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Bacillus licheniformis isolated from Mozambican soil capable of producing 2, 3-butanediol

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Declaration

I declare that '***Bacillus licheniformis* isolated from Mozambican soil capable of producing 2, 3-butanediol**' is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Date: 01 October 2018

Layla Hendricks

Abstract

Due to the current fossil fuel sources rapidly depleting as a result of the increased global need, alternative, sustainable and renewable sources are required. Biofuels, which are environmentally friendly, meet all the requirements as they can be generated from the biomass of biodegradable waste. Selected species of yeast, bacteria and algae are capable of producing biofuels from a host of substrates. Microorganisms, historically used to manufacture valuable products ranging from the pharmaceutical to food industry, are now employed to generate biofuels.

Several bacteria are native producers of biofuels and do so without requiring any manipulation. Some of the most effective biofuel producers are pathogenic organisms, therefore industrialisation is complicated due to the associated health risks. Generally regarded as safe (GRAS) microorganisms are preferred vehicles for the production of biofuels as they do not pose any risks when manufactured at industrial levels. 2, 3-Butanediol (2, 3-BD) was identified as being a favourable biofuel due its heating value being more favourable than methanol and ethanol. This compound exists in three stereoisomeric forms and organisms often produce a mixture thereof depending on the fermentation conditions.

In this study, five bacterial samples isolated from a hot spring were screened for the production of acetoin, a 2, 3-BD precursor. As mesophilic industrial processes often result in contamination, the hot spring was an ideal screening environment to by-pass the contamination issue. The isolates were identified as *Bacillus licheniformis* with two isolates being closely related to *Bacillus licheniformis* ATCC 14580. The *B. licheniformis* isolate is a GRAS organism known to produce a mixture of meso and dextro-2, 3-BD at a wide range of temperatures while using several different substrates and carbon sources. Nutrient broth (NB), Luria Bertani (LB), Beef extract (BE) and Zymobilis media (ZM), an in-house media, were compared to determine which yielded the highest growth rate. Based on the literature and the results generated in the comparative analysis, LB was selected to determine the effect of various carbon sources on the growth rate of the isolates. Un-supplemented Luria Bertani was compared to LB supplemented with either sucrose, fructose, starch and glucose. A marked increase in cellular density was detected in the carbon-supplemented media. High performance liquid chromatography was used to determine the compounds produced in the glucose-enriched media. We were able to identify 2, 3-BD at 37°C in cultures of all five isolates. Four of the isolates produced only meso-2, 3-BD, which is significant and of great

industrial importance as no downstream applications would be required to separate the two isoforms. Further work can be performed to examine production of 2, 3-BD at elevated temperatures.

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Dedication

Mom and Dad, your support, faith and prayers allowed me to reach this point. I am eternally grateful for all the sacrifices you made so that I could attain my dream.

I love you always.

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List of abbreviations

2, 3-BD	2, 3-butanediol
2, 3-BDH	2, 3-butanediol dehydrogenase
2, 3-butylene	2, 3-butanediol
2, 3-dihydroxybutane	2, 3-butanediol
(2S, 3S)-2, 3-BD	dextro
(2R, 3R)-2, 3-BD	levo
(2R, 3S)-2, 3-BD	meso
16S rRNA	16s ribosomal ribonucleic acid
AACR	acetylacetoin reductase
AACS	acetylacetoin synthase
AHAS	acetohydroxyacid synthase
ALDC	acetolactase decarboxylase
ALS	acetolactase synthase
alsD	acetolactate synthase decarboxylase gene
alsS	acetolactate synthase gene
AR	acetoin reductase
Amp	ampicillin
ARDRA	amplified rDNA restriction analysis
BCAA	branch chain amino acid
BDH	butanediol dehydrogenase
BE	beef extract
Bp	base pair
BSA	bovine serum albumin
cALS	catabolic acetolactase synthase
CCR	carbon catabolite repression
EDTA	ethylendiaminetetraacetatic acid
FAD	flavin adenine dinucleotide
GDH	glycerol dehydrogenase
GRAS	generally regarded as safe
HPLC	high pressure liquid chromatography

IDT	integrated dna technology
IMBM	Institute for Microbial Biotechnology and Metagenomics
IPTG	isopropyl β-d-thiogalactosidase
JA	jerusalem artichoke
MDR	medium chain reductase
MEK	methyl ethyl ketone
NAD^+/NADH	nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
A	absorbance
PCR	polymerase chain reaction
PGPR	plant growth promoting rhizobacteria
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulfate
TAE	tris-acetate-EDTA
ThDP	thiamine diphosphate
Tris	tris-hydroxylmethyl-aminomethane
TSB	tryptone soy broth
X-gal	bromo-chloro-indolyl-galactopyranoside

Chapter 1: Literature Review

1.1. Introduction

In a time where energy resources, specifically fossil fuels, are rapidly depleting (Wang and Yin, 2018), alternatives are required to ensure that the standard of living that we have become accustomed to, can be maintained. At present, fossil fuels account for more than 80% of the energy sources. The combustion of fossil fuel leads to high volumes of pollution and was reported to contribute 42% (Show *et al.,* 2017) and 73% (Berhe and Sahu, 2017) of the total global CO_2 emissions which negatively impacts the environment (Antoni *et al.,* 2007; Chapagain *et al.,* 2009; Lin and Xu 2013; Bialkowska *et al.,* 2015). The use of fossil fuels is therefore causing health-related concerns to living organisms (Fatma *et al.,* 2018). Furthermore, the United States Energy Information Agency estimates a 28% energy consumption increase by 2040 (EIA, 2017).

In response to the need for alternative fuel sources that are renewable, researchers identified biofuels, which can be defined as fuel products generated by using biomass and the biodegradable waste products generated during industrial processes i.e. (Barnard *et al.,* 2010; Buchholz and Collins, 2013; Bialkowska *et al.,* 2015). Additionally, biofuels are environmentally friendly and extensive research has been conducted to produce these compounds at industrial levels. Several research studies are underway to determine ways in which biofuel production can be maximised while remaining cost effective and not posing any risks to the human population. Various organisms such as yeast, bacteria and fungi (Martinez *et al.,* 2015) are able to produce biofuels from different substrates (Hu *et al.,* 2013; Javidnia *et al.,* 2016).

The bacterial production of biofuels is sustainable, environmentally friendly and often more cost-effective than chemical synthesis (Jenkins and Alles, 2011; Green, 2011; Li *et al.,* 2014a; Bialkowska, 2016). This is due to the potential of using low-value waste as substrates (Kandasamy *et al.,* 2016). Waste products such as glycerol, agricultural residues, excess biomass such as wood hydrolysates (Bialkowska, 2016), corncob molasses, whey permeate, starch hydrolysate, sugarcane molasses, inulin and cassava powder (Li *et al.,* 2014c) may be used in the microbial production of biofuels.

Several bacterial strains that produce high yields of biofuels are pathogenic (Bialkowska, 2016; Fu *et al.,* 2016; Yang and Zhang, 2018), e.g. *Klebsiella pneumonia* and *Klebsiella oxytoca* which produce biodiesel (Yang *et al.,* 2016; Rahman *et al.,* 2017). To ensure that there are no negative effects when organisms

are produced at industrial levels, strict regulations are in place (Kim *et al.,* 2016b). Alternatively, researchers employ strategies where the virulence factors of pathogenic strains are removed (Jung *et al.,* 2014), or perform genetic manipulation and transfer the genes responsible for biofuel production to non-pathogenic strains. Various approaches are employed to increase the yields of GRAS strains and these include optimising fermentations and/or performing genetic manipulation. (Rados *et al.,* 2015; de Oliviera and Nicholson, 2016).

1.2. Biofuels

Biofuel sources include staple foods such as maize and other food crops which attracted negative publicity as many resource-limited countries would not be able to produce sufficient crops for food as well as fuel (Koizumi, 2014). In response to this problem, attention was directed at using waste products as substrates such as the corn of the cob as substrates to generate alternative biofuels, which would not impact on food supply (Chemier *et al.,* 2009; Li *et al.,* 2014c).

As countries recognise the importance of biofuels, billions are being spent on developing clean and renewable technologies (Mallick *et al.,* 2016). The United States and Brazil have been driving the need for policies to secure energy since 1970 (Chung and Yang, 2016). Brazil is the second largest producer of bioethanol (Cassman *et al.,* 2018) and launched the Brazilian National Program of Production and Use of Biodiesel (PNPB) in 2014 (Cerri *et al.,* 2017). While United States mainly use soybean in the production of biodiesel (Hood, 2016) and corn in the production of bioethanol (Schwietzke *et al.,* 2009), Brazil relies on sugarcane (Cassman *et al.,* 2018).

The United States has a total of 94 biodiesel plants with an annual capacity to produce 2.4 billion gallons of biodiesel (EIA, 2018). In December 2017, the United States produced 148 million gallons of biodiesel from 1132 million pounds of feedstocks, of which 594 million pounds were derived from soybean oil. A total of 56 million gallons was sold as B100 (100%) diesel and 88 million gallons of B100 was sold in biodiesel blends with diesel fuel derived from petroleum (EIA, 2018). In 2009, Schwietzke *et al*. (2009) reported the production of 7 billion gallons of bioethanol and predicted that this may soon reach 10 billion gallons. In 2014, a total of 14 billion gallons of ethanol was produced from cornstarch in the United States. It is evident that that the production of both bioethanol and biodiesel is extremely high in the United States (Hood, 2016).

The state of Sau Paulo has 5.7 million hectares of land dedicated to the growth of sugarcane, which makes Brazil the highest sugarcane producer with more than 300 processing plants (Cassman *et al.,* 2018). Sugarcane production levels were escalated in response to an increased demand for its use in biofuel production (Cherubin *et al.,* 2016) as it is used as a substrate in the production of butanol and ethanol (Shen *et al.,* 2016). Soil quality is assessed and the sustainability monitored to ensure that the processing plants are able to meet the ever-increasing demands (Cherubin *et al.,* 2016). For example, a total of 13 900 m^3 ethanol was produced in the 2013/2014 season (Cassman *et al.,* 2018).

Biodiesel and bioethanol are the main biofuels produced and contributed to more than 90% of the biofuels being generated industrially (Balat and Balat, 2009; Barnard *et al.,* 2010). Biofuels can be divided into five groups (Figure 1); biohydrogen, biogas, biobutanol, biodiesel, and bioethanol (Barnard *et al.,* 2010; Evanie *et al.,* 2012). An additional subdivision for biofuels is based on the feedstock as well as the production method; described as first, second and third generation biofuels (Ortiz-Marquez *et al.,* 2012).

1.2.1. First generation biofuels

First generation biofuels, which are particularly in high demand in arable areas, are produced from the storage organs of agricultural plants and include cereal grains and sugar cane (Walker, 2011; Gabrielle, 2008). Microbial production of first generation biofuels relies on the fermentation of sugars to produce alcohols such as ethanol, propanol and butanol (Barnard *et al.,* 2010). First generation bioethanol is derived from sugars or starch e.g. corn and grains (Lennartsson *et al.,* 2014). To alleviate the conflict of using food sources for fuel, non-food lignocellulosic materials were introduced to produce biofuels (Tempels and Van den Belt, 2016).

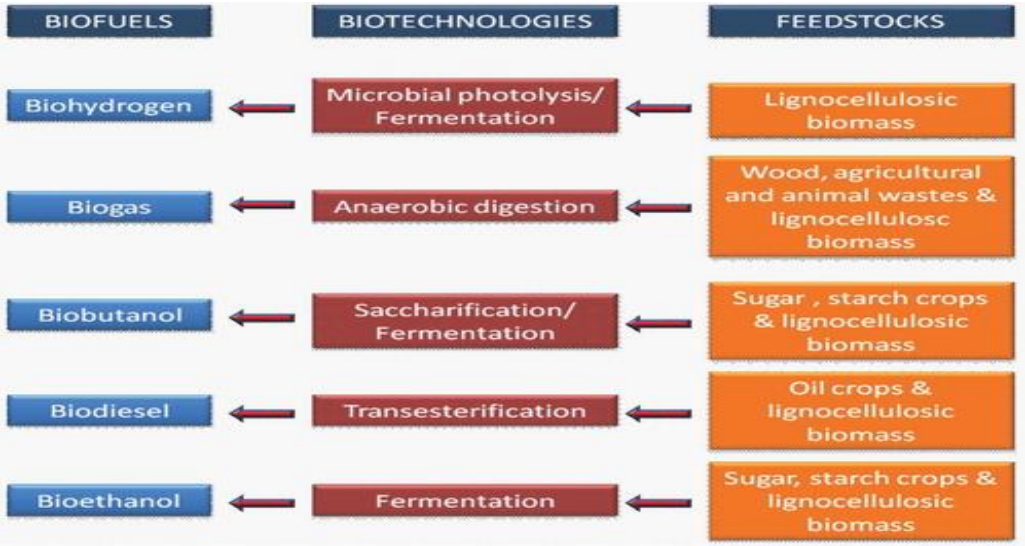


Figure 1: Diagram illustrating biofuel production from different feedstocks using different technologies (Barnard *et al.*, 2010)

1.2.2. Second generation biofuels

Second generation biofuels are produced using generic biomass (Amore *et al.*, 2012; Gabrielle, 2008), which includes cost-effective, abundantly available non-food materials (Balat and Balat, 2009; Gomez *et al.*, 2008; Walker, 2011), e.g. bioethanol is derived from miscanthus (Evanie *et al.*, 2012). The most commonly used biomass for these purposes are plant materials composed largely of lignocellulosic material, including cereal straw, sugar cane bagasse and organic municipal waste. Due to the abundance of cellulose, it also serves as an attractive second generation biofuel (Jia *et al.*, 2017b). Over time, it became evident that the use of lignocellulose was not economically attractive, as land is needed for producing the non-food lignocellulosic materials, resulting in indirect competition with food production, particularly where agricultural waste is not plentiful (Swain *et al.*, 2017). Additionally, lignocellulose pretreatment is costly as multiple enzymes are required for the hydrolysis to soluble sugars (Jia *et al*, 2017b).

1.2.3. Third generation biofuels

Third generation feedstocks include seaweed (Goh and Lee, 2010) and microalgal biomass (Oritz-Marquez *et al.*, 2012). Cyanobacteria have been identified as being capable of converting sunlight and atmospheric CO_2 into biofuels (Atsumi *et al.*, 2009; Quintana *et al.*, 2011). One of the major strengths of using cyanobacteria for biofuel production is that it does not compete for arable land required for food production (Asato, 2003; Bandyopadhyay *et al*., 2010; Quintana *et al.*, 2011). More recently, algae have

been considered as a potential biofuel. The cultivation of microalgae is environmentally friendly as it complements carbon dioxide sequestration and bioremediation of wastewaters (Show *et al.,* 2017).

1.3. 2, 3-Butanediol (2, 3-BD)

2, 3-BD (2, 3-BD EC 1.1.1.4), also known as 2, 3-butylene glycol, dimethylene glycol and 2, 3-dihydroxybutane (Kim *et al.,* 2016a; Kim *et al.,* 2016b), is an industrially relevant chemical that can be used as a biofuel (Bialkowska, 2015). Since there are multiple uses of 2, 3-BD and the need for the compound is still increasing (Song, *et al.,* 2018), manufacture at industrial levels has been explored (Kim *et al.,* 2016a; Kim *et al.,* 2016b; Bae *et al.,* 2016b). However, the production cost of 2, 3-BD at industrial levels is high as 2,3-butene oxide is hydrolysed at a temperature range of 160°C to 220°C and 50 bar pressure with several catalytic reactions. An economically attractive alternative is the microbial production of 2, 3-BD (Javidnia *et al.,* 2016) with waste substances used as feedstock.

Microbial production of 2, 3-BD from *Klebsiella pneumonia* was identified as early as 1906 by Harden and Walpole (Bialkowska, 2016) and used on a commercial scale during World War II for the production of synthetic rubber (Ge *et al.,* 2011). *K. pneumonia, Klebsiella oxytoca, Paenibacillus polymyxa* and *Enterobacter* species are able to produce high levels of 2, 3-BD. The industrial application is hindered due to biofilms containing expolysaccharides being formed, optical impurity of 2, 3-BD, as well as by-products including succinate, lactate, acetate and ethanol being generated (Kim *et al.,* 2016a, Kim *et al*., 2016b). However, there are various processes to get around this matter, which are discussed in greater detail in section 1.7 and 1.8. The fermentative process can be manipulated to yield the compound at high purity, lower temperatures and normal pressures (Song *et al.,* 2018). Additionally, genetic manipulation may be employed to ensure high purity levels of the compound (Qui *et al.,* 2016). These two points are also discussed in greater detail in sections 1.7 and 1.8, respectively.

Organisms capable of producing 2, 3-BD include *Bacillus subtilis, Paenbacillus polymyxa*, *Bacillus licheniformis* (Du *et al.,* 2011; Li *et al.,* 2014c), *K. oxytoca, Serratia marcescens* and *K. pneumoniae* (Li *et al.,* 2014b; Yang *et al.,* 2016). Some of these microorganisms, such as *K. oxytoca, S. marcescens* and *K. pneumonia,* are classified as risk group 2 pathogens by the World Health Organisation, (de Oliviera and Nicholson, 2016), therefore, their industrial scale production is not desirable (Qi *et al.,* 2014). There are several GRAS microorganisms, including *B. licheniformis, B. subtilis* and *P. polymyxa (*Li *et al.,* 2014c; Bialkowska *et al.,* 2015), which are considered as possible candidates for industrial applications.

1.4. 2, 3-BD Structure

2, 3-BD has a molecular weight of 90,12 g/mol and appears as a colourless and odourless liquid or in a crystalline form (Syu, 2001; Yang *et al.*, 2013a). Furthermore, 2, 3-BD has a four-carbon chain with a hydroxyl group on the second and third carbon. 2, 3-BD has 2 chiral centres (also known as a chiral bivalent alcohol) resulting in three stereoisomeric forms.

The three isomeric forms are (2S, 3S)-2, 3-BD (dextro), (2R, 3R)-2, 3-BD (levo) and the optically inactive (2R, 3S)-2, 3-BD (meso) forms (Figure 2) (Rados *et al.*, 2015). Each stereoisomer has a slightly different boiling point ranging from 7 to 182°C (Celinska and Grajek, 2009) and each are used in different applications (Qui *et al.*, 2016).

