University of Reading

Production of Poly-D-Lactic Acid from Wheat Dried Distiler's Grains with Solubles (DDGS)

A thesis submitted for the Degree of Doctor of Philosophy

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Abstract

Wheat Dried Distiller's Grains with Solubles (DDGS) is a by-product of bioethanol and distillery industries, currently marketed as animal feed. However, DDGS is a carbohydrate- and protein-rich material that could be converted into an alternative feedstock for microbial fermentations. The present study focused on exploiting DDGS as a starting material for the production of the biodegradable polymer, polylatic acid (PLA). DDGS was firstly subjected to alkaline pretreatment to facilitate enzymatic hydrolysis (Accellerase® 1500) of carbohydrate polymers to monosaccharides by removing hemicellulose and increased the surface area inside the DDGS matrix. Improved hydrolysis yields were observed in alkaline treated DDGS compared to untreated DDGS. Secondly, the hydrolysates of DDGS were used as carbon sources for lactic acid fermentation. Here, three *Lactobacillus* sp. were evaluated for their lactic acid production using DDGS hydrolysates in Separate Hydrolysis and Fermentation (SHF). Among the tested strains, *L. coryniformis* subsp. *torquens* showed promising characteristics producing D-lactic acid with 99.9% of optical purity and at high conversion yields. D-Lactic acid production from alkaline treated DDGS was also evaluated using Simultaneous Saccharification and Fermentation (SSF) approach, where high concentrations, yields and productivities of D- lactic acid were achieved. Furthermore, D-lactic was subjected to broth decolorisation with activated carbon, followed by acidification and D-lactic acid separation using ion exchange chromatography. High recovery and purity of D-lactic acid with complete removal of sugars and protein from the

fermentation broth was obtained at the end of the process. The purified D-lactic acid was then concentrated and used as monomer to synthesise poly-D-lactic acid (PDLA). Azeotropic polycondensation process was used to polymerise purified D-lactic acid leading into a clear, solid PDLA with 3010 Da molecular size at the end of polymerisation process.

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Author's Declarations

'Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged'.

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Chapter 1 - General Introduction

Agri-industrial biomass is considered as by-product of low or zero cost that is commonly utilised as animal feed and or for bioenergy purposes. The abundant existence of biomass is often associated with environmental issues related to its inappropriate management, which leads in turn to major problems such as the release of gas pollutants (carbon monoxide, nitrogen dioxide and methane) during biomass combustion and the accumulation of landfill waste. Extensive research has been conducted in the past few years targeting agri-biomass valorisation, aiming to expand its utilisation in other fields such as biomaterials and bioethanol production. Concurrently, the demand for polymers made from renewable sources has increased, representing an alternative way to reduce the dependence on fossil fuel sources. Polylactic acid (PLA) is a bioplastic that has gained attention due to its potential to replace petroleum-based polymers. It has similar properties to synthetically synthesized polymers, combined by the additional advantage of its biodegradable nature (Achmad et al., 2009; Nampoothiri et al., 2010). In 2002, US Food and Drug Administration (FDA) classified PLA as generally recognized as safe (GRAS) compound, enabling its application as food packaging / contact-material.

PLA can be manufactured from the D- and L- forms of lactic acid or from its racemic mixture (DL-). The principal research focus was initially given on L-lactic acid for PLA synthesis, until Ikada et al. (1987) reported that polymer blends of poly-L-lactic acid (PLLA) and poly-D-lactic acid (PDLA) exhibited higher melting points (230 ^{o}C) compared to PLLA (170 ^{o}C) alone. The improved thermostability of PLA has broadened its applications within the food industry, especially as heat-resistant food contact material, i.e. instant noodle

containers, coffee cups and kitchen utensils. Lactic acid can be produced via chemical or biological routes. The biological route involves microbial fermentation and is preferable as it is "greener" and leads to the production of specific forms of lactic acid when appropriate microorganisms are used (Wright & Axelsson, 2012).

Generally, PLA production from agricultural biomass consists of four processing steps: i) hydrolysis of biomass, ii) fermentation using suitable microorganisms, iii) recovery and purification of lactic acid and iv) polymer synthesis. In order for lignocellulosic biomass to be used as feedstock, it needs to undergo hydrolysis into monosaccharides that are directly assimilable by microorganisms during fermentation. However, the structure of lignocellulosic materials is very complex and pretreatment processes are often needed prior to enzymatic hydrolysis. Another important parameter that affects the final quality of PLA is the purity of the monomer. Effective purification strategies need to be developed to ensure high purity of lactic acid, combined with a cost-effective production process.

A potential renewable carbon source for microbial lactic acid production (monomer of PLA) is dried distiller's grains with solubles (DDGS). DDGS is a by-product from bioethanol industry that is currently being used as animal feed. The recent opening of two new bioethanol plants in the United Kingdom, Crop Energies AG (formerly known as Ensus) in 2010 and Vivergo, in 2013, has led into DDGS production surplus, exceeding its demand as animal feed in the UK (Renewable Fuels Association, 2015b).

1.1 Research Challenges

a. The global demand for fossil fuels replacement continues to grow rapidly. Consequently, the production of DDGS (by-product of bioethanol production process) is expected to exceed its demand as animal feed within the UK. In addition, consistency and predictability of feed ingredients is an important parameter for animal feed supply. However, in the case of DDGS, variations in its nutritional composition due to differences in wheat varieties, geographic growth locations and processing conditions may reduce its nutritional quality and market value as feed for ruminant's diets. There is also raised concern on the high levels of mycotoxins in DDGS that may also impact negatively on its value as animal feed. Thus, alternative routes for DDGS valorisation need to be developed.

b. Polylactic acid (PLA) is on the top list of biodegradable plastics as it decomposes to lactic acid, an organic acid naturally occurring in the environment (Sin et al., 2013a). The commercially available PLLA is produce from solely L-lactic acid, which is associated with a relatively low melting point (~ 170 ^{o} C). This limits the utilisation of PLA in other applications such as high heat packaging materials.

c. Successful PLA synthesis requires high purity of its monomer (lactic acid). Thus, several isolation and purification steps are necessary in order to obtain highly pure lactic acid prior to polymer synthesis. A conventional method to recover lactic acid is via its precipitation as calcium lactate. However, this method results in high lactic acid loss and environmental problems due to the formation of gypsum. Other methods such as distillation, esterification and crystallisation are also of interest,

as they can result into a satisfactory purity of lactic acid. However, these processes, are energy-consuming, have relatively small recovery rate and thus, represent a large part of the total cost in PLA production.

