO_4$ on extraction ability in two different compositions of fermentation broths

| Bacterial strains | AC (%w/v) | IL/salt system (%wt) | $Y_{BD}$ | $K_{BD}$ | R | $S_{SU}$ | $S_{AC}$ | $Y_{IL}$ |
|---|---|---|---|---|---|---|---|---|
| | 2 | 35/35 (%wt) | 97.28±0.5 | 24.32±4.6 | 1.50±0.1 | 50.63±1.1 | 1.36±0.4 | 90.3 |
| | 2 | 35/30 (%wt) | 97.31±0.3 | 24.68±3.4 | 1.48±0.1 | 56.61±5.4 | 1.11±0.1 | 90.0 |
| ***B. subtilis*** | 2 | 30/35 (%wt) | 95.14±0.7 | 14.16±2.3 | 1.40±0.1 | 30.15±6.7 | 1.05±0.2 | 89.7 |
| | 0 | 30/35 (%wt) | 94.30±1.1 | 10.87±2.2 | 1.55±0.1 | 25.69±5.5 | 0.70±0.2 | ND |
| | 1.5 | 35/35 (%wt) | 95.92±0.5 | 24.36±3.8 | 0.98±0.03 | 91.23±1.9 | 2.10±0.2 | 96.6 |
| | 1.5 | 35/30 (%wt) | 95.47±0.3 | 21.39±0.3 | 0.99±0.1 | 74.29±5.2 | 1.45±0.1 | 97.8 |
| ***S. plymuthica*** | 1.5 | 30/35 (%wt) | 95.63±0.2 | 24.55±1.7 | 0.89±0.1 | 84.61±9.6 | 1.54±0.2 | 96.1 |
| | 0 | 30/35 (%wt) | 93.10±0.1 | 17.54±0.3 | 0.77±0.1 | 64.54±2.3 | 1.21±0.1 | ND |

The IL-based salting out extraction systems comprised of ionic liquid, salt, and the fermentation broths with and without the treatment with activated charcoal. Indicated values are reported as means ± standard deviation. AC: activated charcoal; $Y_{BD}$: recovery of 2,3-BD; $K_{BD}$: partition coefficient; R: phase volume ratio; $S_{SU}$: selectivity of BD over sucrose; $S_{AC}$: selectivity of BD over acetoin; $Y_{IL}$: recovery of IL; ND: not determined.

The separation of bio-based chemicals with ATPS using ILs and organic solvents has been reported by several research teams and their results are summarised in Table 5.6.

**Table 5.6** Aqueous two phase systems and extraction efficiency in the literature for recovery of bio-based chemicals

| Solution component | ATPS | Target product | Y (%) | K | R | References |
|---|---|---|---|---|---|---|
| Fermentation broth | 6%[EOAB] - 38%$K_3PO_4$ | Acetoin, 2,3-BD | $Y_{AC}$ (92.7) $Y_{BD}$ (86) | $K_{AC}$ 40.5 | ND | [61] |
| Fermentation broth | 35%[$C_4$mim][Cl] - 8%$K_2HPO_4$ | 2,3-BD | 96 | 5.2 | 5 | [33] |
| Artificial solution of 5% 1,3-PD, 0.5%acetic acid, 0.5%butyric acid | [$C_4$mim][$CF_3SO_3$] - Phosphate | 1,3-PD | ND | 3.4 | ND | [26] |
| Solution of 10%2,3-BD and 5% 1,3-PD | [$P_{66614}$][$C_8SO_3$] | 2,3-BD, 1,3-PD | ND | $K_{BD}$ (0.39) $K_{PD}$ (0.22) | ND | [23] |
| Fermentation broth (50-150 g/L 1,3-PD) | Ethanol/$(NH_4)_2SO_4$ | 1,3-PD | ND | 4.77 | ND | [62] |
| Fermentation broth | 32%Ethanol -16%$(NH_4)_2SO_4$ | 2,3-BD | 91.7 | 7.10 | ND | [63] |
| Fermentation broth | 30%[$C_2$mim][$CF_3SO_3$]-35%$K_2HPO_4$ | 2,3-BD | 95.6 | 24.6 | 0.89 | This study |
| | 35%[$C_2$mim][$CF_3SO_3$]-30%$K_2HPO_4$ | 2,3-BD | 97.3 | 24.3 | 1.5 | |