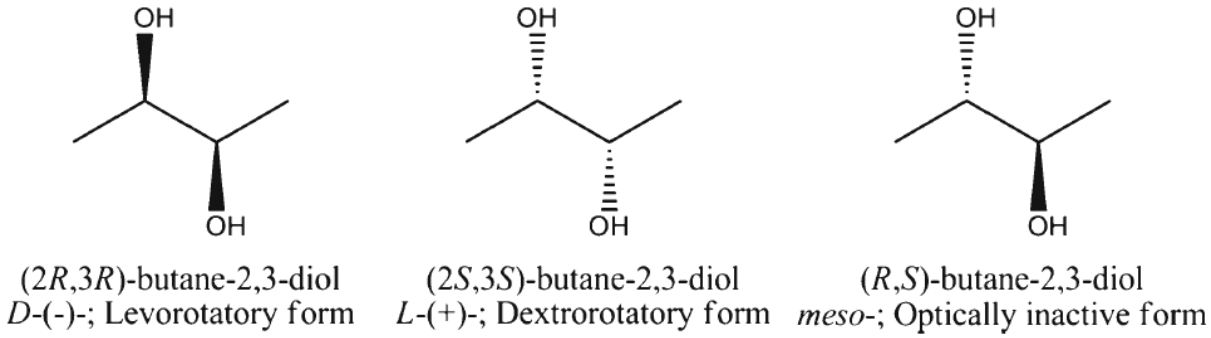


Figure 2: 2, 3-butanediol stereoisomers: levorotatory, dextrorotatory and meso optically inactive forms (Jurescho *et al.*, 2013)

In the optically inactive meso form, the methyl groups are gauche (Voloch *et al.*, 1985; Saha, 2003). The optically active levo and dextro forms are anti-gauge, display increased stability (Voloch *et al.*, 1985; Saha, 2003) and are used as building blocks in the synthesis of chiral compounds. Organisms produce various combinations and concentrations of the stereoisomers based on the fermentation conditions (Table 1) (Fu *et al.*, 2016; Kim *et al.*, 2016a; Kim *et al.*, 2016b; Liang *et al.*, 2017a).

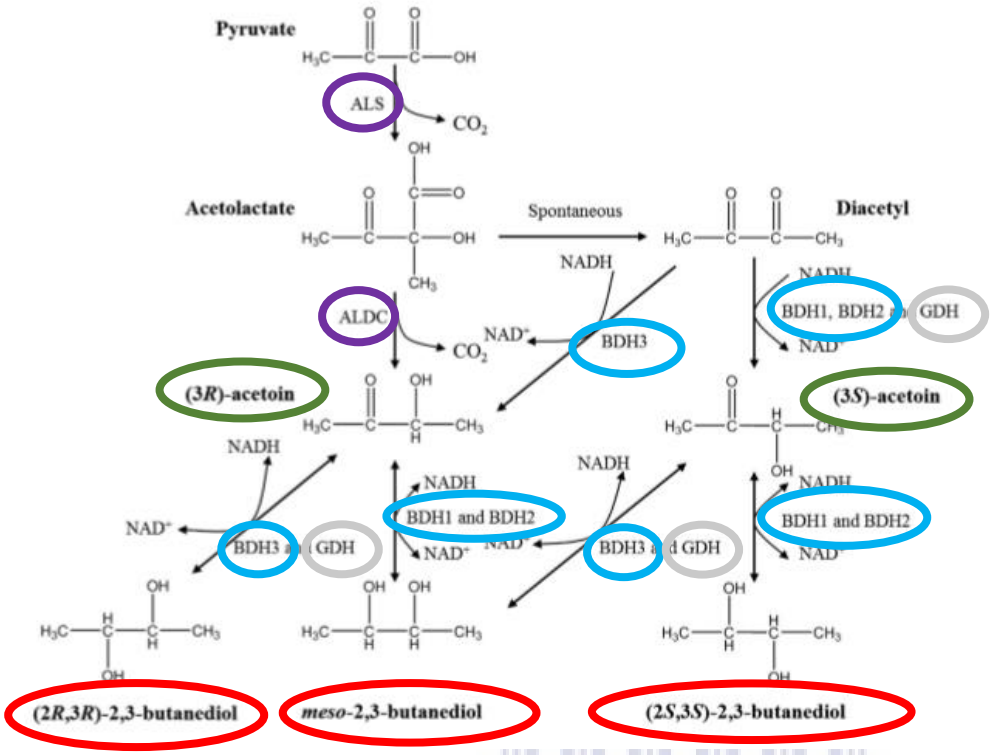
Table 1: Stereoisomeric isoforms of 2, 3-BD produced by various organisms

Organism	Meso	Levo (2R, 3R)	Dextro (2S, 3S)	Reference
*Paenibacillus polymyxa**	+	+		Zhang *et al.*, 2016
Bacillus subtilis	+	+	+	Fu *et al.*, 2016
Serratia marcescens	+		+	Zhang *et al.*, 2016
Klebsiella pneumonia	+ (a, b)	+ (b)	+	a) Zhang *et al.*, 2016 b) Fu *et al.*, 2016
Klebsiella oxytoca	+ (a, b)	+ (b)	+	a) Zhang *et al.*, 2016 b) Fu *et al.*, 2016
Enterobacter aerogenes	+	+		Fu *et al.*, 2016
Bacillus licheniformis	+		+	Qiu *et al.*, 2016
Enterobacter cloacae			+	Li *et al*, 2015

*previously *Bacillus polymyxa*

There are several theories explaining the production of the various forms of 2, 3-BD (Figure 3). One theory suggests that stereospecific 2, 3-butanediol dehydrogenase (2, 3-BDH) exists (Figure 3), each producing different 2, 3-BD isomers with varying combinations and concentrations (Voloch *et al.*, 1985; Qi *et al.*, 2014; Qui *et al.*, 2016; Zhang *et al.*, 2016). The conversion of (3R)-acetoin by specific BDH yields either (R)-2, 3-BD or meso-2, 3-BD (Kandasamy *et al.*, 2016).

In *K. pneumonia*, *Serratia marcescens* and *Enterobacter cloacae*, the meso-2, 3-BDH belongs to the short-chain dehydrogenase/reductase family and catalyses (3R)-acetoin to *meso*-2, 3-BD and (3S)-acetoin to (2S, 3S)-2, 3-BD. *Paenibacillus polymyxa, Bacillus subtilis* and *Saccharomyces cerevisiae*'s (2R, 3R)-2, 3-BDH belong to the medium-chain dehydrogenase/reductase family (MDR) and catalyse the conversion of (3R)-acetoin to (2R, 3R)-2, 3-BD and (3S)-acetoin to meso-2, 3-BD (Wang *et al.*, 2014; Zhang *et al.*, 2016).



KEY

ALS=acetolactase synthase
ALDC=acetolactase decarboxylase
BDH1=meso-2, 3-BDH
BDH2=(2S, 3S)-2, 3-BDH
BDH3=)2R, 3R)-2, 3-BDH
GDH=glycerol dehydrogenase

Figure 3: Various production pathways of 2, 3-BD stereoisomers (Zhang *et al.*, 2016). Purple circles indicate α-acetolactase synthase and α-acetolactase decarboxylase enzymes. Blue circles indicate various 2, 3-BDH isomers, green circles indicate acetoin isomers; red circles indicate various 2, 3-BD isomers.

1.5. 2, 3-BD Function

The precise metabolic function of intracellular 2, 3-BD is not well-known, but it has been speculated that its production is required for maintaining internal pH. Intracellular acidification may be prevented by switching from acid production to the production of neutral metabolites, such as 2, 3-BD, which counteracts the acidic products formed (Blomqvist *et al.*, 1993; Bialkowska, 2016). This proposed function is supported by the observation of 2, 3-BD pathway enzymes being induced in the presence of acetic acid (Bialkowska, 2016). The production of 2, 3-BD also helps regulate the nicotinamide adenine dinucleotide (NAD^+)/(NADH) ratio. When acetoin is reduced to 2, 3-BD, NAD^+ is regenerated from NADH. The reaction is reversible and thus assists in maintaining the intracellular NAD^+/NADH balance (Saha, 2003; Celinska and Grajek, 2009; Liang and Shen, 2017). It was proposed that upon production of 2, 3-BD, carbon and energy are stored that may be reutilised during the stationary phase when reserves are depleted (Xiao and Xu, 2007; Celinska and Grajek, 2009; Ji *et. al*, 2011; Bialkowska, 2016).

The (R, R)-2, 3-BD isomer promotes plant defence against disease and drought (Park, 2016). While the metabolic function of 2, 3-BD is still unclear in bacteria, its role is well understood in plant growth. *K.*

pneumonia is a plant-growth-promoting rhizobacteria (PGPR) that colonize the roots and enhance plant growth due to the production of 2, 3-BD (Ryu *et al.,* 2003).

1.6. Microbial Production of 2, 3-Butanediol

Research has shown that 2, 3-BD can be produced by three interrelated pathways (Figure 4); the cyclic pathway (Hosaka *et al.,* 2001; Ui *et al.,* 2002), metabolic pathway (Chiam *et al.,* 2012; Qi *et al.,* 2014; Qui *et al.,* 2016) and the mixed-acid fermentation pathway (Celinska and Grajek, 2009; Ng *et al.,* 2012; Vivijs *et al.,* 2015). As depicted in Figure 4, both the cyclic and mixed-acid fermentation pathways branch off from the 'main' metabolic pathway, therefore the enzymes involved in the metabolic pathway form part of the cyclic pathway as well as the mixed-acid fermentation pathway (Table 2). The cyclic and mixed-acid pathways are distinguished from the metabolic pathway by the presence of diacetyl reductase (DR) and acetylacetoin synthase (AACS) and acetylacetoin reductase (AACR), respectively.

Table 2: Enzymes involved in 3 pathways for 2, 3-BD production

Pathway	Substrate	Enzyme	Product	Comment
Metabolic pathway[1]	Pyruvate	α-acetolactate synthase (ALS)	α -acetolactate	Involved in cyclic and mixed-acid fermentation pathway
	α-acetolactate	α -acetolactate decarboxylase (ALDC)	acetoin	
	acetoin	2, 3-butanediol dehydrogenase (2, 3-BDH)	2, 3-butanediol	
Cyclic pathway[2]	α-acetolactate	N/A	Diacetyl	Spontaneous conversion
	Diacetyl	Acetylacetoin synthase (AACS/AAS)	Acetylacetoin	Hypothetical pathway
	Acetylacetoin	Acetylacetoin reductase (AACR/AAR)	Acetylbutanodiol	
Mixed-acid fermentation pathway[3]	α-acetolactate	N/A	Diacetyl	Spontaneous conversion
	Diacetyl	Diacetyl reductase (DR)	acetoin	High oxygen conditions

[1]Qui *et al.*, 2016, [2]Ui *et al.*, 2012, [3]Liang and Shen, 2017

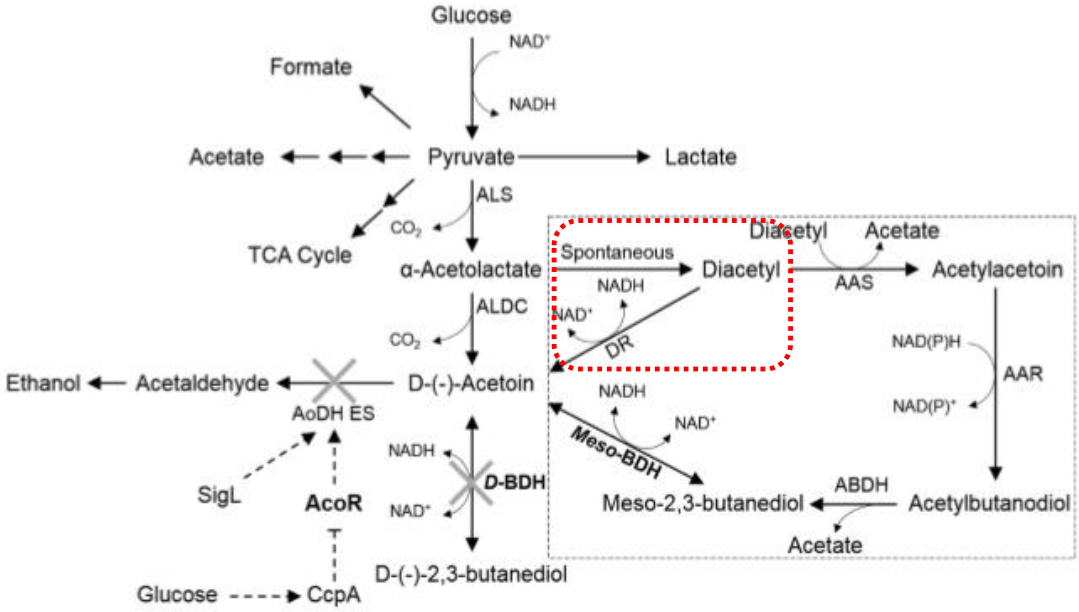


Figure 4: Schematic representation of 2, 3-BD metabolic pathway in *B. licheniformis* WX-02. ALS α-acetolactate synthase, ALDC α-acetolactate decarboxylase, BDH 2, 3-butanediol dehydrogenase, AAS acetylacetoin synthase, AAR acetylacetoin reductase, DR diacetyl reductase, AoDHES acetoin dehydrogenase enzyme system. The dashed black square represents the hypothetical 2, 3-BD cycle. The red dashed rounded-square represents the mixed-acid fermentation pathway (modified from Qiu *et. al*, 2016).

1.6.1. 2, 3–BD metabolism: Hypothetical 2, 3-BD Cyclic Pathway

The cyclic pathway (Figure 4) was detected in *B. cereus*, *B. subtilis*, *Micrococcus urea* (Hosaka *et al.*, 2001), *Bacillus stearothermophilus* (Giovannini *et al*., 2008) and yields several additional end products including ethanol, lactic acid, hydrogen and CO_2 (Celinska and Grajek, 2009). Although the cyclic

pathway was initially identified through the presence of acetylacetoin synthase (AACS) and acetylacetoin reductase (AACR), further investigation proved that all strains producing AACS, co-produced AACR. However, not all strains synthesising AACR had AACS functionality, therefore indicating that AACS accumulation is the true indicator of the 2, 3-BD cycle. In 2002, Ui *et al.* (2002) found that AACS and AACR accumulate intracellularly and suggested that this complicated their characterization (Ui *et al.*, 2002). This was confirmed by Sadaharu *et al.* (2002) who found that the identification and characterization of this pathway in other organisms was complicated due to intermediates accumulating in the growth medium (Sadaharu *et al.*, 2002). It was, however, noted that acetoin induced AACS and glucose induced AACR (Ui *et al.*, 2012). Due to the various challenges associated with characterising these enzymes, not much work was performed, this is therefore referred to as a hypothetical pathway.

In 2015, Bernacchia *et al.* (2015) cloned and overexpressed the acetoin:dichlorophenolindophenol oxidoreductase enzyme from *B. licheniformis* and proposed that this is the AACS enzyme (Bernacchia *et al.*, 2015). Unfortunately, there is no additional literature available to either support or disprove this notion.

1.6.2. 2, 3-BD metabolism: 2, 3-BD Mixed-Acid Fermentation Pathway

The mixed-acid fermentation pathway (Celinska and Grajek, 2009) (Figure 4) was identified in the early growth phase of *Enterobacter aerogenes, K. oxytoca, K. pneumoniae, S. marcescens* and *B. polymyxa* (Ng *et al.*, 2012). In the *Enterobacteria* family, there is a division based on the end-products yielded; 2, 3-BD fermenters result in neutral end products and mixed-acid fermenters result in acidic products. Vivijs *et al.* (2015) describes 2, 3-BD production observed during the early growth phase by 2, 3-BD fermenters as a 'switch' from the mixed-acid fermentation pathway to the metabolic pathway. This 'switch' result in the production of neutral products. As mentioned above and depicted in Figure 4, the mixed-acid fermentation pathway follows the same process as the metabolic cycle where pyruvate is converted to α-acetolactate by ALS. Furthermore, during an anoxic state, the metabolic cycle is followed as ALDC is responsible for the conversion of α-acetolactate to acetoin. However, the mixed-acid fermentation pathway is 'activated' in the presence of oxygen when α-acetolactate is spontaneously decarboxylated to produce diacetyl. Diacetyl reductase (DR) subsequently converts diacetyl to acetoin, which is reduced to 2, 3-BD by butanediol dehydrogenase (Ng *et al.*, 2012; Liang and Shen, 2017).

1.6.3. 2, 3-BD metabolism: 2, 3-BD Metabolic Pathway

The metabolic pathway for the production of 2, 3-BD from pyruvate was identified in *B. cereus*, *B. subtilis*, *B. licheniformis* and *M. urea* (Qi *et al.*, 2014). Three key enzymes exist in the 2, 3-BD metabolic pathway (also involved in the hypothetical cyclic pathway and mixed-acid fermentation pathway); catabolic acetolactate synthase (ALS, EC 2.2.1.6), acetolactase decarboxylase (ALDC, EC 4.1.1.5) and butanediol dehydrogenases (BDH, EC 1.1.1.76; EC 1.1.1.4) (Rados *et al.*, 2015) (Figure 4). Two pyruvate molecules are converted to α-acetolactate by ALS (Zhang *et al.*, 2016; Bae *et. al.*, 2016; Zhang *et al.*, 2017a). ALDC subsequently reduces α-acetolactate to acetoin, which is converted to 2, 3-BD by acetoin reductase (AR)/2, 3-butanediol dehydrogenase (2, 3-BDH) (Figure 4). The reaction of acetoin to 2, 3-BD by AR is reversible, allowing AR to act as both a reductase and dehydrogenase (BDH) (Ji *et al.*, 2008; Qi *et. al.*, 2014; Yang *et. al.*, 2016). The key enzymes involved in the 2, 3-BD metabolic pathway are encoded by various genes in different organisms (Table 3).

Table 3: Various genes responsible for 2, 3-BD production in the metabolic pathway

Organism	Gene		
	α-acetolactate synthase	**α-acetolactate decarboxylase**	**2,3-butanediol dehydrogenase/acetoin reductase**
Bacillus licheniformis ATCC 14580	*TRNA_RS40425*	*budA*	*TRNA_RS31725/gdh*
Bacillus cereus ATCC 14579	*BC0883*	*BC0884*	*BC0668*
Bacillus subtilis	*alsS*	*alsD*	*bdhA*
Bacillus coagulans DSM1	*BF29_RS07225*	*BF29_RS07220*	*R,R-BDH*
Bacillus amyloliquefaciens DSM7	*BAMF_RS38045*	*BAMF_RS38040*	*BAMF_RS29820/*
Bacillus thuringiensis YBT-1518	*YBT1518_RS05750*	*budA*	*YBT1518_RS04445/*
Klebsiella pneumoniae KCTC2242	*budB*	*budA*	*budC*
Klebsiella oxytoca	*AB185_RS9475*	*AB185_RS19480*	*ADB185_RS19470/*
Lactococcus lactis subsp. Lactis II1403	*als*	*aldB*	*butB/butA*

(NCBI_gene_searchhttp://www.ncbi.nlm.nih.gov)

a) α-Acetolactate synthase (ALS)

In the production of 2, 3-BD by the three pathways, α-acetolactate synthase - a thiamine diphosphate (ThDP)-dependent enzyme - is responsible for the conversion of two pyruvate molecules to α-acetolactate, which is unstable (Kim *et al.,* 2016b; Jia *et al.,* 2017a). In *K. pneumonia*, the acetolactate synthase enzyme is encoded by the *budB* gene and in *Lactococcus lactis* by the *als* gene (Table 3).