1.2 Research Objectives

The main aim of this research was to develop a biotechnological process for the production, purification and polymerisation of D-lactic acid into PDLA. DDGS, a by-product from bioethanol / distiller's industry, was selected as a starting material for this purpose. The specific objectives that were set out in order to accomplish individual research goals were:

1. Determination of the chemical composition of DDGS and evaluation of the necessity of pretreatment prior to enzymatic hydrolysis of DDGS, in order to enhance assimilable sugar release **(Chapter 3)**.
2. Optimisation of microbial D-lactic acid production using DDGS hydrolysates as fermentation feedstock **(Chapter 4)**.
3. Development of a suitable separation and purification process for the recovery of D-lactic acid from the fermentation broth **(Chapter 5)**.
4. Synthesize poly-D-lactic acid (PDLA) by employing previously purified D-lactic acid as building block **(Chapter 6)**.

1.3 Research Hypotheses

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

Pretreated DDGS will be more susceptible to enzymatic hydrolysis, leading into the generation of a fermentation feedstock that is suitable for lactic acid production. D-lactic acid could be effectively recovered from fermentation broth with high yield and purity by multistep purifications techniques, focusing on ion exchange chromatography. The level of D-lactic acid purity will be high enough to allow its polymerisation into PDLA by single step azeotropic polycondensation process.

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Chapter 2 - Literature Review

2. 1 Lactic Acid

Lactic acid ($C_3H_6O_3$) is considered as one of the most useful chemical products and has attracted great attention worldwide due to its widespread applications in food, chemical, cosmetic, textile and pharmaceutical industries. It can exist in two isomeric forms, dextrorotatory (D) and levorotatory (L), and has molecular weight of 90.08 g/mol (Figure 2-1). Lactic acid has a generally recognized as safe (GRAS) status from FDA which can be found under Code of Federal Regulations Title 21 (U.S. Food and Drug Administration, 2015). In the food industry, lactic acid is commonly used as preservative, acidulant, flavouring agent and is also involved in cheese and yogurt production. On the other hand, in the chemical industry, it is used as the basis for the production of lactate ester, propylene glycol, 2-3-pentanedione, propionic acid, acrylic acid, acetaldehyde and dilactide (Abdel-Rahman et al., 2013; Åkerberg & Zacchi, 2000; Wee et al., 2006). John et al. (2007) reported that approximately 85% of the total demand for lactic acid comes from food and food-related applications. Global Market Insights reported that the market demand for lactic acid in 2015 was 952,200 tonnes with total revenue of USD 1.61 billion. At the same time, the market size for biopolymer production accounted for 503,600 tonnes (USD 1.07 billion revenue). The market size of bioplastics is foreseen to increase by 20% per year. Depending on its purity, the market price for lactic acid is within USD 1300 to USD 2300 for chemical or food

purposes and between USD 1300 USD 5000 per tonne as bioplastic ingredient (Cellulac Ltd., 2015; Global Industry Analysts Inc., 2014). The world top three lactic acid manufacturers are PURAC Corbion (Netherlands), Cargill (USA) and Henan Jindan Lactic Acid Technology Co., (China) with an estimated total production of 505,000 tonnes in 2013 via fermentation technology (PR Newswire, 2014).

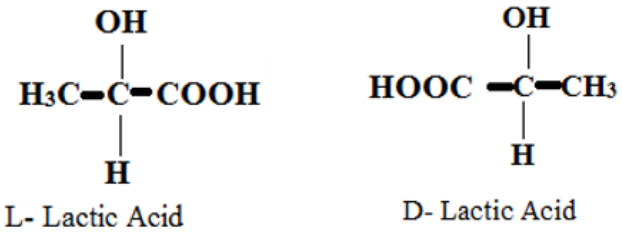

Figure 2-1: Isomeric form of lactic acid

2.1.1 Lactic Acid Production

Lactic acid was first isolated in 1780 by CW Scheele from sour milk and was first commercially produced in 1881 by CE Avery in Littleton, MA, USA (John et al., 2007; Vickroy, 1985). It can be produced by chemical synthesis or microbial fermentation (Figure 2-2). However, the microbial route attracts more attention since it is more environmental-friendly and produces either the optically pure forms of D, L or a racemic DL lactic acid mixture, when specific strains are used. It also offers advantages due to the utilisation of cheap renewable substrates such as distillers' by-products and biomass from agricultural processing (Bai et al., 2015; Nguyen et al., 2013b). In contrast, the limited supply of

petrochemical resources, the use of hazardous solvents and the generation of a racemic mixture of lactic acid isomers (DL) are among the factors that limit the use of chemical processes (Abdel-Rahman et al., 2013; Nguyen et al., 2013a; Wee et al., 2006).