[EOAB]: [hydroxylammonium][Butyrate]; [C4mim][Cl]:1-butyl-3-methylimidazolium chloride; [$P_{66614}$][$C_8SO_3$]: trihexyl(tetradecyl) phosphonium octanesulfonate

## 5.4 Conclusion

Activated charcoal and $[C_2mim][CF_3SO_3]$-based salting out extraction were investigated for 2,3-BD recovery from two fermentation broths (*S. plymuthica* and *B subtilis* cultures). Macromolecules and colour were removed from *S. plymuthica* and *B. subtilis* fermentation broths with 1.5% (w/v) and 2.0% (w/v) activated charcoal, respectively. High recovery yield of 2,3-BD (95.6% to 97.3%) was achieved, whereas untreated fermentation broths still exhibited satisfactory results (93 to 94% recovery yield). The ionic liquid system composed of $[C_2mim][CF_3SO_3]$ - $K_2HPO_4$ demonstrated a prospective extraction system for the recovery of 2,3-BD from diversified fermentation broths. This method gives emphasis towards high efficiency and less toxicity, and holds potential for novel green separation process, thus promoting cleaner production of bio-based 2,3-BD.

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# Chapter 6

# General Discussion and Future Perspectives

## 6.1 General Discussion

The present work demonstrates an efficient strategy of exploiting two non-pathogenic wild-type bacterial strains, namely *B. subtilis* and *S. plymuthica* for 2,3-BD production. The work further advanced towards the evaluation of integrated processes based on activated charcoal treatment and IL-based salting out extraction of 2,3-BD from the fermentation broths. As research studies on specific 2,3-BD stereoisomer production using non-pathogenic wild-type microorganisms have rarely been conducted, this research provided a useful knowledge of possible biotechnological approaches that could be applied for the production of highly pure 2,3-BD stereoisomers, and for efficient separation of 2,3-BD. Specifically, in Chapter 3, five different bacterial strains from the culture collection of the Department of Food and Nutritional Sciences (University of Reading, UK) were evaluated for their abilities to convert sucrose into 2,3-BD. *B. subtilis* and *S. plymuthica* were selected as promising 2,3-BD producing strains for further studies. A number of microorganisms belonging to the family of *Enterobacteriaceae* such as *Klebsiella pneumoniae* and *Enterobacter aerogenes*, have been reported as efficient 2,3-BD producers. However, their pathogenic nature limits their applications on an industrial-scale. Genetic engineered microorganisms have been extensively investigated to improve 2,3-BD production, however, some drawbacks, such as plasmid stability, are still a concern. Another focus of the work in Chapter 3 was the evaluation of alternative low-cost nitrogen sources to substitute for expensive ones.