The ALS enzyme has the ability to function both catabolically (cALS) and anabolically (acetohydroxyacid synthase – AHAS) (Rados *et al.,* 2015; Liu and Wang 2016; Jia *et al.,* 2017b). In *Bacillus subtillis,* the presence or absence of O_2 and the environmental pH drive the activity of the enzyme, i.e. at low pH and low oxygen concentrations, the enzyme functions catabolically in 2, 3-BD production and at high pH and high oxygen concentrations, the enzyme acts anabolically in branch chain amino acid (BCAA) synthesis (Rados *et al.,* 2015).

The cALS enzyme is involved in butanediol metabolism and is not present in all bacteria (Rados *et al.,* 2015). The AHAS enzyme is the key component responsible for the first step in the BCAA biosynthesis from either pyruvate or 2-aceto-2-hydroxybutyrate (Liu *et al.,* 2017). Furthermore, the enzyme is flavin adenine dinucleotide (FAD)-dependent and contains a large catalytic and a small regulatory subunit. Applications of AHAS include its use in herbicides and as a potential target of new antimicrobial drugs (Lonhienne *et al.,* 2017). Extensive studies were conducted at pH 8 to elucidate the function of the AHAS enzyme, which plays a pivotal role in BCAA (Figure 5). The catalytic activity of this enzyme is strongly inhibited by the accumulation of the end products associated with the BCAA pathway, which includes valine, leucine and isoleucine. High concentrations of its co-factors, FAD and Mg^{2+} also inhibit the catalytic activity of the enzyme (Kaushal *et al.,* 2003; Rados *et al.,* 2015; Evans and Joshi, 2016; Kim *et al.,* 2016b; Liang *et al.,* 2017b).

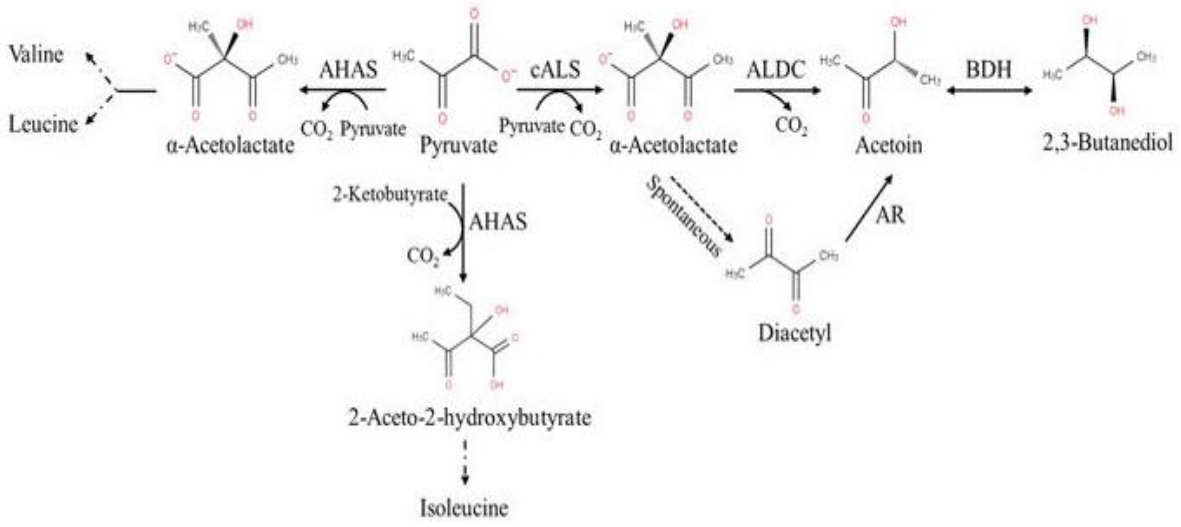


Figure 5: Pyruvate metabolism, 2, 3-BD pathway and Branched amino acid synthesis (BCAA). cALS: catabolic α-acetolactate synthase, AHAS: anabolic α-AHAS (Taken from Jia *et al.,* 2017a).

b) α- Acetolactate Decarboxylase (ALDC)

The α-acetolactate decarboxylase enzyme is responsible for the decarboxylation of α- acetolactate to acetoin. This conversion is considered to be a rate-limiting step in the biosynthesis of 2, 3-BD (Jia *et al.,* 2017a). Acetoin is a high-value compound used in natural and artificial flavourants (Zhang *et al.,* 2016; Shen *et al.,* 2016; Kim *et al.,* 2016b) as well as the manufacture of various chemicals such as alkyl pyrazine, diacetyl and acetylbutanediol (Bae *et al.,* 2016). In *Lactococcus lactis subsp lactis*, the ALDC enzyme is encoded by the *aldB* gene, which is situated within the BCAA operon. Goupil-Feuillerat *et al.* (1997) hypothesised that the *aldB* gene is allosterically activated by leucine, transcribed from two promoters and constitutively expressed in growing cells, regardless of branch chain amino acid (BCAA) synthesis. Furthermore, the presence of one of the two *aldB* gene promoters situated within the BCAA operon could indicate that it plays a role in directing synthesis of α-acetolactate to 2, 3-BD (Goupil-Feuillerat *et al.,* 1997). Later on, Goupil-Feuillerat *et al.* (2000) showed that two *aldB* gene promoters are induced during BCAA starvation, which results in inhibition of ALDC at the translational level. A third promoter acts independently of this scenario and continues to produce ALDC at low levels, even during activation of pyruvate catabolism. This differs from transcriptional regulation observed in other organisms (Goupil-Feuillerat *et al.,* 2000).

c) Acetoin Reductase (AR)/ 2,3-Butanediol Dehydrogenase (BDH)

Acetoin reductase (AR)/2, 3-BDH is responsible for the reversible reaction between 2, 3-BD and acetoin. Although this reaction is performed by the same enzyme, the enzyme is referred to as 2, 3-BDH in the reverse reaction (Qi *et al.*, 2014). In the forward reaction, acetoin is reduced to 2, 3-BD and this enzymatic reaction requires the reduced nicotinamide adenine dinucleotide (NADH) or its phosphate form (NADPH) as electron donor (Liang and Shen, 2017). To understand the interplay between the forward and reverse reactions, one may refer to the study performed in *B. subtilis* indicating that high concentrations of dissolved oxygen and glucose result in acetoin being reduced to 2, 3-BD. Conversely, when glucose is depleted and dissolved oxygen limited, 2, 3-BDH catalyses the reverse reaction, i.e. the conversion of 2, 3-BD to acetoin (Qi *et al.*, 2014).

It was also noted that various 2, 3-BDH isomers exist and these were identified and characterized (Pu *et al.*, 2017; Zhang *et al.*, 2016) with the overall classification resulting in three types based on stereospecificity for substrates and their products (Ui *et al*, 1984);

- (2S, 3S)-BD → (2S, 3S)-BDH (S, S-BDH) → 3S-AC
- (2R, 3R)-BD → R, R-BDH → (3R)-AC
- meso-BD → meso-BDH → (3R)-AC

This classification was used in extensive research studies to determine which genes are responsible for the production of the various isomers. In 2012, Gao *et al.* (2012) identified that *B. subtillis***,** meso-AR/BDH is encoded by the *bdh*A gene and genetic manipulation of this gene can lead to increased levels of D-2, 3-BD, a more desirable isomer (Goa *et al.*, 2012). Two years later, Qi *et al* (2014) knocked out the *bud*C gene in *B. licheniformis* (WX-02Δ*bud*C) to ascertain its function. In the absence of the *bud*C gene, there was no production of meso-2, 3-BD, while D-2, 3-BD production was increased from 13.77 g/L to 30.76 g/L. This indicates that the *bud*C gene is responsible for the production of meso-2, 3-BD from acetoin. However, trace amounts of meso-2, 3-BD were detected towards the end of fermentation. This may be due to other genes encoding meso-2, 3-BDH (Qi *et al.*, 2014). Qui *et al.*, (2016) identified the glycerol dehydrogenase (*gdh*) gene as the catalyst for the conversion of D-acetoin to D-2, 3-BD in *B. licheniformis* (Qui *et al.*, 2016). This indicates that different genes are responsible for the production of

various isomers within the same organism, i.e. the *budC* gene is responsible for meso-2, 3-BD and *gdh* gene for D-2, 3-BD.

Based on the above, it is evident that while several pathways exist for 2, 3-BD production, each pathway and its components allow room for manipulation both genetically and by fermentation conditions to increase the production yields. Researchers ardently explore microorganisms and upon understanding the native function of the 2, 3-BD pathway, attention is directed towards increasing production levels. This is the focus of the next two sections.

1.7. Fermentation as a tool for microbial production of 2, 3-BD

As previously mentioned, the chemical synthesis of 2, 3-BD is a costly and harsh process, therefore microbial technologies such as fermentation are employed to reduce production costs (Xiu and Zeng, 2008; de Oliviera and Nicholson, 2016; Kim *et al.*, 2017). Furthermore, during fermentation, microorganisms produce various concentrations of mixtures of 2, 3-BD stereoisomers. Several separation techniques are used for recovery of the desired isomers, including solvent extraction and membrane distillation (Syu, 2001, Mazumdar *et al.*, 2013; Bialkowska *et al.*, 2015; Park *et al.*, 2016; Chan *et al.*, 2016). Therefore, when considering the microbial production of 2, 3-BD, it is critical to explore the downstream purification processes as this will affect the overall cost. Additionally, optimisation of fermentation conditions can lead to increased production of desired isomers (Yu and Saddler, 1983; Green, 2011; Jurchescu *et al.*, 2013; Li *et al.*, 2014a; Chan *et al.*, 2016). The 2, 3-BD yields and cost (Li *et al.*, 2014b) obtained through microbial production may be influenced by several fermentation factors including carbon source, temperature and substrate usage. These factors, along with examples are discussed below.

Carbon source: The comparison of 2, 3-BD production yields in *B. licheniformis* from glucose, fructose, cellobiose, sucrose, starch and mannose, resulted in glucose producing the highest yields (Nilegaonkar *et al.*, 1996). Additionally, the use of glucose as a carbon source resulted in more favourable results than that observed in *K. oxytoca* and *B. polymyxa.* After 72 hours, the 2, 3-BD production from 100g glucose was 47g in *B. licheniformis* compared to 37g/100g in *K. oxytoca* and 24g/100g in *B. polymyxa* (Nilegaonkar *et al.*, 1996).

More recently, Song *et al.* (2018) investigated the use of sugarcane, as it is a commonly used fermentation feedstock containing sucrose, fructose and glucose. Upon testing and comparison of the ability of a *B. licheniformis* strain to utilise these carbon sources to produce 2, 3-BD, no significant difference was noted between sucrose (31.0 g/L), fructose (30.4 g/L) and glucose (33.2 g/L) (Song *et al.*, 2018).

As mentioned above, microorganisms are able to produce 2, 3-BD from various carbohydrate sources. Unfortunately, when organisms are provided with multiple carbon sources, e.g. sugarcane that consists of mixtures of sucrose, fructose and glucose, only the preferred carbon will initially be used as this will result in the fastest growth. Therefore, any other substrates will not be utilised simultaneously due to various regulatory mechanisms being activated, including the repression of certain genes. This is referred to as carbon catabolite repression (CCR) (Jung *et al.*, 2015).

In order to get around this problem, Jung *et al.* (2015) aimed to develop an *Enterobacter aerogenes* mutant capable of effectively fermenting sucrose, glucose and fructose simultaneously to produce 2, 3-BD. This was achieved by removing transcriptional repressors that would allow the mutant strain to ferment all sugars simultaneously. Additionally, the duration and productivity of fermentation was increased. Furthermore, the key genes involved in carbon catabolism were over-expressed to ensure that the sugars would not accumulate in the fed-batch fermentation. The mutant was able to produce 140 g/L 2, 3-BD after 54 hours (Jung *et al.*, 2015).

The above is an indication that carbon source usage needs to be optimised per isolate and strain to determine which result in the best yields, and that CCR needs to be considered. Furthermore, the concentration of carbon source also impacts on product yield (Yu *et al.*, 2017).

Temperature: Another major factor that impacts the success of fermentation is temperature. Most microorganisms capable of producing 2, 3-BD do so at 37°C, which allows room for contamination (Li *et al.*, 2013). To reduce contamination at these (mesophilic) temperatures, the culture media must be prepared at high temperature and pressure to maintain the sterility of media. The infrastructure required to maintain these standards are costly and therefore not economically feasible (Xiao *et al.*, 2012). To circumvent the risk of contamination during fermentation at 37°C, researchers investigated the use of thermophiles as thermostable enzymes are more feasible for bioconversion systems at high temperatures (Jia *et al.*, 2017b) and there will be no need for the costly infrastructure. Fermentation of thermophilic microorganisms can be performed between 50 to 60°C and the yields generated, even within the same

isolate, depend on several factors. As noted in Table 4 below, *B. licheniformis* can generate 12 to 115 g/L 2, 3-BD, depending on the fermentation conditions. While the highest 2, 3-BD yields depicted in Table 4 were achieved at 37°C, the industrial application of *K. oxytoca* is not feasible. Further investigation is therefore required to isolate GRAS thermophilic organisms.

Table 4: Effect of temperature on 2, 3-BD yield

Strain	Temperature (°C)	Concentration (g/L)	Productivity (g/L-h)	Yield (%)
B. amyloliquefaciens	37	92.3	0.96	84
B. subtilis	37	6.1	0.4	67
S. cerevisiae	ND	2.3	0.03	22.6
P. polymyxa	30	111	2.06	ND
Geobacillus sp.	55	14.5	0.30	69
B. licheniformis	30	144.7	1.14	80
B. licheniformis	50	12.1	1.01	90
S. marcescens	30	152	2.67	82
K. oxytoca	37	130	1.64	96
K. pneumonia	37	150	4.21	86
B. licheniformis	50	115	2.4	94

modified from Li *et al.*, 2013

As mentioned above, thermophiles are attractive organisms to reduce fermentation costs and their value is noted for the production of second generation biofuels (e.g. from lignocellulose) and as a measure to reduce contamination.

Lignocellulose, e.g. straw and corncob, are second generation biofuel feedstocks. As lignocellulose is recalcitrant and requires several enzymes for successful hydrolysis to fermentable sugars, the process of simultaneous saccharification and fermentation (SSF) or separate hydrolysis and fermentation (SHF) is employed to increase fermentation efficacy (Jia *et al.*, 2017b). During the SHF process, raw material is pretreated and degraded by biomass-hydrolyzing enzymes to obtain enzymatic hydrolysates. Degradation products, mostly glucose and xylose, are further converted by microorganisms during fermentation (Xiao and Xu, 2007). The SSF approach is employed in response to the inhibition of enzymes used to hydrolyse lignocellulose by the accumulation of end products. In the SSF method, the

enzymes are hydrolysed during fermentation and the resulting sugars are made available to microorganisms for further fermentation. As this process is performed at elevated temperatures, the use of mesophilic organisms for sugar fermentation complicates the SSF process (Choudhary *et al.,* 2016). By employing thermophilic organisms, using SSF is more efficient, and result in increased product yields (Li *et al.,* 2014b).

Although both approaches are useful, deciding which method to choose is dependent on the starting material and organism, among other factors. In *B. licheniformis* ATCC 14580, Li *et al.* (2014) found SSF to be more suitable and produced 103g/l 2, 3-BD from inulin in 30 hours at 50°C. The optimum temperature range of inulinases is 45 to 55°C, which coincides with the use of thermophilic organisms. At this elevated temperature, fermentation can take place without sterilization thereby reducing the cost and making the process more efficient (Li *et al.,* 2014b).

In 2012, Xiao *et al.* (2012) identified the first native thermophilic 2, 3-BD producer, *Geobacillus* (Table 4). Following optimisation of temperature, pH, dissolved oxygen, culture medium, carbon and nitrogen source, a strain capable of producing 14.5 g/L of 2, 3-BD at 55°C was identified. While these yields were found to be low, Xiao *et al.* (2012) believed that the conditions may be further optimised to increase the yield. However, no additional literature on 2, 3-BD production from *Geobacillus* was found.

Li *et al.* (2013) identified a novel thermophilic *B. licheniformis* strain, 10-1-A, capable of producing 115.7 g/l 2, 3–BD with the optimal fermentation conditions at pH 7 and 50°C with glucose as a substrate.

Substrate Usage: The commercial production of 2, 3-BD relies on the careful selection of substrates (Table 5) as this can ensure maximum economic benefit (Li *et al.,* 2014c). Availability of cheap and abundant substrates are key factors to guarantee that microbial production can be economically feasible (Chan *et al.,* 2016).

Table 5: Substrates utilised by different organisms for the production of 2, 3-BD

Organism	Substrates	Reference
Klebsiella oxytoca	Maltodextrin (derived from cassava)	Chan *et al.,* 2016
Paenbacillus polymyxa	Inulin (derived from Jerusalem artichoke tuber)	Cao *et al.,* 2017
Enterobacter cloacae	Glucose and xylose	Li *et al.,* 2015
Bacillus subtilis	Glucose	Fu *et al.,* 2016
Serratia marcescens	Xylose	Zhang *et al.,* 2016
Klebsiella pneumonia	Glycerol	Park *et al.,* 2016
Enterobacter aerogenes	Sugarcane bagasse	Um *et al.,* 2017
Bacillus licheniformis	Glucose	Qui *et al.,* 2016
Bacillus amyliquefaciens	Enzymatic hydrolysates	Sikora *et al.,* 2016

While the above-mentioned aspects are a few examples of methods to increase 2, 3-BD yield, there is no denying the fact that optimised fermentation conditions work best when employing the use of genetically modified microorganisms. The next section focuses on additional methods used to increase 2, 3-BD yields.

1.8. Manipulation of strains to increase 2, 3-BD yields

With the advent of genetic engineering and microbial genome sequencing, it is possible to genetically modify organisms to enhance the production of desired compounds (Du *et al.,* 2011; Bialkowska *et al.,* 2015; Bae *et al.,* 2016). These technologies are useful when considering ways in which to increase the 2, 3-BD production levels in GRAS microorganisms. Strategies employed to increase the yields include genetic modification (Qi *et al.,* 2014; Bialkowska *et al.,* 2015; Bae *et al.,* 2016) or metabolic engineering in fermentation processes (discussed above) amongst other approaches (Li *et al.,* 2013; Qian *et al.,* 2015; Kim *et al.,* 2017).