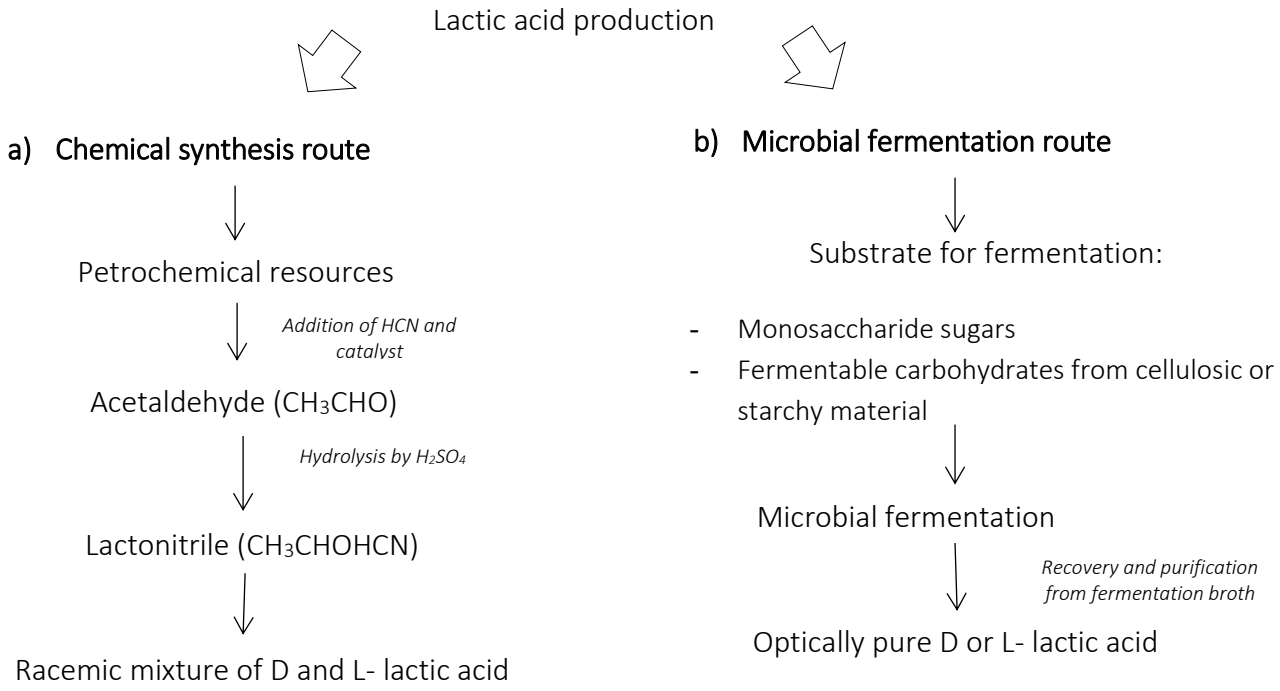

Figure 2-2: Overview of the two manufacturing methods of lactic acid; (a) chemical synthesis and (b) microbial fermentation. Adapted from Wee et al. (2006)

According to Abdel-Rahman et al. (2011) and Ghaffar et al. (2014), almost all lactic acid that is produced worldwide derives from the fermentation route using batch fermentation with neutralizing agents to control the pH. It can be produced from various microbial species, including bacteria such as Lactic Acid Bacteria (LAB), *Bacillus* sp., *Escherichia coli* and *Corynebacterium glutamicum;* fungi such as *Rhizopus* sp.; yeasts such as *Saccharomyces* sp., *Zygosacchromyces* sp., *Candida* sp. and *Pichia* sp.*;* microalgae such as *Nannochlorum* sp. 26A4 (Hirayama & Ueda, 2004) and cyanobacteria (Ducat et al., 2011).

Examples of industrially used bacteria for lactic acid production are *Lactobacillus acidophilus* and *Streptococcus thermophilus* (Nattrass & Higson, 2010). Among all lactic acid producers, the genus *Lactobacillus* is most commonly used (Ghaffar et al., 2014).. The produced lactic acid is then collected and purified using techniques such as membrane filtration, crystallization or evaporation. Normally, commercial lactic acid is sold at purities ranging from 80% to 93% (Jem et al., 2010). The selection of a suitable microbial strain is key factor for producing high amounts of lactic acid (Abdel-Rahman et al., 2013), and as such, important criteria for strain selection include high productivity, high conversion yields and strain robustness to minimise the likelihood of culture contamination (Groot et al., 2011). Most LAB, and especially *Lactobacillus* sp., are considered to be safe for industrial lactic acid production without any report on adverse health effects on consumers. They are also identified as high acid tolerant, exhibit high productivities and are amenable to genetic engineering modifications towards the production either D or L lactic acid (Abdel-Rahman et al., 2013; Groot et al., 2011; Kylä-Nikkilä et al., 2000).

2.1.2 Biochemical Synthesis of Lactic Acid by LAB

Among LAB species, *Streptococcus* sp., *Pediococcus* sp. and some species of *Lactobacillus* sp. convert ≥ 85% glucose to lactic acid, while, *Leuconostoc* sp. and other *Lactobacillus* sp. species only convert 50% of glucose to lactic acid (Garvie, 1980). This conversion of glucose to end-product(s) has led into the grouping of LAB to either homofermentative or heterofermentative bacteria. Homofermentative LAB employ the

Embden-Meyerhof-Parnas pathway (EMP) and utilise glucose to produce two molecules of pyruvic acid which are then reduced to two molecules of lactic acid (Garvie, 1980; Reddy et al., 2008). On the other hand, heterofermentatitve LAB commonly use the Phosphoketolase pathway (PK), where other products such as ethanol, formate, diacetyl, acetic acid or carbon dioxide are produced together with one molecule of lactic acid (Abdel-Rahman et al., 2011; Wang et al., 2011). In addition to this, the assimilation of hexose and pentose sugars during fermentation is also carried out by employing different pathways. Hexose sugars, such as glucose and fructose, are normally catabolised via EMP, while pentose sugars such as xylose and arabinose are assimilated via the PK pathway to produce lactic acid as shown in Figure 2-3 (Garde et al., 2002; Reddy et al., 2008).