Corn steep liquor (CSL) was effectively employed as an alternative nitrogen source. This could be beneficial for large-scale 2,3-BD production in terms of nutrient cost reduction; additionally, it adds economic value to waste or by-products from agri-food industries, and reduces industrial waste discharge to the environment. Oxygen supply is a crucial parameter for microbial 2,3-BD production. Different DO control regimes were applied to evaluate 2,3-BD production in batch bioreactor cultures (Chapter 3). Limited dissolved oxygen supported maximum (R,R)-2,3-BD production at high purity (100%) in *B. subtilis*, whereas high dissolved oxygen conditions promoted bacterial growth and acetoin formation. *S. plymuthica* was reported for the first time (Chapter 4) as capable producer of highly pure (95%) meso-2,3-BD. The influence of dissolved oxygen on the metabolic pathways involved in 2,3-BD biosynthesis was further supported by monitoring the activity of key enzymes involved in 2,3-BD production. A 2 - 2.5-fold higher of AR activity was detected in *B. subtilis* and *S. plymuthica* compared to that of BDH in low dissolved oxygen conditions, indicating that the reductive reaction for acetoin conversion to 2,3-BD was more active in both strains than the oxidative reaction, thus promoting 2,3-BD production. However, it was also noted that in both strains, BDH exhibited reversible conversion activity, converting 2,3-BD back to acetoin under carbon limited conditions, as a cell maintenance mechanism. These findings were further used to inform fed-batch cultivation strategies, aiming to maximise 2,3-BD, by maintaining optimum DO conditions to suppress the oxidative pathway. Both strains improved 2,3-BD production, with *B. subtilis* achieving a production yield of 0.52 g per g sucrose, accounting for 99% of the maximum theoretical yield.

Even though alternative low-cost nitrogen source, i.e. corn steep liquor was successfully employed to replace expensive nitrogen source (peptone, yeast extract, beef extract), which could reduce the cost of nutrient of the proposed process for 2,3-BD

production. However, a commercial sucrose was used as carbon source for 2,3-BD production and the concentration of 2,3-BD obtained from this process was still lower than a targeted level (80 g/L). Thus, 2,3-BD production by the proposed process might be not economically feasible compared to that of conventional chemical synthesis. Thus, future work on utilization of alternative feedstocks, strain improvement and process development should be performed to improve product concentration, minimize production cost and make the bioprocess more sustainable and economically viable.

Despite the optimization of the fermentation stage, the separation of 2,3-BD from the fermentation broth still represents a challenge, mainly due to its physical properties (high boiling point and high polarity) [1], as well as due to the presence of residual nutrients and fermentation by-products in the fermentation broth. To this end, the development of separation processes for downstream recovery of 2,3-BD from fermentation broth was investigated in Chapter 5. The unavoidable yellowish-brown colour of the culture broth was successfully reduced through treatment with activated charcoal. Subsequently, ionic liquid-based salting out extraction was applied to recover 2,3-BD from two different compositions fermentation broths (*S. plymuthica and B. subtilis*). In case of *S. plymuhica* broth, 95.6% of 2,3-BD was successfully recovered from the fermentation broth with system composed of 30% $[C_2mim][CF_3SO_3]$ - 35% $K_2HPO_4$ . Moreover, 35% $[C_2mim][CF_3SO_3]$ - 30% $K_2HPO_4$ extraction system provided high 2,3-BD recovery (97.3%) from *B. subtilis* broth, thus verified the effectiveness of this extraction method. In addition, it was demonstrated that IL could be effectively recovered (90% to 98%), which would be beneficial in terms of reuse or recycle towards driving costs during downstream process. The use of activated charcoal and IL-based salting out extraction is considered an attractive option, as it provides a safe, less toxic, recyclable and practical operation process with potential for commercial applications.