Furthermore, having a clear understanding of the 2, 3-BD production pathway has allowed genetic manipulation to direct synthesis towards the desired product. As previously mentioned, various genes are responsible for the production of key enzymes used during 2, 3-BD metabolism. In *B. licheniformis,* the glycerol dehydrogenase (GDH) enzyme is encoded by the *gdh* gene and is responsible for catalysing the conversion of D-AC to D-2, 3-BD (Li *et al.,* 2013). A strong sequence similarity was observed when

comparing the *B. licheniformis* WX-02 putative *gdh* gene to genes in other organisms in which the function is known, e.g. *K. pneumonia* and *K. oxytoca* (Qiu *et al.,* 2016). In a study by Qiu *et al.* (2016), the *B. licheniformis* strain was engineered to ensure that only meso-2, 3-BD was produced by knocking out the glycerol dehydrogenase gene. To ensure that there was sufficient acetoin (2, 3-BD precursor), the *acoR* gene responsible for degrading acetoin was also deleted. This double-deletion mutant produced 50% more 2, 3-BD than its parent strain (Qiu *et al.*, 2016).

There are four distinct strategies to improve 2, 3-BD metabolism at industrial levels; reduction of by-product formation, engineering cofactor recycling, enhanced glycolysis and 2, 3-BD synthesis pathway and heterologous expression of 2, 3-BD synthesis pathway. These approaches will be discussed by means of examples in the next few sections.

a) Reduce by-product formation

In 2, 3-BD production, high by-product formation results in product loss during the purification process (Anvari and Motlagh, 2011) reduced 2, 3-BD yields (Xu *et al.,* 2009; Chen *et al* 2011; Jung *et al.,* 2014). The former problem was addressed in *K. pneumonia* when Anvari and colleagues (2009) investigated the use of organic solvent, oleyl alcohol, with liquid-liquid extraction to remove products during fermentation, thereby minimizing downstream recovery. When using 80 g/l glucose, 2, 3-BD production levels increased from 9 g/l to 23.01 g/l (Anvari *et al.,* 2009). With the latter, the acids produced limited 2, 3-BD yield since they cannot be metabolized, subsequently resulting in competition with acetoin for NADH (Yang, 2007). The reduction of by-product formation as well as the *Klebsiella* family is discussed below.

Klebsiella is a rod-shaped, Gram-negative bacterium with a large polysaccharide capsule that covers the surface area and offer resistance to many host defence mechanisms (Yang, 2003). This organism is one of the most favoured for the production of 2, 3-BD, therefore extensive genetic manipulation and attempts to create favourable fermentation conditions have been explored (Anvari and Safari Motlagh, 2011). As *Klebsiella* is a risk group 2 pathogen, industrial levels of production is not encouraged. Furthermore, *Klebsiella* produces more than one isomer of 2, 3-BD, therefore separation techniques will be required (Wang *et al.,* 2011). However, one cannot ignore the organism's ability to produce high levels of 2, 3-BD (de Oliviera and Nicholson 2016; Kim *et al.,* 2017). In the *Klebsiella* family, *K. pneumonia* is one of the most favoured strains capable of producing 2, 3-BD due to the broad substrate range, complete

fermentation and ease with which it may be cultured (Garg and Jain, 1995; Ma *et al.*, 2009; Park *et al.*, 2016).

K. oxytoca uses carbohydrates as a substrate to produce 2, 3-BD and several by-products including acetoin, ethanol, lactic and acetic acid (Ji *et al.*, 2008). When acetic acid is not dissociated, it inhibits bacterial growth and increases the energy requirement of the cell (Yang, 2007). Ji *et al.* (2008) created a mutant by UV mutation coupled with diethyl sulfate (DES). These mutants were screened by use of a proton-suicide method that was based on a lethal mixture of NaBr and NaBRO. *K. oxytoca* mutants with a deficiency in the lactate producing pathway, produced 7.3% increase of 2, 3-BD with decreased acid synthesis (88% lactic acid, 92% acetic acid). A total of 39 g/L 2, 3-BD was produced by one of the mutants (Ji *et al.*, 2008).

By-products generated during 2, 3-BD production by *K. pneumonia* include acetate, lactate, ethanol, succinate and formate. In 2009, Xu *et al.* knocked out the *ldhA* gene encoding fermentative D-lactate dehydrogenase (LDH) as lactate is one of the main by-products formed by *K. pneumoniae*. The mutant displayed 89-98% reduction in LDH activity and 95.93% increase in 2, 3-BD production after 48 hours fed-batch fermentation. Interesting to note, was a 50% increase in $NADH/NAD^+$ in the late exponential phase and at the time, Xu *et al.* (2009) postulated that this may have contributed to the increased concentration of final metabolites. Based on recent literature, it is now known that regeneration of cofactors does indeed increase 2, 3-BD yield (discussed below).

Due to the complexity of the cellular mechanisms, the approach of removing multiple genes to reduce by-product formation was found to generate several unexpected problems. One such example is the removal of alcohol dehydrogenase gene (*adhE*), which not only produces ethanol, but also plays a role in alleviating oxidative stress. When knocking out *adhE* in *E. coli*, the cell growth was hampered and morphological defects were noted as a result of accumulation of reactive oxygen species (Echave *et al.*, 2003). Therefore, when considering genes to be disrupted, researchers selected genes which would have little or no effect on the microbial growth (Jung *et al.* (2014). Jung *et al* (2014) disrupted genes encoding glucosyltransferase (*wabG*), lactate dehydrogenase (*ldhA*) and pyruvate formate-lyase (*pflB*) in an attempt to decrease by-product formation, while maintaining a stable cellular network within *K. pneumoniae*. The *wabG* gene was disrupted as it plays a role in the organism pathogenicity. To redirect the carbon flux towards 2, 3-BD instead of formate, acetate and ethanol, the *pfB* gene was disrupted. As previously mentioned, the *ldhA* gene encodes lactate which is the main by-product produced. The mutant

yielded 92.2% of the theoretical maximum and a significant reduction in by-product formation (Jung *et al.,* 2014).

b) Engineer cofactor recycling

Nicotinamide adenine dinucleotide (NADH) or its phosphate form (NADPH) are cofactors in several metabolic pathways. While NADH plays a role in catabolism, NADPH is involved in anabolism, which includes the production of biopolymers (Cai *et al.,* 2017). The reduction of acetoin to 2, 3-BD requires NADH or NADPH as an electron donor. Therefore, the availability of these cofactors greatly influences effective production (Liang and Shen, 2017). These cofactors are costly and to keep costs at a minimum, regeneration or recycling is required (Wang *et al.,* 2013). While glucose is used for internal cofactor regeneration, this process is not always fast or efficient. Glucose dehydrogenase (GDH) and formate dehydrogenase (FDH) are NADH regeneration enzymes. GDH is able to oxidise glucose by regenerating NAD(P)H in an inexpensive and stable manner. The conversion of formate to carbon dioxide by FDH results in the generation of NADH.

Saccharomyces cerevisiae is used extensively in industrial applications; however, ethanol is produced as its major fermentation product. To effectively use *S. cerevisiae* for the production of 2, 3-BD, the pyruvate decarboxylase gene (*pdc*) was deleted, resulting in an accumulation of pyruvate – a precursor to 2, 3-BD (Kim *et al.,* 2016b). Unfortunately, the *pdc*-deficient strain displayed defective growth when using glucose due to a lack of acetyl-CoA in the cytosol as well as a redox imbalance as a result of NADH accumulating in the cytosol. Additionally, *pdc*-deficient *S. cerevisiae* is unable to synthesise acetyl-CoA from glucose as the mutant no longer has C_2-compounds such as acetate and ethanol. Acetyl-CoA is needed to produce lysine and fatty acids in the cytosol. Kim *et al.* (2016) addressed C_2-auxotrophy, insufficient cytosolic acetyl-CoA and the redox imbalance by employing a metabolic engineering approach. 2, 3-BD yield was increased five-fold (154.3 g/L 2, 3-BD produced) by directing carbon flux from glycerol to 2, 3-BD. This was achieved by altering the NADH/NAD^+ ratio by the expression of NADH oxidase (Kim *et al.,* 2016b).

Ben and colleagues (2017) increased the levels of 2, 3-BD in *Serratia marcescens* by manipulating the cofactors at an intracellular level and engineering the organism as a whole-cell biocatalyst. This was achieved by employing the use of quorum sensing, an auto-inducing system, as well as a NADH regeneration system. Quorum sensing is the bacterial way of communicating, thereby allowing the

coordination of target gene expression in response to population density. In *S. marcescens*, the *swr* system is used to control many functions including the production of 2, 3-BD. The auto-inducing expression system was developed to express 2, 3-BD based on the pET system and *swr* quorum sensing in *S. marcescens*. FDH and 2, 3-BD were co-expressed to construct a NADH regeneration system to aid conversion of diacetyl to 2, 3-BD. The combination of these technologies resulted in the production of 53.6 g/L 2, 3-BD in *S. marcescens* (Ben *et al.,* 2017).

c) Enhance glycolysis and 2, 3-BD synthesis pathway

MTH1 is a transcription factor involved in glucose sensing and pyruvate decarboxylases (PDC) are structural genes in *S. cerevisiae (*Lian *et al.,* 2014*).* In an attempt to increase glycolysis and subsequent 2, 3-BD production, the PDC gene was inactivated to redirect the flux from ethanol and the *MTH1* gene was overexpressed. The mutant produced than 100 g/L enantiopure meso-2, 3-BD, which was more than 70% of the theoretical yield from a mixture of glucose and galactose. Furthermore, red algae was identified as a suitable renewable feedstock for biofuel production and consists of these two major components (glucose and galactose), making it useful for meso-2, 3-BD production by *S. cerevisiae* (Lian *et al.,* 2014).

In *K. pneumonia,* the α-acetolactate synthase, α-acetolactate decarboxylase and acetoin reductase genes were overexpressed to increase 2, 3-BD yield (Guo *et al.,* 2014). These mutants were cultured in media containing glucose, galactose, fructose, sucrose and lactose. Upon comparison of 2, 3-BD yield in different carbon sources, the mutants consistently achieved higher concentrations than the parental wild-type strain, except when cultured on lactose. The productivity observed in the mutants was 12% higher than that of the parental strain (Guo *et al.,* 2014).

In an attempt to increase the production of acetoin, Zhang and colleagues (2017b) deleted the 2, 3-BDH (*bdhA*) gene in *B. subtillis* 168, which meant that acetoin would not be converted to 2, 3-BD. When supplementing the media with 20 g/L glucose, almost no 2, 3-BD was detected. However, when the glucose concentration was increased to 100 g/L, a total of 2.4 g/L 2, 3-BD was detected. Upon further analysis of the chiral structure, it became apparent that meso-2, 3-BD was the primary isomer formed in the recombinant strain. The deletion of the *bdhA* gene abolished the production of (2R, 3R)-2, 3-BD, but not the meso form, which may indicate that another pathway exists for the production of the latter (Zhang *et al.,* 2017b).

Rahman and colleagues (2017) created an aerobic *K. pneumoniae* mutant capable of converting 55.0 g/L glycerol to 32.3 g/L of 2, 3-BD and 77.51 g/L of 2, 3-BD from batch fed and batch fermentations, respectively. In the batch process, the pH was assessed between 4 and 9 with pH 8 being the optimal condition for maximum 2, 3-BD production (Rahman *et al.*, 2017).

d) Heterologous expression of 2, 3-BD synthesis pathway

As mentioned in section 1.6 and Table 3 above, 2, 3-BD is produced from pyruvate by employing the use of three key enzymes. In *B. subtilis*, 2, 3-BD is produced by *alsS, alsD* and *bdhA* genes. Furthermore, the *alsS* and *alsD* genes form a bicistronic operon (*alsSD*), which is under positive transcriptional control by the divergent AlsR protein. The *alsR* gene is responsible for production of the AlsR protein, which indirectly regulates the monocistronic *bdhA* gene. *B. subtilis* produce low levels of 2, 3-BD, therefore de Oliviera and Nicholson (2016) engineered the *alsS, alsD* and *bdhA* genes into a single tricistronic operon under regulation of the isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible Pspac promoter in a shuttle plasmid. The shuttle plasmid successfully expressed 2, 3-BD in *B. subtilis* as well as *E. coli*. Although the genes occur naturally in *B. subtilis*, 2, 3-BD production was increased by 2.8-fold following introduction of the shuttle plasmid (de Oliveira and Nicholson, 2016).

Corynebacterium glutamicum, a GRAS facultative anaerobe, is used extensively as a host strain in industrial applications and produces L-glutamate and L-lysine in large scale (Rados, *et al.*, 2015). Due to the extensive research performed on its genetic make-up, it is an ideal organism to be manipulated for the production of biofuels especially as the wild-type produces low levels of 2, 3-BD (Wendisch *et al.*, 2006). The 2, 3-BDH gene of *C. glutamicum* is promiscuous as it recognises diacetyl and acetoin as substrates. This is also observed in *L. lactis*.

In 2015, Rados and colleagues successfully engineered *C. glutamicum*, resulting in increased 2, 3-BD yield. This organism produced very low amounts of 2, 3-BD as can be seen in the wild type control in the study. A two-pronged approach was used that consisted of genetic engineering as well as optimising the fermentation conditions. An artificial operon containing the *L. lactis* 2, 3-BD pathway genes was cloned into pEKEx2 expression vector under the control of isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter and produced 6.3 g./L 2, 3-BD (Rados *et al.*, 2015). While the yields achieved in this study were lower than that observed in other organisms, this study provided a basis on which future work can be performed in this suitable microorganism. In order to increase these yields in this

mutant, a higher density bioreactor may be used which will increase the yield by the order of one magnitude. This will make the yield comparable with natural GRAS 2, 3-BD producers (Rados *et al.*, 2015).

Clostridia is used extensively for the production of acids and alcohols, which act as building blocks in industrial applications or biofuels. *Clostridium acetobutylicum* does not naturally produce 2, 3-BD due to the absence of the *alsD* gene, but it can produce acetoin (Raedts *et al.*, 2014). Shen and colleagues (2016) screened the *alsD* gene of *B. subtilis* and overexpressed it in *C. acetobutylicum.* The ALDC enzyme does not naturally occur in this organism, therefore the 10 and 50-fold increase in activity observed at 12 and 48 hours, respectively, is compared to no ALDC being produced in the wild type. While no 2, 3-BD was detected, 12.9 g/L butanol was produced. Furthermore 6.5 g/L acetoin was produced, which is a marked increase from the original acetoin production of 1.5 g/L (Shen *et al.*, 2016).

In some instances, researchers employ multiple approaches to increase 2, 3-BD yield in their study design. Yang and colleagues (2013) embarked on a long-term investigation to determine ways to increase 2, 3-BD yield by *B. amyloliquefaciens*. *B. amyloliquefaciens* is able to produce 83.3 g/L of 2, 3-BD from biodiesel-derived glycerol with beet molasses as a co-substrate in a fed batch system. Large amounts of acetoin, lactate, acetate and succinate were formed during this process that negatively regulate 2, 3-BD yield and result in costly downstream separation processes (Yang *et al.*, 2013b). In a follow-up study in 2015, Yang *et al.* (2015) successfully increased 2, 3-BD yields to 102.3 g/L using biodiesel-derived glycerol as a substrate. This was achieved by using a three-pronged approach; manipulating the carbon flux to 2, 3-BD, overexpressing $NADH/NAD^+$ regeneration and optimisation of fermentation conditions. A decrease in by-product formation was also observed (Yang *et al.*, 2015).

1.9. Applications of 2, 3-BD

There are several applications for 2, 3-BD (Figure 6) that include its use as an anti-freeze agent due to the low freezing point (Qi *et al.*, 2014; Qui *et al.*, 2016; de Oliviera and Nicholson, 2016; Xie *et al.*, 2017). The dehydration of 2, 3-BD results in 1, 3-butanedene (Li *et al.*, 2014c), which has a low melting point (0 to -86°C) and is therefore used as an anti-freeze agent. Dehydrogenation of 2, 3-BD produces acetoin and diacetyl, which are used as flavouring agents in dairy products and cosmetics (Syu, 2000; Qi *et al.*, 2014). Additionally, 2, 3-BD may be used as feedstock for the production of chemicals, cosmetic products, explosives, plasticizers and pharmaceuticals (Wu *et al.*, 2008). Further uses of 2, 3-BD include

rubber production, solvent for resin, lacquer, bacteriostatic food additive (Celinska and Grajek, 2009; Qi *et al.*, 2014), drugs, explosives and perfumes (Li *et al.*, 2013; Bialkowska *et al.*, 2015).

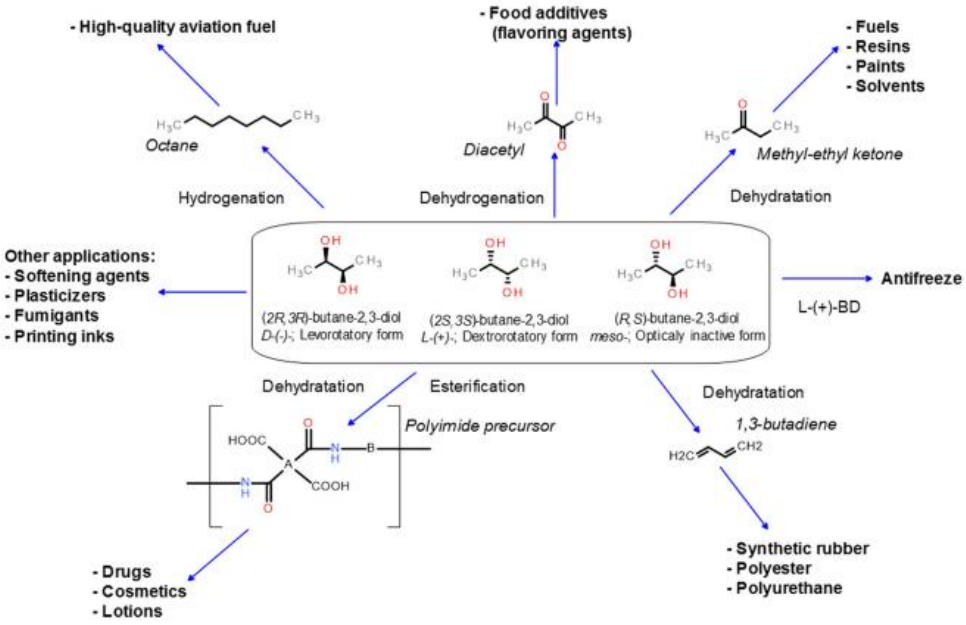


Figure 6: The industrial applications of 2, 3-BD: Chemical reactions resulting in various commercially attractive products (Bialkowska, 2016).

However, the main focus of this review is biofuels and 2, 3-BD has great potential as a liquid fuel additive for a petroleum replacement. This is due to the heating value (27.2 kJ/g) being more favourable than that of methanol (22.1 kJ/g) and ethanol (29.1 kJ/g) (Li *et al.*, 2014a; Bialkowska *et al.*, 2015; Qui *et al.*, 2016).