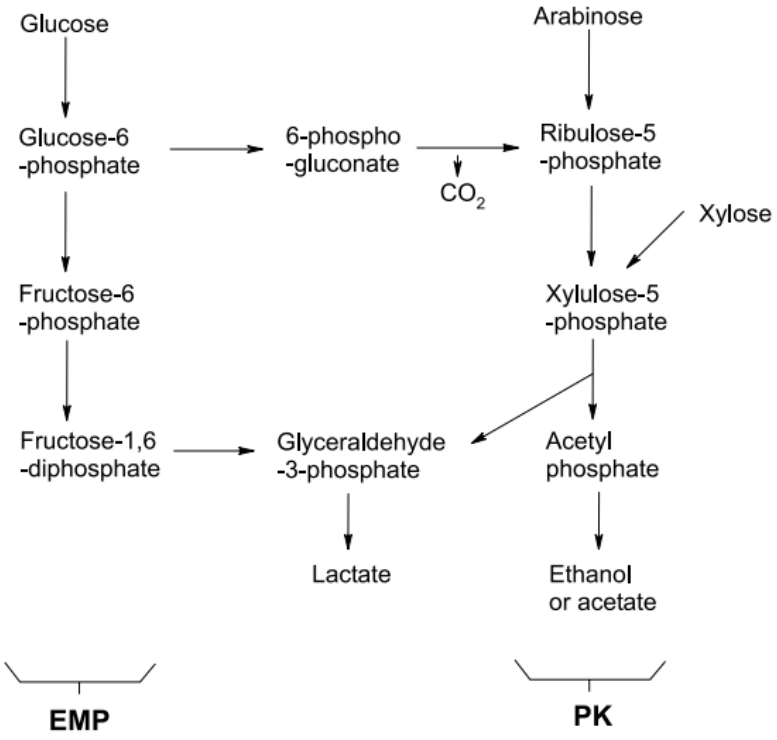

Figure 2-3: Homo- and hetero- fermentation of lactic acid through Embden-Mayerhof pathway (EMP) and Phosphoketolase pathway (PK). Adapted from Garde et al. (2002)

For both pathways, two molecules of nicotinamide adenine dinucleotide (NAD^+) are regenerated from nicotinamide adenine dinucleotides (NADH) that are formed during glucose assimilation in the earlier stages of the glycolysis pathway. NAD^+ functions as a coenzyme to lactate dehydrogenase (nLDH), the enzyme that is responsible for converting pyruvate to L- or D- lactic acid, as well as for the reverse reaction (Garvie, 1980; Jun et al., 2013). NAD-dependent L-lactate dehydrogenase (L-nLDH; EC 1.1.1.27) or NAD-dependent D-lactate dehydrogenase (D-nLDH; EC 1.1.1.28) are encoded by *ldhL* or *ldhD*, respectively (Garvie, 1980; Gu et al., 2014; Simon et al., 1989; Taguchi & Ohta, 1991; Zheng et al., 2012). These two enzymes are the key factors for the production of either L or D lactic acid (Mirasol, 1999). LAB can produce L or D lactic acid depending on type of nLDH present. In case where both D and L lactic acid are produced from the strain, this is likely due to the presence of both *ldhD* and *ldhL* genes (Salminen et al., 2004; Wright & Axelsson, 2012).

2.1.3 Lactic Acid Producing Microorganisms

The levels of D, L or racemic DL lactic acid that are produced depend on the type of substrates and microorganism employed during fermentation. LAB ferment sugars using different pathways to produce homo-, hetero or mixed acid fermentation. Table 2-1 summarizes the common genera of LAB and type of lactic acid produced.

Table 2-1: LAB genera and type of lactic acid produced

Family	***Genera***	**Type of Lactic Acid Produced**
Aerococcaceae	*Aerococcus*	L
Carnobacteriaceae	*Carnobacterium*	L
Enterococcaceae	*Enterococcus*	L
Lactobacillaceae	*Lactobacillus*	D, L, DL
	Pediococcus	L, DL
	Sporolactobacillus	L, D
Streptococcaceae	*Lactococcus*	L, DL
	Streptococcus	L
Leuconostocaecae	*Leuconostoc*	D
	Oenococcus	D
	Weissella	D, DL

Source: Kitahara and Suzuki (1963); Wright and Axelsson (2012)

In order to produce optically pure D or L lactic acid, homofermentative producers offer several advantages in terms of purity, activity and productivity compared to heterofermentative producers. This is due to the fact that the production of lactic acid from homo-producers is more than 85% and they do not produce substantial amounts of by-products. Therefore, the cost to purify lactic acid is considered lower due to the low interference by other compounds which are regarded as impurities. Even though the presence of D-lactic acid is known for centuries, the interest on investigating this compound only increased recently, compared to L-lactic acid, which has been studied for many years. Table 2-2 lists the key D-lactic acid producing microorganisms of the Lactobacillaceae family that have been studied so far.

Table 2-2: Homo D-lactic acid producing strain from the Lactobacillaceae family

Microorganisms	Strain No.	Optical Purity of D-Lactic Acid Produced (%)	References
Lactobacillus coryniformis subsp. *torquens*	ATCC 25600 / DSMZ 20004	99.36	Nguyen et al. (2013a)
		95.8-99.6	Nguyen et al. (2012)
		NA	Yañez et al. (2003)
		99.5	Nguyen et al. (2013b)
Lactobacillus delbrueckii subsp. *delbrueckii*	ATCC 9649/ DSMZ 20074/ IFO 3202	99.8	Zhang and Vadlani (2013)
		95	Tanaka et al. (2006)
		> 97.5	Fukushima et al. (2004)
Lactobacillus delbrueckii subsp. *lactis*	ATCC 4797/ DSMZ 20076/ JCM 1148 / JCM 1106	> 98	Prasad et al. (2014)
		97.2-98.3	Calabia and Tokiwa (2007)
		95-96	Nakano et al. (2012)
Sporolactobacilllus inulinus	ATCC 15538/ DSMZ 20348/ CGMCC No. 2185/ JCM 6014	NA	Zhao et al. (2010)
		98.9	Fukushima et al. (2004)
		96.4	Sawai et al. (2011)
Sporolactobacillus laevolacticus	ATCC 23492/ DSM 442/ JCM 2513	99.3	Li et al. (2013)
		98.2	Sawai et al. (2011)