## 6.2 Future Perspectives

- The research work presented in this thesis has demonstrated the successful production of highly pure (R,R)-2,3-BD (100%) and meso-2,3-BD (95%) from non-pathogenic wild-type *B. subtilis* and *S. plymuthica* strains, respectively. Also, an efficient downstream separation for 2,3-BD recovery from diverse compositions of fermentation broths was demonstrated. The work carried out in this thesis can set the foundation for future research on a number of different avenues. Firstly, even at fed-batch cultures under optimum DO conditions, the maximum 2,3-BD produced was 42.3 g/L; ideally, concentrations higher than 80 g/L are required for commercial scale production and cost-effective downstream processing. As such, future work could be directed towards investigating genetic modification of 2,3-BD producing strains, focusing on improving 2,3-BD production, whilst minimising the generation of metabolite by-products. This could be achieved through driving carbon flux towards desired pathways, deleting pathways responsible for by-product formation, as well as increasing substrate uptake rate with the aim of improving 2,3-BD productivity. Additionally, another strand of future work could represent a pilot scale study, in order to verify the results obtained in 2-L benchtop bioreactor, and obtain more reliable data on scale that could inform commercial scale studies.
- In case of *B. subtilis* (Chapter 3), 2,3-BD concentration (43 g/L) obtained from fed-batch process was still lower than the ideal level of 80 g/L. The limitation from fed-batch process was that sugar consumption rate was slow when sugar dropped below 10 g/L, leading to low 2,3-BD production. Thus, 2,3-BD concentrations could be improved and achieved an ideal level by increasing initial sucrose concentration to 50-80 g/L,

while maintaining sucrose at higher level of 30-50 g/L, and feeding more sugar when the concentration dropped below 20 g/L.

- Also, sucrose contains glucose and fructose sugars that can be used as carbon sources in microbial fermentations. However, glucose is a preferable carbon source for most native microorganisms and is generally catabolized first, while other sugars (i.e. fructose in this study) remained unconsumed in the fermentation media, particularly in case of *S. plymuthica* (Chapter 4). In efficient fermentation media, a complete utilisation of all available carbon sources is required. Thus, future work could be directed towards genetic modification of native bacteria through the deletion of catabolite repressor genes as demonstrated by Jung et al. [2], which could improve fermentation efficiency and rectify the limitation of the strain.

- Furthermore, the economic viability of industrial 2,3-BD production is highly dependent on raw material cost [3]. The carbon source is major raw material for 2,3-BD production. The carbon source employed for 2,3-BD production in this work is sucrose (a commercial carbon source). However, the high cost of sucrose ($0.4 per kg) [4] could contribute to relatively high production costs. Hence, alternative abundant and cheap renewable carbon sources, particularly those containing high amounts of assimilable sugars such as sugarcane molasses, sugar beet molasses, sweet sorghum juices, cane sugar or even fruit wastes, could be considered as potential substrates for 2,3-BD production in future work to achieve economically viable process.

- With regards to downstream process, this work demonstrated that a considerable amount of IL could be recovered (90% to 98%), denoting recyclability potential of IL. The latter could reduce IL costs, as they are currently more expensive compared to conventional organic solvents.

- Moreover, as CSL was successfully employed as a nitrogen source in this work, a techno-economic evaluation should be carried out to determine whether CSL replacement could offer an overall media cost reduction in 2,3-BD production. Also, a techno-economic evaluation study is needed to evaluate the cost-effectiveness of the whole process and establish minimum 2,3-BD concentrations and supply and demand scenarios whereby the suggested process could be economically viable. Moreover, a life-cycle assessment (LCA) study is required to evaluate the sustainability of the suggested approach [5].
- Additionally, future work could investigate the biological activities of 2,3-BD, especially of the optically pure isomers, in order to identify future applications. Several research studies have reported that meso-2,3-BD has antibacterial activity; thus, it could be potentially applied as a natural preservative in cosmetic applications [6]. Linked to its antimicrobial activity, meso-2,3-BD could be used to alleviate and relieve from skin diseases such as eczema [7]. Moreover, meso-2,3-BD was suggested for applications as a natural humectant for cosmetic and skin care products [6]. However, the mechanisms conferring these properties are poorly understood, and could be investigated further. 2,3 BD has also applications in agriculture; Ju et al. [8] reported that (R,R)-2,3-BD produced by *Paenibacillus polymyxa*, could promote the growth of soybean and strawberry seedlings. Kong et al. [9] reported the ability of R,R-BD and meso-2,3-BD to induce plant resistance against pathogenic virus in pepper field trials. To this end, future research should also aim to investigate the mechanisms of actions, which will help to broaden the applications of 2,3-BD.

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