Furthermore, the dehydration of 2, 3-BD results in methyl ethyl ketone (MEK) and further hydrogenation of MEK results in high octane isomers that are required for high quality aviation fuels (Liang *et al.*, 2017b).

1.10. Aims and Objectives

Bacteria previously isolated from hot spring sediment showed potential for use in the generation of biofuels due to their ability to produce acetoin. The main aim of this study was to assess these isolates for their ability to produce 2, 3-BD, a useful biofuel. The following objectives were established:

- Identify previously isolated organisms by employing the use of 16S rRNA sequence analysis
- Screen isolates for the *alsD* gene which is involved in acetoin metabolism
- Identify optimal fermentation conditions for cell growth by investigating the effect different media has on cellular density
- Assess 2, 3-BD production under best growth conditions

Chapter 2: Materials and Methods

2. Introduction

Based on the review of the literature, the need to identify bacterial isolates capable of producing single stereoisomers of 2, 3-BD was recognized. This chapter describes the process followed to identify isolates as well as determine the most suitable conditions to enhance their growth and 2, 3-BD production. To elucidate one of the genes in the 2, 3-BD pathway, a bio-analytic review was performed on the *Bacillus* spp.

2.1. Chemicals and Reagents

Analytical grade chemicals and reagents were supplied by Merck KGaA (Darmstadt, Germany), Sigma-Aldrich Chemical Company (Deissenhofen, Germany, now Merck) and Kimix Chemical and Laboratory supplies (South Africa). Culture media was supplied by Oxoid Ltd and Biolabs (Cheshire, United Kingdom). DNA size markers and all DNA modifying enzymes (polymerases and restriction enzymes) were purchased from Fermentas Life Sciences Ltd (Vilnius, Lithuania), unless otherwise stated. Primers for Polymerase Chain Reaction (PCR) were synthesised by Inqaba Biotechnical Industries (Pty) Ltd. (Johannesburg, South Africa).

2.2. Growth Media

Media was sterilised by autoclaving at 121°C for 20 minutes. Where necessary, ampicillin and supplements such as bromo-chloro-indolyl-galactopyranoside (X-gal) and isopropyl-β-D-1-thiogalactopyranoside (IPTG) were added prior to use (liquid media) or after autoclaving (solid media) as stated. Unless otherwise stated, all media were prepared according to Sambrook and Russell (2011). The following concentrations of antibiotics and supplements were used in the growth medium: ampicillin 100 µg/ml, X-gal 80 µg/ml, ITPG 1mM and 2% glucose.

Luria-Bertani (LB) broth was constituted as follows (g/L): yeast extract, 10.0; tryptone, 5.0; NaCl, 10.0; pH adjusted to 7. For Luria-Bertani agar, 15 g/L agar was added. Tryptone Soy Broth (TSB) was constituted as follows (g/L): Tryptone, 17.0; Peptone, 3.0; Glucose, 2.5; NaCl, 5.0; KH_2PO_4, 2.5; pH adjusted to 7.4. Beef Extract (BE) was constituted as follows (g/L): Beef Extract, 10.0;

Peptone, 10.0; NaCl, 5.0; pH adjusted to 7. The ZM broth was constituted as follows (g/L): Yeast Extract, 10.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5; Ammonium sulphate, 1.0; pH adjusted to 5.4. A final concentration of 2% glucose was added to the media after autoclaving. For ZM agar, 15 g/L agar was added. Glucose was added aseptically to a final concentration of 2% after autoclaving. Nutrient Broth (NB) was constituted as follows (g/L): Peptone, 5.0, Meat Extract, 3.0; pH adjusted to 7. For Nutrient Agar, 15 g/L agar was added.

2.3. Screening for Acetoin-Producing Isolates

In 2006, Professor D. Cowan (principal investigator at the IMBM) collected soil samples from a hot spring in the Pungwe River Mozambique. Bacterial isolates were tested for acetoin production using a colorimetric Petri plate assay. Acetoin is a precursor to 2, 3-BD, and a colorimetric assay was performed according to Mallonee and Speckman (1988). Briefly, the assay is based on the organisms' ability to metabolise glucose and the subsequent detection of acetoin, a neutral product. In an alkaline environment where oxygen is available, acetoin is oxidised to diacetyl, which produces a red colour.

Isolates were screened by Dr Desiree Barnard on nutrient agar plates containing 0.5% D-glucose and incubated at 37°C for 24 hours. The cells were fixed by swirling with a 1.2 ml mixture of 5% α-napthol and 95% ethanol for a few seconds, followed by the addition of 400 µl 40% KOH and swirling for 15 seconds. After decanting the excess fluid, the plates were incubated at room temperature for 2 hours. Five colonies exhibiting a red halo were identified as being putatively positive for acetoin production (unpublished work, Dr Desiree Barnard, IMBM). These isolates were named ZM1, ZM2, ZM3, ZM4 and ZM5. A lyophilised sample of *Bacillus licheniformis* ATCC 14580 was obtained from the United States Department of Agriculture (Research, Education and Economics Agricultural Research Service) and was used as a positive control throughout the project. The sample was aseptically revived in TSB in a 37°C incubator with agitation for a period of 24 hours.

2.4. Preparation of *E. coli* competent cells and transformation

The *Escherichia coli* GeneHogs strain (genotype: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G *fhu*A:IS2), used for the preparation of competent cells, was obtained from Invitrogen (USA).

2.4.1. Chemically competent cells ($CaCl_2$)

The *E. coli* GeneHogs strain was aseptically cultured in 5 ml LB broth and incubated at 37°C with agitation at 250 rpm for a period of 24 hours. The overnight culture was added to 500 ml LB broth and incubated with agitation at 250 rpm at 37°C until the A_{600} was between 0.4 - 0.6. Cells were harvested by centrifugation for 10 minutes at 5 000 rpm (Eppendorf 5810R) at 4°C. The cells were kept on ice, resuspended in 11 ml pre-chilled 100 mM $MgCl_2$ and incubated for 30 minutes on ice. The cells were pelleted at 4000 rpm at 4°C for 10 minutes and stored on ice. The cells were resuspended in 1 ml ice cold 100 mM $CaCl_2$-15% glycerol and 100 µl aliquots were dispensed into pre-chilled 1.5 ml microcentrifuge tubes and stored at –80°C (Sambrook and Russell, 2001).

2.4.1.1. Transformation using chemically competent cells

Transformation was conducted by adding 1 µl of ligation reaction to 100 µl competent cells and vortexing briefly. The reaction was incubated on ice for 1 minute followed by heat-shock at 42°C for 45 seconds. The cells were incubated on ice for 1 minute, transferred to room temperature for two minutes and resuscitated in 900 µl LB broth by briefly vortexing and incubating at 37°C for 30 minutes. Cells were harvested by centrifugation at 9000 rpm for 30 seconds while 800 µl of the supernatant was discarded and the remaining cells were resuspended and spread onto LB plates supplemented with ampicillin (100µg/ml), IPTG (20 µg/ml) and Xgal (30 µg/ml) before incubating at 37°C for 24 hours. White colonies representing putative recombinant transformants were selected, inoculated into 5 ml LB broth containing 100 µg/ml ampicillin and incubated for 24 hours at 37°C. Following overnight incubation, the cultures were harvested for plasmid isolation.

2.4.2. Electrocompetent cells

E. coli GeneHogs was aseptically cultured in 5 ml LB broth, and incubated at 37°C with agitation at 250 rpm for a period of 24 hours. The overnight culture was added to 500 ml LB broth and incubated at 37°C with agitation at 250 rpm until the $A600_{nm}$ was between 0.4 - 0.6. Cultures were incubated on ice for 20 minutes and harvested by centrifuging at 4000 rpm at 4°C for 15 minutes. Equal volumes of 10% glycerol were used to resuspend the pellet which was then centrifuged at 4 000 rpm and 4°C for 15 minutes. This step was repeated with 0.5 x volume of the 10% glycerol. The pellet was resuspended in 20 ml cold 10% glycerol and centrifuged at 4 000 rpm at 4°C for 15 minutes. Finally, the pellet was resuspended in 2 ml cold 10% glycerol. Aliquots (100 µl) were stored at –80°C for future use (Sambrook and Russell, 2000).

2.4.2.1. Transformation of electrocompetent *E. coli* cells

Electroporation cuvettes (Bio-rad Laboratories, Hercules, California, USA) were chilled on ice an hour prior to transformation. Electrocompetent cells were thawed on ice and mixed with 1 µl ligation reaction. The mixture was transferred to pre-chilled electroporation cuvettes and pulsed (1.8 kV, 25 µF, 200 Ω) using the Bio-Rad Gene Pulser electroporator (Hercules, California, USA). The cells were resuscitated by adding 1 ml LB broth and incubating for 30 minutes at 37°C. Aliquots (100µl) were plated onto LB-AMP-IPTG-Xgal agar and incubated at 37°C for 24 hours. White colonies representing putative recombinant transformants were selected, inoculated into 5 ml LB broth containing 100 µg/ml ampicillin and incubated for 24 hours at 37°C. Following overnight incubation, the cultures were harvested for plasmid isolation.

2.5. Agarose Gel Electrophoresis

The integrity of total chromosomal DNA fragments and PCR products were verified on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide in 1 X TAE buffer (40 mM Tris-HCl, 1 mM EDTA, 10 mM glacial acetic acid, pH 8.5). Samples were prepared by the addition of 6x concentrated loading buffer (20% (v/v) glycerol and 5 mg/ml bromophenol blue). Electrophoresis was performed in 1 X TAE buffer at 100 V. Gels were visualised via ultraviolet illumination and the image captured with a digital imaging system (Alphaimager 2000, Alpha Innotech, San

Leandro, CA). DNA fragments were sized by comparison to the migration of the DNA molecular weight marker bands in the agarose gel.

2.6. Identification of Bacterial Isolates

The hypervariable regions within the 16S rRNA gene are highly conserved (Clarridge, 2004), therefore comparing the sequence of unknown isolates to a database of known species is used in the identification process (Rajendhran and Gunasekaran, 2011). The next few sections outline the procedure followed to identify the five bacterial isolates, ZM1 to ZM5.

2.6.1. Genomic DNA extraction

A modified protocol of Miller *et al.* (1999) was utilized to extract genomic DNA from bacterial strains ZM1 to ZM5, which were cultured on ZM agar at 37°C for 24 hours. A single colony of each isolate was inoculated into 5 ml ZM broth and cultured at 37°C with agitation for 24 hours. The cells were recovered via centrifugation at 13 000 rpm for 2 minutes and resuspended in 10 mg autoclaved quartz sand, 300 µl of 100 mM cold sodium phosphate buffer pH 8, 300 µl SDS lysis buffer (100 mM NaCl, 500 mM Tris pH 8, 100 g/L SDS) and vortexed briefly. A total of 300 µl chloroform/isoamyl alcohol (24:1 v/v) was added, mixed vigorously for 60 seconds and centrifuged at 15 000 rpm for 10 minutes to pellet the cellular debris. The supernatant was transferred to a sterile microcentrifuge tube and mixed with 0.5 volumes 2.5 M ammonium acetate (filter sterilised and pre-chilled at -20°C) by inversion. Following centrifugation at 15 000 rpm for 5 minutes, the supernatant was retained and 0.6 volumes of isopropanol was added. The microcentrifuge tube was inverted several times and held at room temperature for 15 minutes. The pellet was recovered by centrifugation at 15 000 rpm for 5 minutes and resuspended in 1 ml pre-chilled 70% ethanol. The DNA was collected by centrifugation at 15 000 rpm for 2 minutes and allowed to air-dry for 30 minutes. The DNA was then resuspended in 30 µl dH_20 (Miller *et al.*, 1999) and the integrity of the DNA was determined by gel electrophoresis (Section 2.5).

2.6.2. 16S rRNA gene amplification

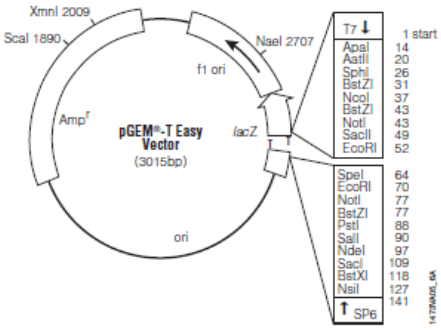
16S rRNA gene PCR was performed in a final volume of 50 µl with final concentrations of 50 – 100 ng DNA; 1 X Dream Taq Buffer; 1 U/µl Dream Taq; 200 µM dNTP each; 1 mg/ml Bovine Serum Albumin (BSA); 2 mM $MgCl_2$, 5 µM E9f (Farrelly *et al.,* 1995) Forward Primer (AAGTCGTAACAAGGTAACCG) and 5 µM U1510R (Reysenbach and Pace, 1995) Reverse Primer (AGAGTTGATCATGGCTCAG). The cycling conditions were as follows: 98°C for 5 minutes, 30 x (98°C for 30 seconds, 52°C for 30 s, 72°C for 90 seconds), 72°C for 7 minutes. Following successful amplification of the 16S rRNA gene, further analysis was performed to characterise and identify the isolates.

2.6.3. Amplified Ribosomal DNA Restriction Analysis (ARDRA)

ARDRA was used to dereplicate strains ZM1 to ZM5, in comparison to the positive control, *Bacillus licheniformis* ATCC 14580. The 16S rRNA amplicons were excised from the gel and purified using the Illustra GFX PCR DNA and Gel Band Purification kit (AEM Amersham, South Africa) as recommended by the manufacturer. A total of 5 µl of the purified amplicon was digested with 1 U *Alu*I and 1 U *Hae*II, incubated at 37°C for 24 hours and resolved on a 2.5% agarose gel in 1 X TAE (Section 2.5). In order to determine the nucleotide composition of the isolates, the 16S rRNA amplicons were cloned as described below.

2.6.4. Ligation

The PCR product was ligated into the pGEM®-T-Easy vector (Figure 7) following the recommendations of the manufacturer. The ligation reactions were halted by incubating the mixtures at 65°C for 15 minutes before the constructs were transformed into chemically competent *E. coli* GeneHogs cells (Section 2.4.1).



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Figure 7: The pGEM-T-Easy® vector with restriction enzyme and recognition sites displayed (https://worldwide.promega.com/resources/product-guides-and-selectors/protocols-and-applications-guide/cloning/)

2.6.5. Plasmid miniprep

Plasmid DNA was extracted using a modified alkaline lysis method of Sambrook and Russell (2001). A volume of 2 ml of an overnight culture was pelleted at 5000 rpm for 30 seconds at 4°C. The supernatant was decanted and the pellet was resuspended in 100 µl Solution 1 (50 mM glucose, 25 mM Tris:Cl pH 8, 10 mM EDTA pH 8). This was followed by the addition of 200 µl of Solution 2 (0.2 M NaOH, 1% (w/v) SDS), mixing by inversion and incubation for 2 minutes at room temperature. Solution 3 (5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml dH_20) was chilled and 150 µl was added and mixed by inversion until the solution coagulated. The solution was incubated at –80°C for 10 minutes. Following a 10 minute centrifugation step at 5000 rpm, the supernatant was transferred to a sterile microcentrifuge tube containing 1 ml 100% ethanol, mixed by inversion, incubated at room temperature for 2 minutes and incubated at –80°C for 10 minutes. The DNA pellet was obtained by centrifuging at 5000 rpm for 10 minutes and was allowed to air-dry. The plasmid DNA was then solubilised in 100 µl dH_20 containing a final concentration of 20 µg/ml RNase and stored at -20°C for future use.

2.6.6. Plasmid construct verification

Two methods were used to confirm successful constructs; M13 PCR and restriction digestion.

a) M13 PCR

M13 PCR is used to determine whether an insert (16S rRNA PCR product) is successfully cloned into the vector and is based on the presence of specific recognition sites on the pGEM®-T-Easy vector. The M13 primers bind and amplify a specific region within the vector, on either side of the multiple cloning site. A final PCR volume of 50 µl contained the following components (final concentration); DNA template, 50 – 100 ng; Lab Taq Buffer, 1 X; Lab Taq, 1 U/µl; dNTP, 200 µM, each; BSA, 1 mg/ml; $MgCl_2$, 2 mM; M13 Reverse Primer (CAGGAAACAGCTATGAC), 5 µM; M13 Forward Primer (GTAAAACGACGGCCAGT) and 5 µM, dH_20 to final volume. Lab Taq and its corresponding buffer were prepared in-house at the Institute of Microbial Biotechnology and Metagenomics. The cycling conditions were as follows; 98°C for 30 seconds, 30 x (94°C for 30 seconds, 52°C for 30 seconds, 72°C for 90 seconds), 72°C for 7 minutes.

b) Restriction digestion

The correct insertion of PCR products into the pGEM-T-Easy vector was verified by analysis of restriction digests. The pGEM-T-Easy plasmid contains an *EcoR*I restriction site on both sides of the Multiple Cloning Site (MCS). By selecting *EcoR*I for the restriction enzyme analysis, one can easily identify successful clones based on the *EcoR*I restriction profile.

Restriction digestion was performed by adding 500 – 1000 ng plasmid DNA to 1 X Tango buffer, 2U *EcoR*I and dH_2O to a final volume of 10 µl. The reaction was allowed to proceed at 37°C for 2 hours and resolved on a 1% agarose gel (Section 2.5).

Based on M13 PCR and restriction digestion analysis, representative clones of the five isolates were selected and submitted to the University of Cape Town sequencing facility (South Africa) (http://www.mcb.uct.ac.za/DNA_sequencing/index.htm) for sequence analysis.

Initially, isolates ZM1 to ZM5 were screened for acetoin, a precursor of 2, 3-BD, the compound of interest in this project. The *alsD* gene is responsible for the conversion of alpha acetolactate to acetoin, a high a value compound. The next step was to identify the *alsD* gene in isolates ZM1 to ZM5 as well as the positive control, *B. licheniformis* ATCC 14580.

2.7. Acetolactate decarboxylase (*alsD*) gene screening

Primer 3 (http://primer3.sourceforge.net) and Integrated DNA Technology (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer) platforms were used to design primers specific for the *alsD* gene in *B. licheniformis* ATCC 14580, yielding a 812 bp product. Recognition sites were included in the forward and reverse primers for *Nde*I (CATATG) and *Hind*III (TTCGAA), respectively. The components of the *alsD* gene PCR in a final volume of 50 µl (final concentration) were as follows; DNA template, 50 – 100 ng; Dream Taq Buffer, 1 X; Dream Taq, 1 U/µl; dNTP, 200 µM each; BSA, 1 mg/ml, $MgCl_2$, 2 mM; *alsD* Forward Primer (CCGCCATATGGGAGTGAAAAGAGGAAAATGAAAAGTGC) 5 µM; *alsD* Reverse Primer (GGCGTTCGAAGGGCTTTTTCATTTACTCGGG) and dH_20. The cycling conditions for the touch-down PCR were as follows; 94°C for 5 minutes; 30 x (94°C for 30 seconds, 55°C – 45°C: 30 seconds, decreasing with 1 degree for every second cycle, 72°C for 90 seconds); 72°C for 7 minutes.