NA- Not –Available

2.1.4 Critical Fermentation Parameters for D-lactic Acid Production

Several factors are known to influence the fermentation of lactic acid including pH, temperature and substrate concentration. The growth of LAB is generally negatively affected by low pH due to the accumulation of lactic acid during fermentation. Researchers have reported that the optimum pH value for D- lactic acid production is found to be between 6 and 7. Since lactic acid is the primary metabolite of LAB, its productivity is linked with the bacterial growth. In non-pH control fermentations, lactic acid production usually stops at pH values below 4.5 as a result of growth inhibition. Lactic acid exists in its protonated form, which can cross the cell membrane and cause the accumulation of lactic acid within the cell, leading to cell membrane disruption (Komesu et al., 2017). Hofvendahl and Hahn–Hägerdal (2000) reported that higher lactic acid production was achieved when the pH of fermentation was controlled. Neutralizing agents are used to control the pH of the culture throughout the fermentation process. Commonly used neutralizing agents are sodium hydroxide (NaOH), ammonium hydroxide (NH_4OH), potassium hydroxide (KOH) and calcium carbonate ($CaCO_3$).

Another critical factor that influences the production of D-lactic acid during fermentation is temperature. Most of D-lactic acid producing microorganisms have an optimum temperature between 34 and 40 ^oC. However, *Lactobacillus delbrueckii* HG 106 and *Lactobacillus delbrueckii* subsp. *lactis* QU 41 that have been used in research studies, have been reported to exhibit thermophilic properties by being able to grow and produce lactic acid at 45 ^oC and 43 ^oC, respectively (Lu et al., 2009). Therefore, the optimum temperature condition for lactic acid production is largely dependent on the strain selection.

Microbial growth and product formation are also influenced by substrate concentration. At very high substrate concentrations, bacterial growth is inhibited due to increased osmotic pressure that is exhibited by the fermentation medium (Nancib et al., 2015). Besides, prolonged lag phase and low specific growth rate may also be observed in these conditions. One of the approaches to minimise the effect of substrate inhibition is by using fed-batch fermentation (Lee et al., 2007). However, in the case of complex media (e.g. biomass hydrolysates), this approach might not be suitable, since it is likely that the concentration of non-assimilable compounds (e.g phenolic acids, lignin derivatives in the fermentation medium will increase over time, leading to potential growth inhibition phenomena.

2.1.5 Alternative Feedstock for Microbial Fermentations

Nowadays, commercial lactic acid is produced from sugar cane, sugar beet, corn and cassava. In September 2015, Purac Corbion reported the successful production of high grade lactic acid from alternative or second generation feedstocks from biomass (Lovett & Harten, 2015). One example of potential feedstock sources that has gained recently attention is that of lignocellulosic biomass including agricultural residues, wood chips, bagasse, or by-products of the bioethanol industry. The use of such materials is of interest as they do not interfere with the use of land for the production of food (Villegas-Torres et al., 2015).

Wakil and Ajayi (2013) suggested that renewable materials such as food processing and agricultural wastes that are rich in starch, cellulose and hemicellulose could be excellent

resources for lactic acid production. Starchy material can be hydrolyzed to glucose or maltose, while, cellulosic materials can be hydrolyzed to glucose and cellobiose. On the other hand, hemicellulosic materials (e.g. xylans, glucuronoxylan, arabinoxylan, glucomannan, galactomannan) and pectins can be hydrolysed into galactose, mannose, xylose and arabinose after a suitable thermal/chemical or enzymatic treatment (Hofvendahl & Hahn–Hägerdal, 2000). Previous works have reported a number of starchy raw materials for lactic acid production using *Lactobacillus* sp., such as sweet sorghum, sweet potato, wheat, cassava, rice and barley resulting in lactic acid concentrations higher than100 g/L (Eric et al., 1994; Nguyen et al., 2013a; Wee et al., 2006). In addition to this, agri-industrial by-products that have readily fermentable sugars such as cheese whey (Coelho et al., 2011), molasses, sugar cane and sugar beet juices are also of interest (Calabia & Tokiwa, 2007). Cellulosic and hemicellulosic materials such as cereal bran, spent grains, wheat straw and corn stover have been also evaluated as feedstock for lactic acid production (Wakil & Ajayi, 2013; Wee et al., 2005). Table 2-3 lists some of the raw materials that have been employed as the basal fermentation medium, providing fermentable sugars for D-lactic acid production.

However, biomass resources are naturally very complex in their composition and structure. Therefore, enzymatic, chemical, physical or thermal treatments are necessary prior to fermentation aiming to hydrolyse the complex polysaccharides into fermentable sugars. For example, alkaline pretreatment combined with cellulase actions had been used to prepare pulp and corn stover hydrolysate by Zhang and Vadlani (2013). The hydrolysate was then used on *Lactobacillus delbrueckii* ATCC 9649 as carbon sources, and other required nutrients such as nitrogen (yeast extract), vitamins and salts were supplemented during fermentation, which resulted in the production of 36.3 g/L of D- lactic acid.