Comparative analysis of the *alsD* gene or its homologs in *Bacillus spp* was performed to determine the level of conservation. Identified motifs were further analysed and used to design degenerate primers with a bias towards *B. licheniformis* ATCC 14580.

2.8. Bioinformatic analysis of *alsD* gene in *Bacillus* species

Multiple alignments of the amino acid sequences (accession numbers listed in Table 6) for the *alsD* gene or their homologues were analysed using Mega (version 7.0.26, Pensylvania State University) software to determine the sequence similarity. The ClustalW parameters were set to pairwise alignment: gap opening penalty – 10, multiple alignment: gap opening penalty – 10, protein weight matrix: Gonnet. The online server software MEME (version 4.12.0, Reno and Washington), a *de novo* motif discovery tool was used to identify motifs. The parameters were set as follows: minimum width = 6, maximum width = 50, and the maximum number of motifs to find = 10. The function of the identified motifs were analysed in a prokaryote database using protein sequences in teh TOMTOM Motif Comparison Tool (Version 4.12.0) (Gupta *et al.*, 2007).

Table 6: Accession numbers of organisms used in *alsD* bioinformatic analysis

Organism	Accession number	Gene symbol
Bacillus licheniformis DSM 13 = ATCC 14580	NC_006270.3	*budA*
Bacillus amyloliquefaciens DSM7	NC_014551.1	*BAMF_RS38040*
Bacillus cereus E33L	NC_006274.1	*BC0884*
Bacillus subtilis subsp. subtilis str. 168	NC_000964.3	*alsD*

A degenerate reverse primer (*alsDr2*) was designed using the *alsD* gene sequences of *Bacillus cereus* E33L (NC_006274), *Bacillus subtilis subsp. subtilis str*. 168 (NC_014479.1) and *B. amyloliquefaciens* DSM7 (NC_014551.1) with a bias towards *B. licheniformis* ATCC 14580 (NC_006322) to yield a 921 bp product. The original forward primer was used. The components of the *alsDr2* gene PCR in a final volume of 50 µl (final concentration) were as follows; DNA template, 50 – 100 ng; Dream Taq Buffer, 1 X; Dream Taq, 1 U/µl; dNTP, 200 µM, each; BSA), 1 mg/ml, $MgCl_2$, 2 mM; *alsD* Forward Primer (derived from the *B. licheniformis* ATCC 14580 gene) (CCGCCATATGGGAGTGAAAAGAGGAAAATGAAAAGTGC) 5 µM; *alsDr2* degenerate Reverse Primer (ATAYTCYAAAACGTGTCCGCC) and dH_20. The cycling conditions for the touch-down PCR were as follows; 94°C for 5 minutes; 30 x (94°C for 30 seconds, 55°C – 45°C: 30 seconds, decreasing with 1 degree for every second cycle, 72°C for 90 seconds); 72°C for 7 minutes. PCR products were resolved on a 1% agarose gel. Following successful amplification of the *alsD* gene, the product was ligated into pGEM-T-Easy and transformed into *E. coli* GeneHogs. The presence of the insert was confirmed by performing a restriction digestion analysis and PCR with the *alsDr2* primers on extracted plasmid DNA.

2.9. Fermentation analysis

Bacillus licheniformis is capable of producing 2,3 -butanediol (2, 3-BD) under suitable conditions (Nilegaonkar *et al.,* 1995; Shariati *et al.,* 1995; Celinska and Grajek, 2009). A series of fermentation studies were performed on *B. licheniformis* ATCC 14580 as well as the ZM1 to ZM5 isolates.

2.9.1. Comparative growth curves: media analysis

The *Bacillus licheniformis* ATCC 14580 and ZM1 to ZM5 isolates were cultured on LB agar and incubated at 37°C for a period of 24 hours. Based on the literature, a single colony of each isolate was used to inoculate 5 ml media and cultured for 24 hours at 37°C. The overnight cultures were sub-cultured into 50 ml of the same medium to a starting $A600_{nm}$ of 0.01. Culture growth was monitored through $A600_{nm}$ measurements at hourly intervals for the first 6 hours followed by a final measurement at 24 hours. This was performed in triplicate. Cultures were diluted 1:10 prior to recording absorbance (A) readings.

2.9.2. Carbohydrate supplemented medium

The *B. licheniformis* ATCC 14580 and ZM1 to ZM5 isolates were cultured on LB agar and incubated at 37°C for a period of 24 hours. A single colony was selected and inoculated into 5 ml LB broth containing either 2% glucose, 2% fructose, 2% starch (soluble) or 2% sucrose, and incubated at 37°C for 24 hours. All carbohydrates were sourced from Sigma. These cultures were subsequently used to inoculate 50 ml to a starting A600 of 0.01 in a 250 ml Erlenmeyer flask. Culture growth was monitored through $A600_{nm}$ measurements at hourly intervals for the first 6 hours and again at 24 hours from Day 2 to Day 7 using a spectrophotometer. This was done in triplicate.

2.10. High Performance Liquid Chromatography (HPLC) Analysis

To determine 2, 3-BD production levels of ZM1 to ZM5 and *B. licheniformis* ATCC 14580 in the glucose-enriched media, HPLC analysis was performed as follows: 1 ml of culture was collected at every sampling point throughout the growth curve and the cells pelleted by centrifugation at 5000 rpm for 10 minutes at 4°C. The culture supernatants were injected onto an 8 µm particle size Phenomenex Rezex THM-monosaccharide column (300 mm x 7.8 mm) at a temperature of 48°C. The flow rate of the elutant (5 mM sulphuric acid) was 0.6 ml/min. Chromatograms were processed using the Chromeleon© Dionex (1996-2006) software (V6.80 SP4 Build 2361). Various compounds were identified and their concentrations determined after the peak area was integrated. The identification process is based on the retention times (18.73, 19.79 and 21.34 minutes respectively) of the standards (meso 2, 3-BD 10.122mM and levo 2, 3-BD 10.191) and controls (ethanol 0.268 mM) included in each analytical run.

Chapter 3: Results and discussion

3. Introduction

In order to identify novel bacterial isolates capable of producing desired compounds, samples are collected from different environments. Following successful identification, the aim would be to explore the industrial potential of these organisms and determine their practical applications (Xiao *et al.,* 2012). In 2006, Professor D. Cowan collected soil samples from a hot spring in the Pungwe River, Mozambique. Bacterial isolations were performed and subsequently tested for acetoin production using a colorimetric agar Petri plate assay. Five colonies, named ZM1, ZM2, ZM3, ZM4 and ZM5, exhibited a red halo and were identified as being putatively positive for acetoin production (unpublished work, Dr Desiree Barnard, IMBM). Acetoin is a precursor to 2, 3-BD production, the compound of interest in this project.

This section focuses on the identification of the ZM1 to ZM5 isolates as well as determining whether these organisms are capable of producing 2, 3-BD. As mentioned in the literature review, 2, 3-BD production yields can be influenced by various factors. Before adjusting fermentation factors or employing the use of genetic engineering, this study set out to establish a baseline of 2, 3-BD production in the identified isolates, if any.

3.1. Identification of Novel Bacterial Isolates

Bacterial identification and classification is performed by using the 16S rRNA gene sequence (Rajendhran and Gunasekaran, 2011) due to the conservation in hypervariable regions within the sequence that are unique to each species. Furthermore, the 16S rRNA gene function has not changed over time therefore the gene may be used to study evolution. The size of the amplification product generated, 1500 bp, is adequate for further analysis (Janda and Abott, 2007). By amplifying the 16S rRNA gene through PCR amplification and sequencing the product, it becomes possible to identify unknown isolates (Clarridge, 2004) by comparing their sequences to a database of known species, e.g. National Centre for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov).

To identify the ZM1 to ZM5 isolates, the 16S rRNA gene sequences were amplified with the universal primer set; E9F (Farelly *et al.,* 1995) and U1510R (Reysenbach and Pace, 1995). Amplicons of 1.5 kb were generated and resolved on a 1% agarose gel (Figure 8).

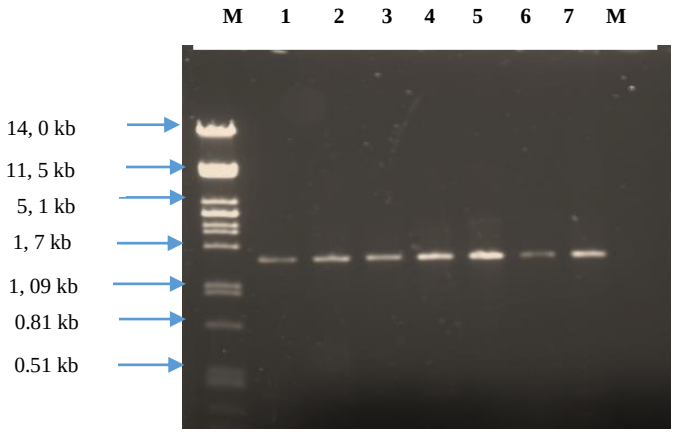


Figure 8: 16S rRNA gene amplification of the ZM1 to ZM5 isolates: The 1.5kb amplicons were resolved on 1% agarose gel. Lane M: *Pst*I digested λ DNA marker, Lane 1: ZM 1, Lane 2: ZM 2, Lane 3: ZM 3, Lane 4: ZM 4, Lane 5: ZM 5, Lane 6: *B. licheniformis* ATCC 14580, Lane 7: Positive control, Lane 8: Negative control

Following successful amplification of the 16S rRNA gene, various molecular tools were used to further characterise the ZM1 to ZM5 isolates.

3.2. Assessing Genetic Diversity: ARDRA

To determine the genetic diversity of ZM1 to ZM5, Amplified Ribosomal DNA Restriction Analysis (ARDRA) was performed. ARDRA is a molecular technique used to differentiate unknown isolates based on restriction digestion patterns obtained from their 16S rRNA PCR products (Gich, 2000). The 16S rRNA gene amplicons were subjected to restriction digestion by *Hae*II and *Alu*I for one hour and subsequently resolved on a 2% agarose gel (Figure 9).

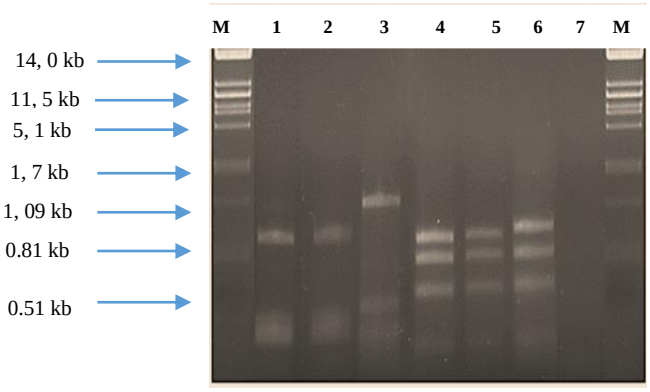
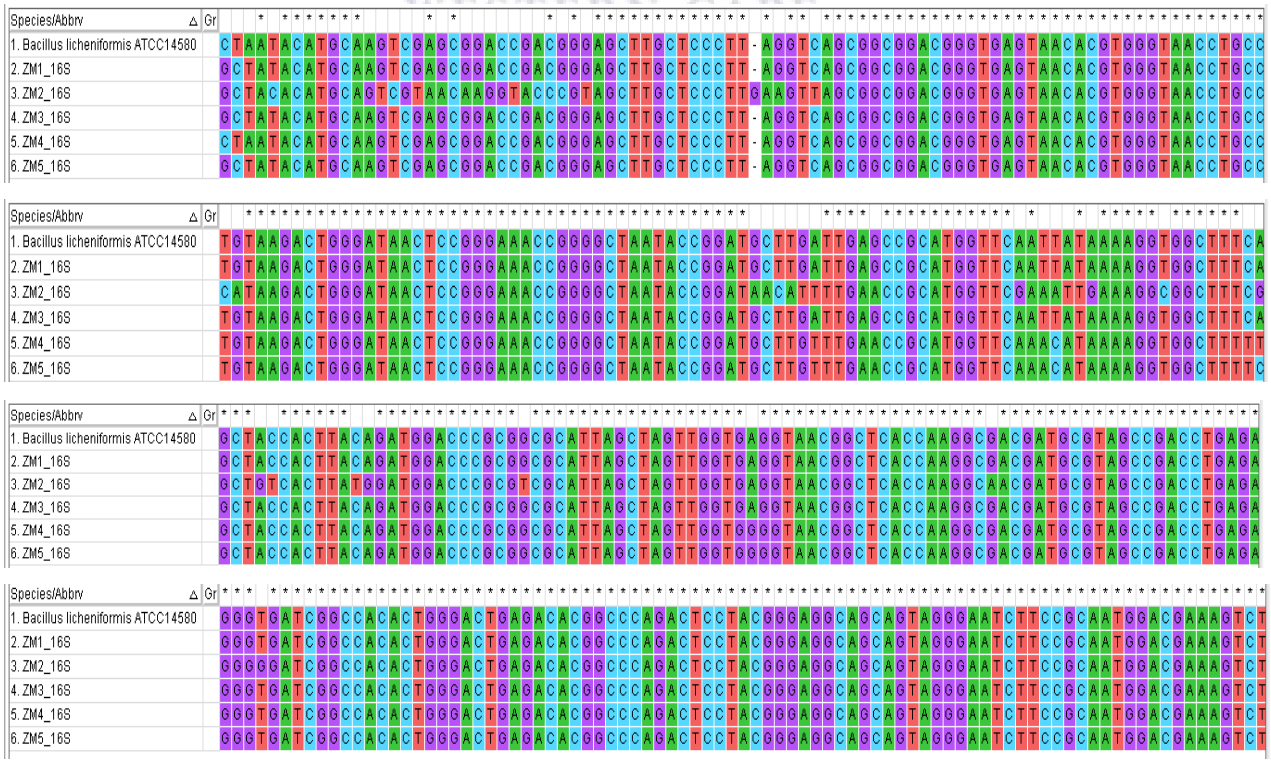


Figure 9: Amplified Ribosomal DNA Restriction Analysis (ARDRA): 16S rRNA amplicons digested with *Alu*I and *Hae*II for an hour and resolved on 2% agarose gel. Lane M: *Pst*I digested λ DNA marker, Lane 1: ZM 1, Lane 2: ZM 2, Lane 3: ZM 3, Lane 4: ZM 4, Lane 5: ZM 5, Lane 6: *B. licheniformis* ATCC 14580, Lane 7: Negative Control

Depending on the restriction enzyme used, the restriction patterns obtained are representative of the species analysed (Jain *et al.*, 2017). Based on the ARDRA results, four different phylotypes were identified. ZM1 and ZM2 displayed similar fragmentation profiles, while ZM4 and ZM5 produced similar banding patterns with four fragments each. While the banding patterns of *B. licheniformis* ATCC 14580 were similar to ZM4 and ZM5, there is a slight difference in the largest band displayed in Figure 9. This is an indication that ZM3, ZM4 and *B. licheniformis* ATCC 14580 are related. The banding pattern of ZM3 was distinctly unique as one of the fragments produced was not close to any of the other fragments. Sequence analysis of the 16S rRNA amplicons was performed to confirm the ARDRA results, as well as to identify the isolates.

3.3. 16S rRNA Sequence Analysis

The 16S rRNA PCR products were gel purified and cloned into the pGEM-T-Easy® vector system. Since *Eco*RI cleaves pGEM-T-Easy® in the multiple cloning site on either side of the insert, resulting in the release of the 1.5 kb product, the recombinant clones were identified by performing restriction digestion with *Eco*RI. For further confirmation, M13 PCR was conducted to confirm the presence of an amplicon of 1.5 kb within the multiple cloning site (results not shown). The pGEM-T-Easy® clones containing the putative 16S rRNA inserts were sequenced to determine the nucleotide composition and to confirm the ARDRA results.



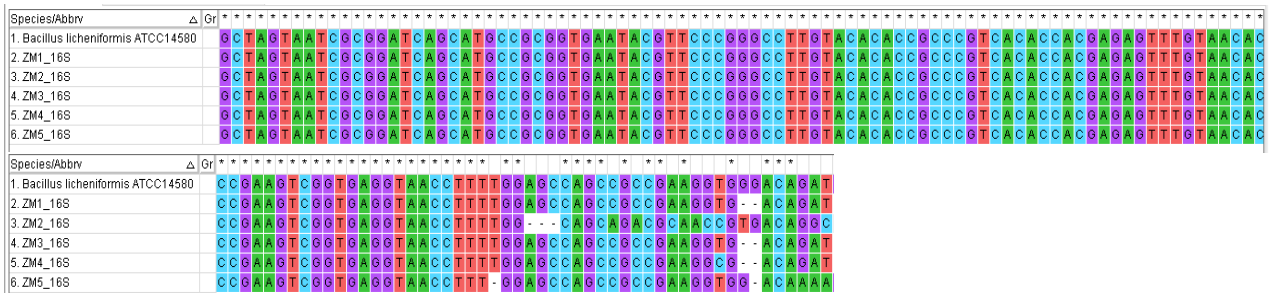


Figure 10: 16S rRNA gene multiple sequence alignment of *B. licheniformis* ATCC 14580 and ZM1 to ZM5 isolates

The ZM1 to ZM5 isolates were identified as *B. licheniformis* (Figure 10) which is a Gram-positive, rod-shaped, facultative anaerobic, spore-forming bacterium. *B. licheniformis* secretes large amounts of extracellular enzymes including proteases, amylases and peptide antibiotics (Veith *et al.*, 2004; Wang *et al.*, 2011; de Oliviera and Nicholson, 2016), has short fermentation cycles and high growth rates, making it attractive as an industrial organism (Sharma and Satyanarayana, 2013). The high growth rate is as a result of its ability to metabolise carbohydrates under various conditions (Sharma and Satyanarayana, 2016) to produce a mixture of D-2, 3-BD and meso-2, 3-BD (Perego *et al.*, 2003; Li *et al.*, 2013; Qi *et al.*, 2014; Li *et al.*, 2014b). The optimal fermentation conditions of *B. licheniformis* is 30 – 40°C, which allows room for contamination and can complicate large-scale fermentation. However, this could be circumvented by identifying thermophilic *B. licheniformis* strains capable of producing 2, 3-BD (Li *et al.*, 2014b). The fermentation temperature of thermophiles range between 50°C and 60°C, thereby significantly reducing the chances of bacterial contamination (Xiao *et al.*, 2012) as noted in Table 4. In recent years, this organism was engineered to improve its ability to breakdown renewable biomass (Anderson *et al.*, 2013).

Due to the slight differences observed in the 16S rRNA sequences of the *B. licheniformis* ZM1 to ZM5 isolates, the decision was made to continue the research study with all five isolates. While ZM1 and ZM2 appeared to be similar, as expected, the 16S sequence identified a greater amount of differences between these two isolates.

The initial phenotypic screen was for acetoin, a precursor to 2, 3-BD, therefore the molecular screening was initiated for the main gene responsible for its production, acetolactate decarboxylase (*alsD*), to establish the amount of genetic diversity in this gene, between the isolates.

3.4. Acetolactate decarboxylase (*alsD)* gene screening

The *B. licheniformis* ZM1 to ZM5 isolates were identified as putative acetoin producers. During favourable conditions, *AlsD* converts α-acetolactate to acetoin via the main metabolic pathway (Qi *et al.*, 2014, Yang *et al.*, 2016). However, due to its unstable nature, α-acetolactate may spontaneously be converted to diacetyl during the mixed-acid or hypothetical pathway (Table 2 and Figure 4). Furthermore, in the mixed-acid pathway, diacetyl is reduced to acetoin by diacetyl reductase. Therefore, if the *alsD* gene is not isolated from these acetoin producing organisms, screening will commence for the diacetyl reductase gene.

As the metabolic pathway is the best understood method of 2, 3-BD production, *B. licheniformis* ZM1 to ZM5 isolates were screened for the presence of the *alsD* gene. Primers were designed using the *alsD* gene in *B. licheniformis* ATCC 14580 based on the 16S sequence identity and due to the presence of the *alsD* gene in this organism.

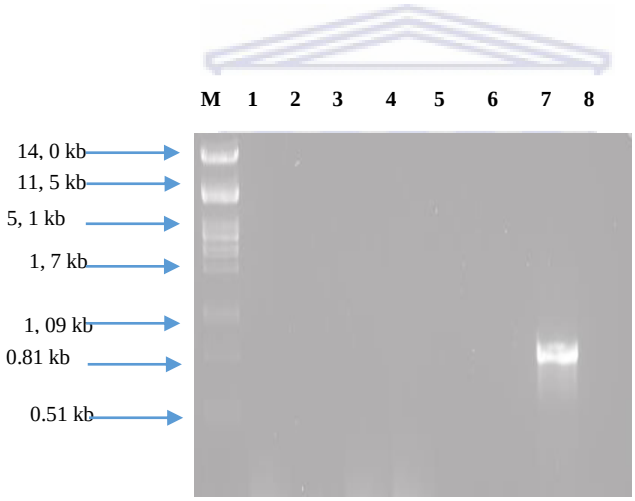


Figure 11: Acetolactate Decarboxylase Synthase (*alsD*) amplification: Lane M: *Pst*I digested λ DNA marker, Lane 1: ZM 1, Lane 2: ZM 2, Lane 3: ZM 3, Lane 4: ZM 4, Lane 5: ZM 5, Lane 6: blank lane, Lane 7: *B. licheniformis* ATCC 14580 Lane 8: negative control

The *alsD* gene was successfully amplified in *B. licheniformis* ATCC 14580 using the primer set designed from this organism (Figure 11). However, the *alsD* gene did not amplify in any of the *B. licheniformis* ZM1 to ZM5 isolates. The lack of amplification may be due to acetoin being produced via the hypothetical pathway or it may be as a result of the genetic diversity between the *B. licheniformis* ZM1 to ZM5 and *B. licheniformis* ATCC 14580 isolates. To eliminate the latter option, further investigation of the sequence diversity of the *alsD* gene in *Bacillus spp* was performed by designing a degenerate reverse primer.

3.5. Bioinformatic analysis of the *alsD* gene

To understand the level of conservation of the *alsD* gene in *Bacillus spp* (Table 5), multiple sequence alignment (Figure 12a) and motifs (Figure 12b) were investigated to determine the best possible location to design the degenerate primers. Marlow *et al.*, (2013) elucidated the AlsD structure and identified highly conserved histidine (194, 196 and 207 – indicated by the blue arrow in Figure 12) and glutamate residues (253 – indicated by the green arrow in Figure 12). However, the region identified to be the most conserved between all the genes and which yielded a compatible primer, did not overlap these conserved residues (Figure 12).

Figure 12: Identification of degenerate reverse primer selected conserved region in *Bacillus spp alsD* gene: 12a; multiple amino acid sequence alignment, 12b; MEME output identifying all motifs in the *Bacillus* spp *alsD* gene

The amino acid multiple sequence alignment identified a highly conserved region (Figure 12a – red block) in the four *Bacillus spp* isolates, therefore this sequence was considered as a good option for the design of the degenerate reverse primer which had a bias towards *B. licheniformis*.

Furthermore, the difference of one and two amino acids for *B. amyloliquefaciens* and *B. cereus* respectively, were taken into account in designing the degenerate primers.

Further analysis of the motif harbouring the sequence selected for the degenerate primer was employed using TOMTOM Motif Comparison Tool (Version 4.12.0) (Gupta *et al.*, 2007). The TRTVELQEK motif was identified in several transcriptional regulation and DNA binding genes. In *Clostridium difficule* 630, the motif was identified in the *ccpA* gene, a global regulator of carbon catabolite repression (CCR). The *ccpA* gene mediates transcription in response to carbohydrate catabolism (Antunes *et al.*, 2012) by directly binding to *cis*-acting catabolite response element (Dubois *et al.*, 2016). In *Pseudomonas protegens* Pf-5, the motif was identified in the zinc uptake regulator (Zur) gene, which has DNA binding transcription factor activity (Lim *et al.*, 2013).

The reverse degenerate primer was used with the original forward primer. Amplicons of the expected size were obtained, suggesting that the conserved region targeted had resulted in the successful amplification of the *alsD* gene from the *B. licheniformis* ZM1 to ZM5 isolates *and B. licheniformis* ATCC 14580 (Figure 13). To confirm this, the amplicons could have been sequenced. However, given that the amplicons obtained were specific (no non-specific amplification), and that all isolates were of the same species, it was deemed unnecessary.

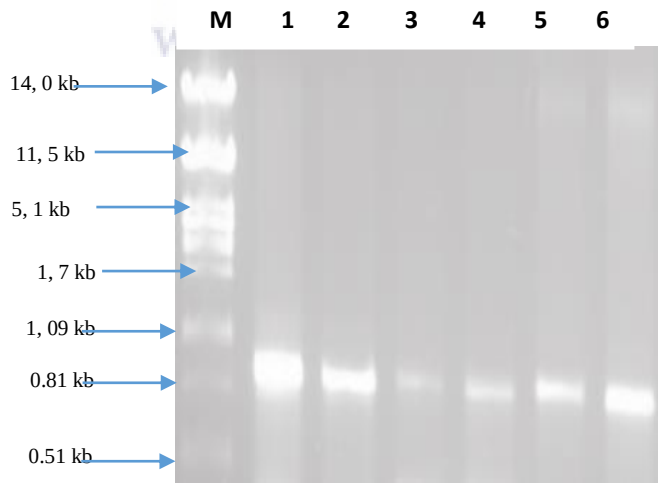


Figure 13: Putative amplification of *alsD* using degenerate primers: The 0.92 kb amplicon resolved on a 1% agarose gel. Lane M: *Pst*I digested λ DNA marker, Lane 1: ZM 1, Lane 2: ZM 2, Lane 3: ZM 3, Lane 4: ZM 4, Lane 5: ZM 5, Lane 6: *B. licheniformis* ATCC 14580

Based on the above, it is evident that various approaches must be employed to amplify desired genes in isolates for which no data is available. Due to the fact that no sequence data was available for the *alsD* gene of ZM1 to ZM5, the design and use of degenerate primers served as

an excellent tool to confirm its presence. Caution needs to be exercised when designing degenerate primers as the level of conservation across isolates may differ and accurately designing the primers in the correct region is key. Failure to do so will not result in amplification of all desired targets and one may incorrectly infer that the gene of interest is absent in the organism, when in fact the region selected for the degenerate primer design was flawed.

3.6. Comparative Growth Analysis: Media types four media formulations

B. licheniformis is capable of producing 2, 3-butanediol (2, 3-BD) under suitable conditions (Nilegaonkar *et al.,* 1995; Shariati *et al.,* 1995; Celinska and Grajek, 2009). In order to determine the optimal conditions for 2, 3-BD production in *B. licheniformis* ZM1 to ZM5 isolates, a series of fermentation studies was performed. As a baseline, cultures of each isolate were grown in shaking incubators at 250 rpm, pH 7 and 37°C for 24 hours in LB (Qiu *et al.,* 2016), ZM (in-house media), NB (Pattison *et al.,* 2003) and BE media (Sarker *et al.,* 2013) (Figure 14) to determine which would result in the maximum growth for the five isolates. The starting $A600_{nm}$ of 0.01 was achieved by recording absorbance readings of the starter cultures and performing dilutions to ensure an even inoculum in all the experiments.

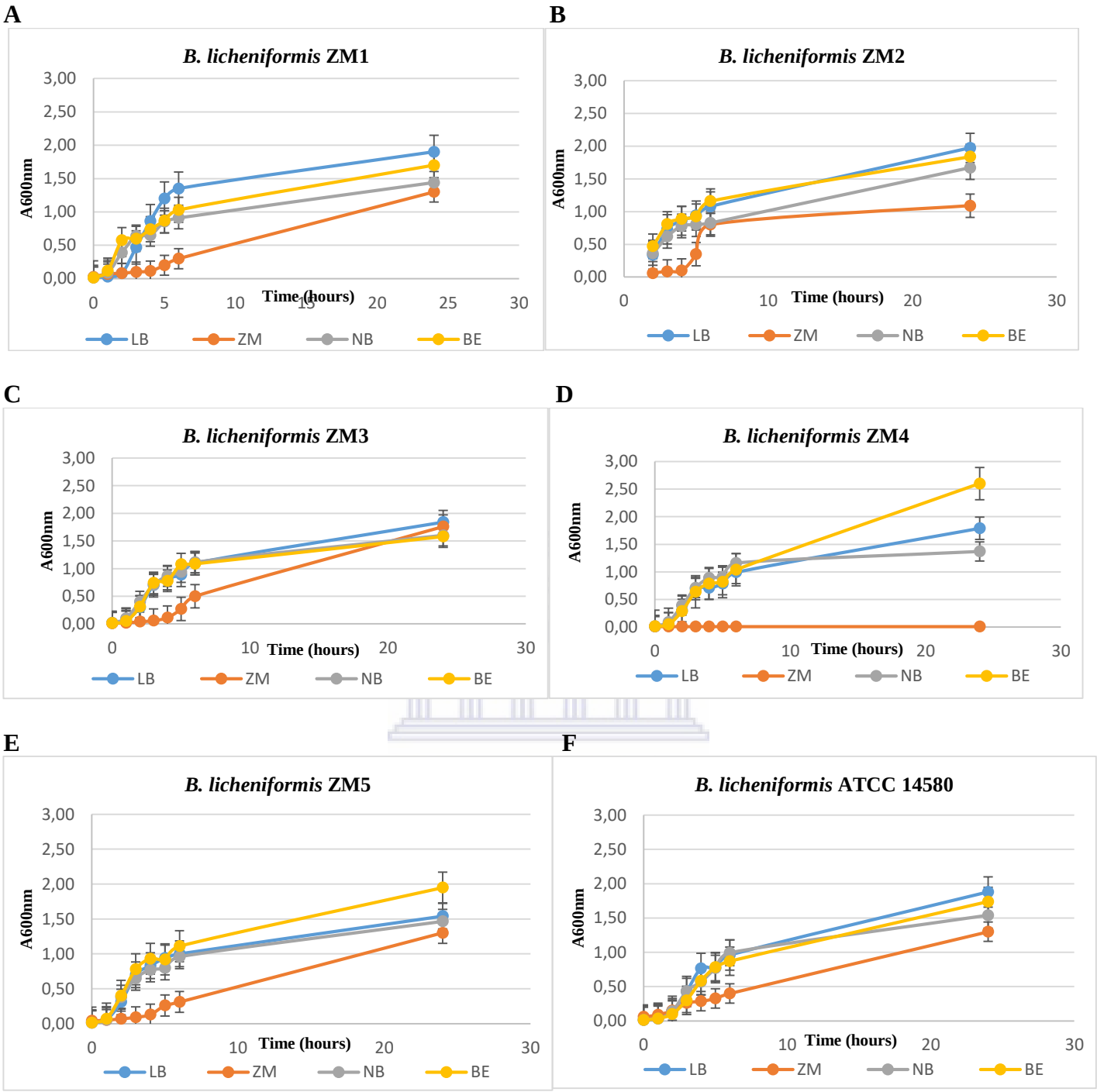


Figure 14: Media analysis: *B. licheniformis* ZM 1 to ZM5 and *B. licheniformis* ATCC 14580 cultured in LB, ZM, NB and BE media – A: ZM1, B: ZM2, C: ZM3, D: ZM4, E: ZM5, F: *B. licheniformis* ATCC 14580. Fermentations were performed in triplicate. Data represents the mean (n=3) ±standard error

Based on the fermentation profiles depicted above (Figure 14), a decision needed to be made as to which media source would be used in phase 2 of the experiment, i.e. carbon source analysis. The growth profiles of ZM1 to ZM5 observed in NB, BE and LB displayed no significant differences and were fairly similar, while all isolates displayed poor growth in ZM media, resulting in its exclusion. In consultation with the literature (Wang *et al.*, 2011; Li *et al.*, 2017), LB media was selected for phase 2 as it was commonly used in *B. licheniformis* fermentation studies.

3.7. Addition of carbon sources

The addition of carbon sources is often used to improve bacterial growth and production of selected compounds. These include sucrose, fructose and glucose, which are found in sugarcane, a commonly used fermentation feedstock (Song *et al.,* 2018). Following mining of the literature to determine which carbon sources to test, glucose, sucrose, fructose and starch (Kabisch *et al.,* 2013) were selected and subsequently added to the LB medium. The correct concentration of carbon source results in accelerated microbial growth, while too high substrate concentrations can have a negative effect (Yu *et al*., 2017). Keeping this in mind, a total of 2% of either glucose, sucrose, starch or fructose was introduced to 50 ml LB media and cultured at 250 rpm, pH 7 for a period of 7 days (Figure 15). In order to determine the growth rate following the supplementation of carbohydrates, 1 ml fractions were collected and analysed at $A600_{nm}$. The effect of glucose, sucrose, starch and fructose on cellular density was determined by comparing the growth present in LB medium to that of the carbohydrate-enriched medium (Figure 15).

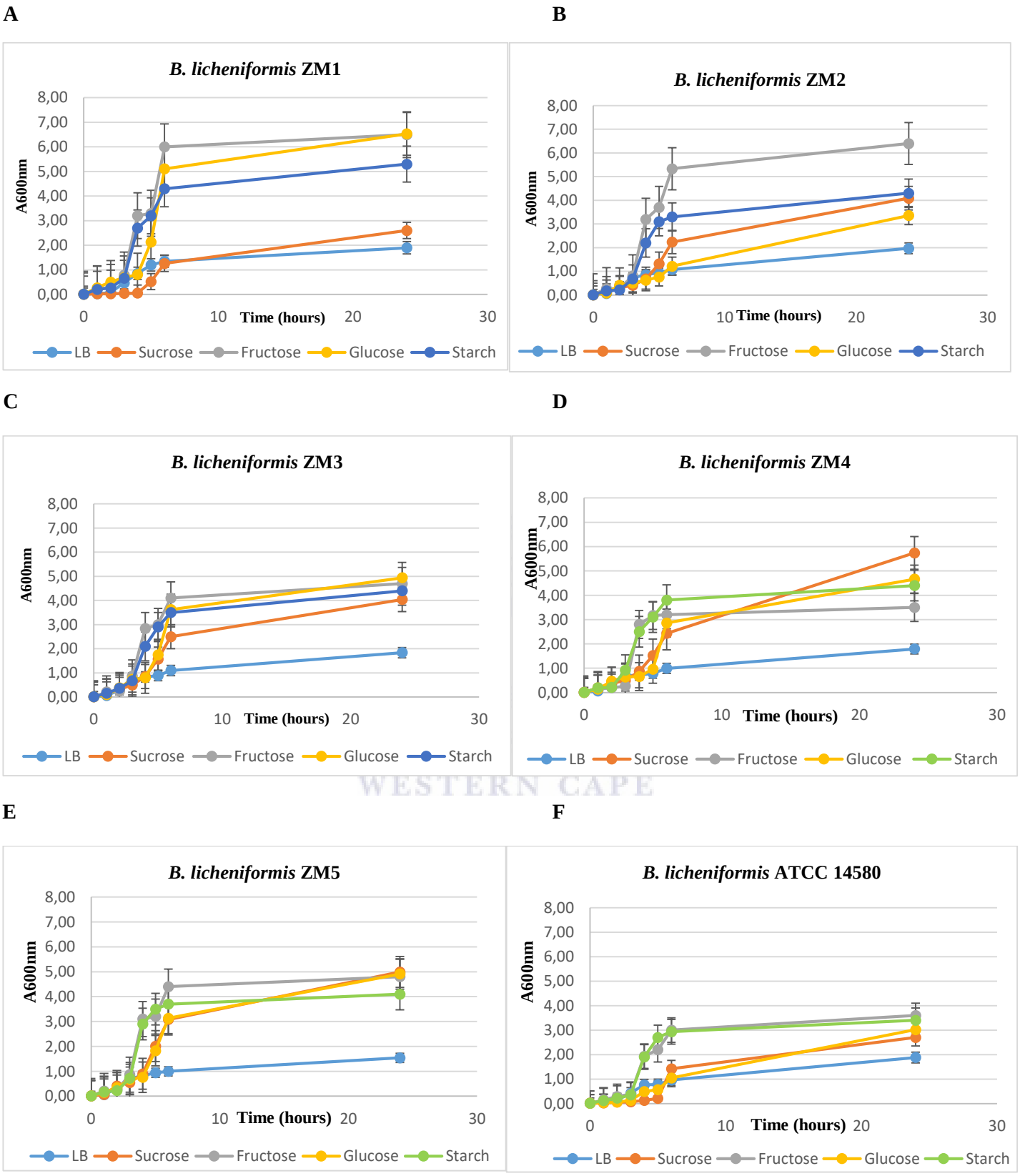


Figure 15: Carbon source analysis: Comparison of cellular density in *B. licheniformis* ZM1 to ZM5 and *B. licheniformis* ATCC 14580 cultured in LB media and LB media enriched with 2% glucose, 2% starch, 2% fructose and 2% sucrose performed in triplicate. Data represents the mean (n=3)±standard error.