It should be noted that the hydrolysis of biomass resources that are rich in cellulosic/ hemicellulosic compounds will produce a mixture of sugars such as glucose, xylose, mannose, arabinose, galactose, etc. Studies have shown that most of homo D-lactic acid producers, i.e. *Lactobacillus* sp. and *Sporolactobacillus* sp., are unable to ferment sugars other than glucose (Bai et al., 2015; Zhang & Vadlani, 2013). Therefore, research on genetically modified microorganisms has been conducted to produce strains that can: i) utilise a variety of carbon sources, especially pentose sugars and; ii) directly ferment carbon sources from starchy and lignocellulosic based materials. For example, Zheng et al. (2013) and Okano et al. (2009a) developed genetically modified *Escherichia coli* SZ470 and *Lactobacillus palantarum* NCIMB 8826 strains to produce D-lactic acid from xylose. However, acetic acid was produced together with lactic acid during fermentation. Moreover, Okano et al. (2009b) developed a genetically engineered *Lactobacillus plantarum* NCIMB 8826 strain to produce D-lactic acid directly from corn starch by deleting the *ldhL* gene and introducing an α-amylase sequence from *Streptococcus bovis* 148. The results showed that 73.2 g/L of D-lactic acid was produced with 99.6% optical purity.

Table 2-3: Potential feedstock and hydrolysing enzymes used for D-lactic acid production

Category	Feed Stocks/ Raw Material	Microorganisms	Pretreatment	Hydrolysis and Fermentation Protocol	D-lactic Acid Concentration (g/L/h)	References
Starch materials	Rice starch	*Lactobacillus delbrueckii LD0025 and LD 0028*	*α*-amylase, *β*-amylase, and pullulanase	SHF	0.89	Fukushima et al. (2004)
	Unpolished rice	*Lactobacillus delbrueckii* HG 106	*α*-amylase and amyloglucosidase	SHF	1.5	Lu et al. (2009)
	Broken rice	*Lactobacillus delbrueckii JCM 1106*	*α*-amylase, *β*-glucoamylase, and protease	SSF	3.59	Nakano et al. (2012)
	Fresh sweet potato	*Lactobacillus coryniformis ATCC 25600*	*α* –amylase, amyloglucosidase	SSF	2.55 – 3.11	Nguyen et al. (2013a)
Readily available fermentable sugars	Molasses	*Lactobacillus delbrueckii* JCM 1148	-	-	2.1	Calabia and Tokiwa (2007)
		Lactobacillus lactis NCIM 2368 mutants	using H_2SO_4 for hydrolysis	-	2.17	Joshi et al. (2010)
	Sugar cane juice	*Lactobacillus delbrueckii* JCM 1148	-	-	2.4	Calabia and Tokiwa (2007)
	Casein whey permeate	*Lactobacillus delbrueckii* subsp. *lactis*	-	-	0.61	Prasad et al. (2014)

(Ligno) cellulosic/ hemicellulosic materials	Biomass of the microalga, *Hydrodictyonreticulatum*	*Lactobacillus coryniformis* subsp. *torqu ens ATCC 25600*	Cellulase, cellobiase, α-amylase, amyloglucosidase	SSF	1.02	Nguyen et al. (2012)
	Defatted rice bran	*Lactobacillus delbrueckii* subsp. *delbrueckii* IFO 3202	amylase and cellulase	SSF	*7.8	Tanaka et al. (2006)
	Biomass of turmeric	*Lactobacillus coryniformis ATCC 25600*	*Cellulase, cellobiase, α –* amylase, amyloglucosidase	SSF	2.08	Nguyen et al. (2013b)
	Waste cardboard	*Lactobacillus coryniformis* subsp. *torqu ens ATCC 25600*	*Alkaline treatment (NaOH), cellulase, β-* glucosidase	SSF	*0.13	Yáñez et al. (2005)
	Corn stover	*Sporolactobacillus inulinus YBS1-5*	*Dilute H_2SO_4, cellulase, β-glucosidase*	SHF	1.96	Bai et al. (2015)
	Corn stover	*Lactobacillus delbrueckii subsp. delbrueckii ATCC 9649*	*Cellulase CTec2*	SSF	0.32	Zhang and Vadlani (2013)

*D-lactic acid concentration = g/kg/h

SSF - Simultaneous Saccharification and Fermentation SHF – Separate Hydrolysis and Fermentation

2. 2 Purification and Recovery of Microbial Lactic Acid

Since lactic acid is used in a wide range of applications in the food, pharmaceutical and chemical area, the production of high purity lactic acid is a key-parameter. An efficient separation and recovery strategy is needed to ensure the quality of purified lactic acid at the end of the fermentation process. Currently, more than 90% of industrially available lactic acid is produced via fermentation route using specific microorganisms (Dusselier et al., 2013; He et al., 2016) . According to Li et al. (2016) the bottle neck for lactic acid production via fermentation route is associated with the high cost of downstream processing. The lactic acid obtained from fermentation process normally contains impurities such as non-utilised sugars, protein, vitamins and phenolic compounds. Other compounds such as alcohols, glycerol and acetic acid that are produced as by-products can also be found in the fermentation medium (Ghaffar et al., 2014). If the feedstock for fermentation process derives from lignocellulose materials, other impurities such as furan, hydroxylmethyl furfural (HMF) and other organic acids might also be present at the end of the fermentation stage (Alvira et al., 2010). In addition, during lactic acid fermentation, the pH of fermentation medium is usually controlled between pH 5 to 7 using alkaline reagents. As a result, lactic acid is as salt (Kim & Moon, 2001). Thus, it is compulsory to convert lactate salt to lactic acid during subsequent purification stages.

Although the production of lactic acid by fermentation is advantageous since renewable materials (lignocellulosic/ hemicellulosic biomass) can be utilised as substrates, the derived hydrolysates usually exhibit a dark colour due to Maillard reactions and

degradation of lignin and hemicelluloses (Jönsson & Martín, 2016; McIntosh & Vancov, 2011). This colour should be also removed in the early stages of purification process. The commonly used compound to adsorb dark-coloured pigments is activated carbon (Ahmedna et al., 2000; Min et al., 2011). Activated carbon has been previously used successfully to decolourise (99% colour removal) fermentation broth containing lactic acid and citric acid with no loss of final product (Qin et al., 2017).