Comparison of the glucose, sucrose, fructose, starch LB-enriched media to the non-enriched media clearly indicates that the presence of some of the additional carbohydrates has a positive effect on cellular density in the isolates (Figure 15). Therefore, the aim was to identify the preferred carbon source for each isolate which could subsequently result in fermentation optimisation in further studies (Figure 15).

The growth observed in the sucrose-enriched medium of ZM1, ZM2, ZM3 and *B. licheniformis* ATCC 14580 displays minimal improvement when compared to the non-enriched media. Furthermore, this carbohydrate yields the lowest cellular density when compared to the other sugars, which implies that sucrose is not favoured under these fermentation conditions. The lag phase of the isolates in the sucrose enriched media appears to be longer than that observed in the other carbon sources. Furthermore, the isolates display a more pronounced exponential phase (data not shown) after 24 hours of fermentation, suggesting that the isolates require more time to efficiently ferment sucrose. After 28 hours, *Bacillus stearothermophilus* ATCC 2027 was able to produce 14.4 g/L 2, 3-BD by using 30 g/L sucrose as a substrate. This was achieved when Giovannini *et al.* (2008) assessed the effect of varying concentrations of sucrose (0, 10, 20, 30 and 40 g/L) at 40°C. Therefore, in order to improve the comparison of sucrose-enriched media to non-enriched media, the isolates will require longer fermentation times.

All isolates displayed increased cellular density in the fructose-enriched media. However, when compared to the other carbohydrate sources, ZM2 and *B. licheniformis* ATCC 14580 favoured the LB-medium supplemented with fructose and resulted in a 2 to 3-fold increase when compared to the non-enriched medium. *B. licheniformis* ATCC 14580's preference for fructose is in line with the study performed by Li *et al.* (2014), where 30 hours of fermentation yielded 103 g/L 2, 3-BD from inulin. Fructose and glucose are hydrolysis products of inulin, an abundant, non-structural storage polysaccharide found in several plants (Li *et al.,* 2014c). Therefore, inulin would be a useful substrate for these two isolates as its hydrolysis would result in fructose and glucose.

While the starch-enriched media was not favoured by any of the isolates, ZM5 displayed the slowest growth at 24 hours of fermentation. However, after 7 days (data not shown), the cellular density of the isolate increased and was more on par with the other isolates. Following 24 hours of fermentation, the growth observed in all isolates was relatively mediocre. Perego *et al.* (2003) optimised 2, 3-BD production in *B. licheniformis* NCIMB 8059 by comparing sucrose, glucose and constarch hydrolysate. While the 2, 3-BD concentration achieved from starch hydrolysate was low (6.4 g/L), this demonstrated that starch could also be used as a carbon source for 2, 3-BD production (Peregro *et al.,* 2003).

Comparison of cellular density observed in LB media to that of glucose-enriched media showed varied results amongst the ZM1 to ZM5 isolates. Based on a study performed by Yu *et al.* (2017), the addition of glucose as a carbon source shortened the lag phase and increased the biomass observed in isolates (Yu *et al.,* 2017). This was not distinctly evident in all the isolates in this study. For *B. licheniformis* ATCC 14580, the addition of glucose arrested cellular growth for the first five hours, which was not expected. The presence of glucose in ZM3 and ZM4 did not result in significant changes in cellular density. The growth of ZM1 was enhanced due to the presence of glucose and following 24 hours of culturing, this isolate displayed the highest biomass. Additionally, in a research study performed by Yu *et al,* (2017), the data generated suggests that glucose was responsible for the activation of the polysaccharide synthesis pathway. This subsequently resulted in an increase in polysaccharide secretion (Yu *et al.,* 2017).

While fructose (ZM2 and *B. licheniformis* ATCC 14580), glucose (ZM1, ZM3) and even sucrose (ZM4 and ZM5) resulted in maximum cellular density in the isolates, the growth observed in the glucose enriched medium was the highest after 24 hours of fermentation therefore HPLC analysis was only performed on the glucose enriched medium.

3.8. HPLC analysis of glucose enriched medium

A total of 1 ml samples were collected from *B. licheniformis* ATCC 14580 and *B. licheniformis* ZM1 to ZM5 isolates cultured in 2% glucose-enriched LB medium. This was performed hourly for the first 6 hours and every 24 hours thereafter for 7 days. The cellular density of these fractions were measured and further analysed by HPLC to detect the presence of 2,3- BD and other fermentation compounds (Table 7). This analysis was performed in triplicate.

Table 7: HPLC analysis: Concentration (mM) of substrate (glucose) and various end products following 7-day culture in 50 ml LB medium supplemented with 2% glucose of *B. licheniformis* ZM1 to ZM5 and *B. licheniformis* ATCC 14580.

Isolate	A600	2, 3-BD1 (mM)	2, 3-BD2 (mM)	Glucose (mM)	Citrate (mM)	Formate (mM)	Ethanol (mM)	Lactate (mM)
ZM1	0h – 0.01 1h – 0.28 2h – 0.51 3h – 0.69 4h – 0.81 5h – 2.13 6h – 5.12 D1 – 6.53 D2 – 6.61 D3 – 7.14 D4 – 8.22 D5 – 9.20 D6 – 9.87 D7 – 10.50	D2 h - 0.002	4h - 0.016	0h – 0.261 1h – 0.242 2h – 0.050 3h – 0.157 4h – 0.109 5h - 0.293 6h – 0.294	NA	0h – 0.291 2h - 0.129 3h – 0.218 4h – 0.590 5h – 0.280 6h – 0.267 D1 – 1.140 D2 – 1.141 D3 – 0.258 D4 – 0.811 D5 – 0.805 D6 – 0.006	0h – 1.33 D1 – 0.055 D2 – 0.162 D3 – 0.227 D5 – 0.217 D6 – 0.235	NA
ZM2	0h – 0.01 1h – 0.09 2h – 0.41 3h – 0.51 4h – 0.63 5h – 0.76 6h – 1.22 D1 – 3.36 D2 – 5.52 D3 – 6.30 D4 – 7.21 D5 – 8.38 D6 – 8.9 D7 -9.50	NA	5h - 0.041 6h - 0.033	0h – 0.253 1h – 0.288 3h – 0.149 D1 – 0.002	NA	0h – 0.010 1h - .0314 4h – 0.381 5h – 0.956 6h – 0.655 D2 – 0.251 D3 – 0.439 D4 – 0.103 D5 – 0.373	4h – 0.046 5h – 0.089 6h – 0.014 D2 – 0.032 D3 – 0.010 D4 – 0.017 D5 – 0.052 D7 – 0.013	NA
ZM3	0h – 0.01 1h – 0.10 2h – 0.38 3h – 0.73 4h – 0.80 5h – 1.75 6h – 3.62 D1 – 4.94 D2 – 5.38 D3 – 5.64 D4 – 6.32 D5 – 7.19 D6 – 7.90 D7 -8.70	NA	4h - 0.221 5h - 0.351 6h - 0.777	0h – 0.232 1h – 0.271 2h – 0.048 3h – 0.142 4h – 0.068	0h – 0.010 1h – 0.082 D2 – 0.001 D3 – 0.001	2h – 0.398 3h – 0.331 4h – 0.692 5h – 0.659 6h – 1.236 D1 – 0.471 D2 – 0.299 D3 – 0.073 D4 – 0.116 D6 – 0.038 D7 – 0.073	2h – 0.160 4h – 0.020 5h – 0.049 6h – 0.196 D1 – 0.033 D2 – 0.166 D7 – 0.039	2h – 0.450 3h – 0.064
ZM4	0h – 0.01 1h – 0.20 2h – 0.50 3h – 0.70 4h – 0.70 5h – 1.00 6h – 2.90 D1 – 4.7 D2 – 5.9 D3 – 6.70 D4 – 7.50 D5 – 8.60 D6 – 8.90 D7 – 9.7	NA	4h - 0.843 5h - 0.295 6h - 0.023	1h – 0.232 2h – 0.048 3h – 0.156 5h – 0.093 6h – 0.102	1h – 0.063	2h – 0.086 3h – 0.699 4h – 1.417 5h – 0.870 6h – 0.781 D1 – 1.640 D2 – 0.714 D3 – 0.103 D6 – 0.341 D7 – 0.302	3h – 0.051 4h – 0.184 5h – 0.143 6h – 0.051 D1 – 0.395 D2 – 0.180 D6 – 0.008	3h – 0.071

Isolate	A600	2, 3-BD1	2, 3-BD 2	Glucose	Citrate	Formate	Ethanol	Lactate
ZM5	0h – 0.01 1h – 0.2 2h – 0.3 3h – 0.7 4h – 0.8 5h – 1.8 6h – 3.1 D1 – 4.9 D2 – 5.4 D3 – 6.5 D4 – 7.5 D5 – 8.4 D6 – 8.7 D7 - 9.3	NA	4h -0.808 5h - 0.166 6h - 0.021	0hr – 0.256 2h – 0.048 3h – 0.122 5h – 0.386 6h – 0.083	0hr – 0.007 3h – 0.068	0h – 0.146 2h – 0.042 3h – 0.060 4h – 1.275 6h – 0.756 D1 – 1.438 D2 – 0.185 D3 – 0.214 D5 – 0.020	0h – 0.018 4h – 0.176 5h – 0.008 6h – 0.048 D1 – 0.372 D2 – 0.172 D3 – 0.170	NA
B licheniformis ATCC 14580	0h – 0.1 1h – 0.1 2h – 0.1 3h – 0.1 4h – 0.5 5h – 0.6 6h – 1.0 D1 – 3.0 D2 – 4.2 D3 – 5.1 D4 – 7.4 D5 – 7.8 D6 – 8.2 D7 - 8.9	NA	NA	0h – 0.214 1h – 0.253 2h – 0.048 3h – 0.241 4h – 0.229 5h – 0.210 6h – 0.201 D4 – 0.002	0h – 0.133 1h – 0.150 3h – 0.198 4h – 0.173 5h – 0.152 6h – 0.157 D3 – 0.001 D4 – 0.001 D5 – 0.001 D6 – 0.002 D7 – 0.002	0h – 0.022 1h – 0.144 2h – 0.031 3h – 0.118 4h – 0.055 D1 – 0.612 D2 – 0.601 D3 – 0.207 D4 – 0.225 D5 – 0.292 D6 – 0.244 D7 – 0.356	3h – 0.007 D1 – 0.064 D2 – 0.103 D3 – 0.117 D4 – 0.112 D5 – 0.095 D6 – 0.116 D7 – 0.195	NA

**Blue and yellow highlights indicate by-products detected at same time as 2, 3-BD1X and 2, 3-BD2X respectively. Similar phylotypes were observed in ZM1 and ZM2 (grey); ZM4 and ZM5 (pink), while ZM3 (blue) and B. licheniformis ATCC 14580 (red) displayed different phylotypes*

Wild-type *Bacillus* strains usually produce 2, 3-BD during log or stationery growth phase (Bialkowska *et al.*, 2015). This was not the case for this study as HPLC analysis indicated *B. licheniformis* ZM1 to ZM5 isolates produced meso-2, 3-BD in the glucose-supplemented media between 4 and 6 hours, which corresponded to the lag phase for all isolates. While this is advantageous as the fermentation time in these isolates is greatly reduced, further optimisation is required to increase the yields. Due to these organisms' ability to completely ferment glucose within 6 hours, with miniscule amounts detected at day 1 (0.002 mM) and day 4 (0.002 mM), the initial glucose concentrations could potentially be increased in order to determine the optimal starting concentration. Alternatively, a different fermentation approach may be used. Jureschu *et al.*, (2013) employed the use of a fed batch system to produce 144 g/L 2, 3-BD from glucose. Bialkowska and colleagues (2015) also employed the use of a fed batch system to produce 113 g/L 2, 3-BD from apple pomace hydrolysate (Bialkowska *et al.*, 2015). The *B. licheniformis* ZM1 to ZM5 isolates could be cultured in a fed batch system so that more glucose may be available in the media for conversion to 2, 3-BD.

Several research studies employed the use of genetic manipulation to produce a single 2, 3-BD isomer in *B. licheniformis*. Qui and colleagues (2016) blocked the gene responsible for converting D-acetoin to D-2, 3-BD

in an attempt to produce high yields of meso-2, 3-BD (Qui *et al.*, 2016). *B. licheniformis* ZM2 to ZM5 naturally produced only meso-2, 3-BD under the fermentation conditions tested. Due to these organisms' ability to produce a single isomer, this would greatly reduce the cost of industrial production as downstream purification processes will not be required. Trace amounts of the levo-2, 3-BD was detected in *B. licheniformis* ZM1 and further optimisation would be useful to increase the yields.

When considering the fermentation temperature, *B. licheniformis* strains are able to produce 2, 3-BD at a broad temperature range as Jureschu *et al.,* (2013) produced 144.7 g/L 2, 3-BD at 30 °C. Li *et al.* (2013) were able to produce 115 g/L of 2, 3-BD at 50°C while Wang and colleagues (2012) were only able to produce 12.1 g/L at the same temperature. However, for the *B. licheniformis* ZM1 to ZM5 isolates, it would be valuable to perform additional optimisation studies and due to the origin of the isolates, it may be effective to increase the fermentation temperatures in order to explore the 2, 3-BD yield. The different performance of organisms, as previously mentioned, indicates that various factors contribute to the 2, 3-BD yields.

A high amount of by-product was noted that negatively affects 2, 3-BD yield. Therefore, further optimisation is required to increase the 2, 3-BD yields and to reduce the by-product formation. This may be accomplished by considering the following factors;

- Fermentation/bioreactor system: By implementing a fed-batch system, the glucose concentration may be kept constant. This will ensure that the initial concentration is not too high, which can inhibit the production of desired compounds (Li *et al.*, 2010), while ensuring that there is sufficient glucose to continue producing 2, 3-BD. As noted, the organism was able to almost completely ferment 20% glucose by 6 hours, therefore additional glucose may be required to continue 2, 3-BD production.
- Optimisation of the pH: pH 7 may need to be maintained as several acidic by-products are generated that affect the pH of the medium. Li *et al.* (2010) previously examined the effect of pH on the development on 2, 3-BD in *B. licheniformis* and found that pH 7 was optimal for enhanced growth while Qiu and colleagues (2016) found pH 6 to be suitable (Li *et al.*, 2010; Qiu *et al.*, 2016; Yu *et al.*, 2017).
- Carbon source: while glucose resulted in the overall maximum growth in the isolates following 24 hours of fermentation, fructose and sucrose also yielded favourable cellular density and may therefore be considered in HPLC analysis. As the isolates were entering the exponential phase at this time, further comparisons to the non-enriched media are required. Additionally, the concentration of the carbon source may also be adjusted to determine the ideal condition per isolate as these isolates completely fermented the 2% glucose by 6 hours.

B. licheniformis ATCC 14580 did not produce 2, 3-BD in the fermentation conditions tested. Bialkowska *et al.* (2015) discovered that *B. licheniformis* NCIMB 8059 was not able to produce 2, 3-BD with only glucose or fructose as a carbon source. The addition of enzymatic apple pomace hydrolysate resulted in 2, 3-BD production while high concentrations of nutrients inhibited 2, 3-BD production of this organism. This may indicate that *B. licheniformis* ATCC 14580 potentially requires additional nutrients to produce 2, 3-BD during the fermentation conditions used in this study (Bialowska *et al.*, 2015). Li and colleagues (2014) reported that the use of inulin hydrolysate as a carbon source for *B. licheniformis* ATCC 14580 resulted in 103 g/L 2, 3-BD in 30 hours. In order to determine the optimal growth temperature in their study, the fermentation was performed at 30°C, 37°C, 42°C, 50°C and 55°C. At 50°C the cells displayed increased growth, consumed fructose at a higher rate and produced 39.0 ± 0.8 g/l 2, 3-BD production after 11 hours (Li *et al.*, 2014). This indicates that while the fermentation temperature of 37°C was sufficient for the production of trace amounts of 2, 3-BD in *B. licheniformis* ZM1 to ZM5, it may not have been optimal for *B licheniformis* ATCC 14580.

When comparing the 2, 3-BD production patterns in *B. licheniformis* ZM1, ZM2, ZM4 and ZM5, the concentration decreased over time until it was no longer present. Conversely, in *B. licheniformis* ZM3, the 2, 3-BD production increased between 4 and 6 hours. This different fermentation profile observed in *B. licheniformis* ZM3 may be attributed to the isolate belonging to a different strain, as indicated in the 16S rRNA results. As no fraction was collected until 24 hours, one is not able to infer whether the 2, 3-BD concentration continued increasing.

Chapter 4: Discussion

To identify novel bacterial isolates capable of producing desired compounds, samples were collected from different environments. Following successful identification, the aim was to better understand the isolated organisms. In this study, isolates collected from soil samples in a hot spring were identified as *B. licheniformis*, which are GRAS organisms (Qui *et al.*, 2016), and that were capable of producing 2, 3-BD under tested fermentation conditions. The preliminary results generated from these isolates is promising as the work described here has laid a foundation for understanding the isolated *B. licheniformis* ZM1 to ZM5 isolates.

Due to the isolates originating from a thermophilic environment, further examination will be required to determine their ability to produce 2, 3-BD at elevated temperatures. As mentioned in the literature review, the use of thermophilic organisms greatly reduces the contamination rates observed during the fermentation process in industrial applications.

We identified putative positive screening of the *alsD* gene in the *B. licheniformis* ZM1 to ZM5 isolates and future work can be performed to confirm this. Furthermore, molecular screening for the acetolactase synthase (*alsS*) and *gdh* genes, which are responsible for the conversion of pyruvate to acetolactate and acetoin to 2, 3-BD, respectively, can be performed. Sequence alignments can be performed to determine the level of sequence similarity or difference between these strains, which can potentially assist in eliminating one or more strains in future characterization work. Identification of the genes involved in 2, 3-BD production will aid in genetic manipulation to enhance the 2, 3-BD production, should this option be considered. Additionally, the levels of by-product formation can be decreased by employing the use of genetic manipulation to ensure that available nutrients are utilised to produce the compound of interest.

Furthermore, we identified *B. licheniformis* ZM1 to ZM5's ability to produce meso-2, 3-BD under the fermentation conditions outlined, hence it will be worthwhile adjusting the fermentation conditions to increase the 2, 3-BD yields. As mentioned, downstream purification applications are costly, therefore the *B. licheniformis* ZM2 to ZM5 strains show industrial potential due to their ability to produce a single isomer. The fermentation study could be stopped at 48 hours as nutrients would be depleted, alternatively, a fed batch system may be used to ensure a constant supply of glucose.

While there is much to be done in optimising 2, 3-BD production in the *B. licheniformis* ZM1 to ZM5 isolates, there is considerable scope, as outlined above. With the global need for alternative renewable biofuels ever increasing, research studies such as this will be the driving force in achieving this goal.

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