Downstream processing is often initiated with separation protocols to separate cell debris or any solid material that may be present in the fermentation broth followed by broth decolourisation. Several techniques such as precipitation, membrane separation, solvent extraction, distillation and adsorption have been proposed for lactic acid purification in subsequent stages (González et al., 2006; Li et al., 2016). Conventionally, lactic acid is purified from the fermentation broth via precipitation using either calcium carbonate or calcium hydroxide, followed by filtration and addition of sulfuric acid to precipitate calcium sulfate (gypsum) and lactic acid is recovered in the filtrate. However, this technique generates large amount of gypsum as by-product, which is an environmental hazard. Besides, the purity of lactic acid produced is only between 22 to 44% (Komesu et al., 2017; Wasewar, 2005) and for subsequent applications, such as PLA synthesis, higher purity of lactic acid is required (> 90%).

2.2.1 Ion exchange chromatography for lactic acid purification

The purification of lactic acid through adsorption chromatography has been studied extensively. Other than effective in fractionation of ionic compounds, ion exchange can also transform lactate salt to lactic acid. Besides, the adsorption is extremely selective, and the desired product can be recovered in a shorter time compared to other purification strategies such as electrodialysis. This process is simple, easier, cheaper and no expensive equipment is needed (Ghaffar et al., 2014; Quintero et al., 2012). Ion exchange adsorption can be divided into two groups: anion exchange and cation exchange. Based on their charge, the adsorbent can be further categorised into weak acid or strong acid cation exchanger, and weak base or strong base anion exchanger that differ in structure, polymer matrix and functional groups (Evangelista & Nikolov, 1996). The mechanism of separation is by formation of ionic bonds between the charged groups of biomolecules with resins that carry the opposite charge. Cation molecules are separated by cation exchange resins and anion molecules are separated with anion exchange resins as shown in Figure 2-4 (Acikara, 2013).

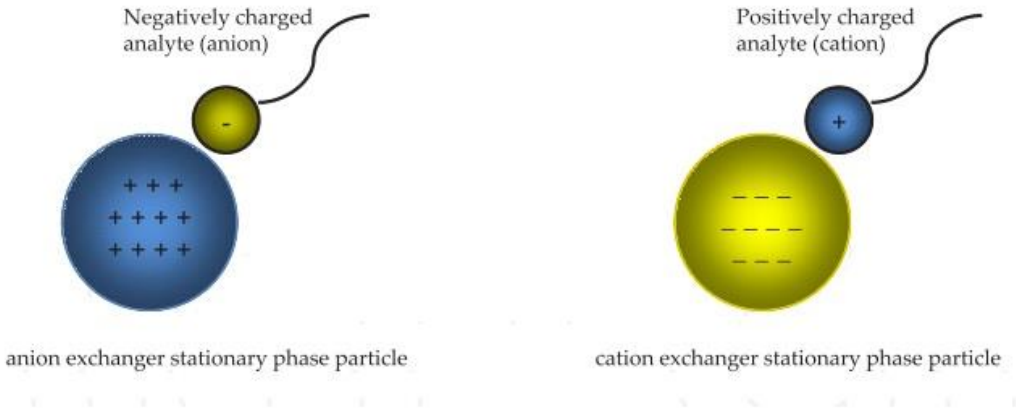

Figure 2-4: Mechanism of ion exchange adsorption. Adapted from Acikara (2013)

In the case of lactic acid, anion exchange resins are used as adsorbent because lactate ion has an anionic charge ($C_3H_5O_3^-$) that is bound to cationic molecule. Commercial weak base and strong base anion exchange resin such as Amberlite® IRA67, Amberlite® IRA96, Amberlite® IRA92, Amberlite® IRA400, Lewatit S3428, DOWEX-XUS 40196 and DOWEX-50 have been previously used to separate lactic acid from defined fermentation media (Evangelista & Nikolov, 1996; Quintero et al., 2012; Tong et al., 2004). The adsorption of lactic acid to resins are influenced by several factors such as pH, contact time and temperature (Gao et al., 2010). The effect of pH on ion exchange binding is related to the pKa value of lactic acid (3.86). Cao et al. (2002) and John et al. (2008) reported that the highest lactic acid binding in anion exchange resin occured when the pH of feed solution was set to 5, which is above its pKa value. While, González et al. (2006) and Evangelista and Nikolov (1996) claimed that when the pH of feed solution was set below lactic acid pKa value, highest lactic acid adsorption was noted. As lactic acid fermentation is conducted at pH 5 – 7, the fermentation broth was first acidified using cation exchange resin, in the latter case. The effect of temperature on lactic acid adsorption has rarely been investigated. However, some researches had reported that increased in temperature, adversely affect the binding of lactic acid in ion exchange column. For example, Gao et al. (2010) found ~18% reduction in lactic acid adsorption when reaction temperature increased from 25 to 50^{o}C, whereas Pradhan et al. (2017) reported 50% reduction in lactic acid binding when temperature was increased from 30 to 80^{o}C.

2. 3 Polylactic Acid (PLA)

Polylactic acid (PLA) is a polymer that has gained attention due to its potential to replace petroleum-based polymers. It has similar qualities to the synthetically derived polymers in addition to its biodegradable nature. Besides that, PLA can be relatively easy modified by controlling the molecular structure of its building block (lactic acid). Because of its degradability and biocompatibility with human body, PLA is extensively used in the biomedical field as suture, drug-delivery material and bone fixation material (Conn et al., 1995; Nampoothiri et al., 2010). In 1999, the European Authorities for Medical Devices approved the use of PLA as a treatment for 'aesthetical correction' of scar tissue and wrinkles (Collins, 2001). In addition, PLA was classified as a generally recognized as safe (GRAS) compound by US Food and Drug Administration (FDA) in 2002, and can be used as food contact-material, e.g. for the production of cutlery, cups, plates and containers, or as food packaging material (Conn et al., 1995; Jamshidian et al., 2010). Apart from the above, PLA is also used in other areas such as textiles and environmental remediation films.

Currently, the worldwide market demand for PLA accounts for 11.4% of the total bioplastic production and is equal to approximately 180,000 metric tonnes per year. The PLA demand is estimated to grow by 28% until 2025 (Aeschelmann & Carus, 2015; Cellulac Ltd., 2015). In 2020, the world PLA market is projected to exceed 5.16 billion USD with 800,000 tonnes of production (Aeschelmann & Carus, 2015; Marketsandmarkets, 2015). The dominant market demand for PLA comes from Europe which accounts for 25,000 tonnes per annum (Nattrass & Higson, 2010). According to a report from National Non-Food Crop

Centre, the demand for PLA in the United Kingdom is projected to grow to 650,000 tonnes per annum by 2025 (Peter Reineck Associates Limited, 2008). Currently, the main producer of PLA are NatureWorks® LLC under trade name Ingeo™ (United States), Cereplast, Inc. (United States), Corbion Purac (Netherlands), Toray Industries (Japan) and Zhejiang Hisun Biomaterial Co., Ltd (China) (Jamshidian et al., 2010; Sin et al., 2013a).

PLA can be manufactured from the D-, L- forms of lactic acid or from its racemic mixture (Yañez et al., 2003). However, poly-L-lactic acid (PLLA) produces a polymer with low melting point (180 ^{o}C) and low crystallisation ability (Xu et al., 2006). In contrast, polymer blends of purified PLLA and purified poly-D-lactic acid (PDLA) produce racemic crystals called stereo-complexes which have high melting point (230 ^{o}C) and distortion temperatures, and offer significant advantages for a number of applications such coffee and instant noodle cups or other high heat packaging materials (Ikada et al., 1987; Ishida et al., 2006; Kim et al., 2014; Nguyen et al., 2013a). Ikada et al. (1987) suggested that the stereo-complex crystalline structure of PLA is formed by the helical chain of PLLA (left-handed chain) and PDLA (right-handed chain) which are linked through van der Waals forces, such as dipole-dipole interactions (Figure 2-5). Strong van de Waals interactions between PLLA and PDLA increase the thermal resistance of PLA (Cheerarot & Baimark, 2015). These improved properties of PDLA and PLLA have increased the interest of polymer industry towards the production of optically pure D- or L- lactic acid.

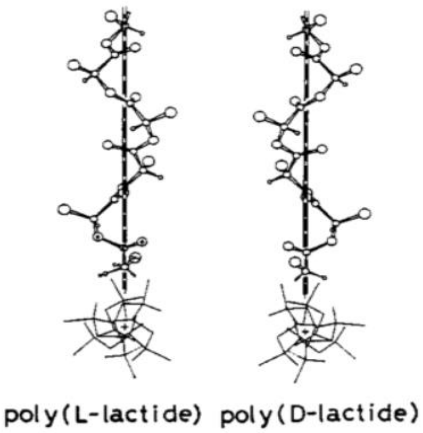

Figure 2-5: Chain model of chain PLLA and PDLA proposed by Ikada et al. (1987)

2.3.1 Synthesis of PLA from lactic acid

There are several ways of synthesizing PLA from lactic acid including ring opening polymerization (ROP) of lactide, direct polycondensation and enzymatic polymerization as shown in Figure 2-6 (Lopes et al., 2012; Orozco et al., 2007). Among this, ROP and direct polycondensation are widely known, with the former one being applied commercially as it produces high molecular weight PLA (Marques et al., 2010). Three steps are involved in ROP polymerisation: (i) conversion of lactic acid to low molecular weight oligomers, (ii) "back-biting" reaction of oligomer to form lactide and (iii) polymerization of lactide to PLA. However, this process is quite complicated, whereas high purity of lactide is required to produce PLA of a good quality. Thus, lactide must undergo a purification step prior to polymerization process. This results in relatively high costs, and it is also time and labour consuming (Hu et al., 2016; Kim & Kim, 1999; Xiao et al., 2012).

On the other hand, direct polycondensation is considered a cheaper, simple and easier process as it involves one polymerization step, but results in the production of low molecular weight polymers (< 5 000 Dalton) (Sin et al., 2013b). In polycondensation process, the lactic acid solution is heated at 130 – 140 ^{o}C. Here, carbonyl and hydroxyl groups of lactic acids are linked and produce water as by-product. However, as the polymerization progresses further, the solution becomes viscous and the removal of water becomes challenging. Even when the reaction temperature is elevated (> 200 ^{o}C) to promote the removal of water, high molecular weight polymers are still not produced because of the decomposition reaction of polymer at high temperature (Achmad et al., 2009). To overcome this disadvantage, supporting methods such as solid-state polymerization and chain extension have been developed. However, additional steps such as melt polycondensation and chain coupling reactions need to take place in order to produce high molecular weight PLA, and these contribute towards the increment of the production costs (Lasprilla et al., 2012). In 1994, Mitsui Toatsu Chemical (Japan) successfully produced high molecular weight PLA (> 300 000 Da) via azeotropic dehydration polycondensation (Enomoto et al., 1994). Here, lactic acid and catalyst were continuously refluxed in organic / aprotic solvent under reduced pressure to produce high molecular weight polymer (Garlotta, 2001; Hu et al., 2016; Lopes et al., 2012). Under azeotropic distillation, the removal of water from the reaction medium becomes easier and drying agents such as molecular sieves are added to help the polymerisation process. Another technique that can be used for direct PLA synthesis is via enzymatic pathways (using lipase), which provide an alternative to chemical routes. During enzymatic polymerization, the reaction takes place under milder conditions (temperature < 60 ^{o}C). However, water needs to be continuously removed during reaction to ensure the

success of polymer synthesis. Besides that, enzymatic polymerization of PLA usually results in low molecular weight PLA (< 5 000 Da) (Lassalle & Ferreira, 2008; Liu, 2015).

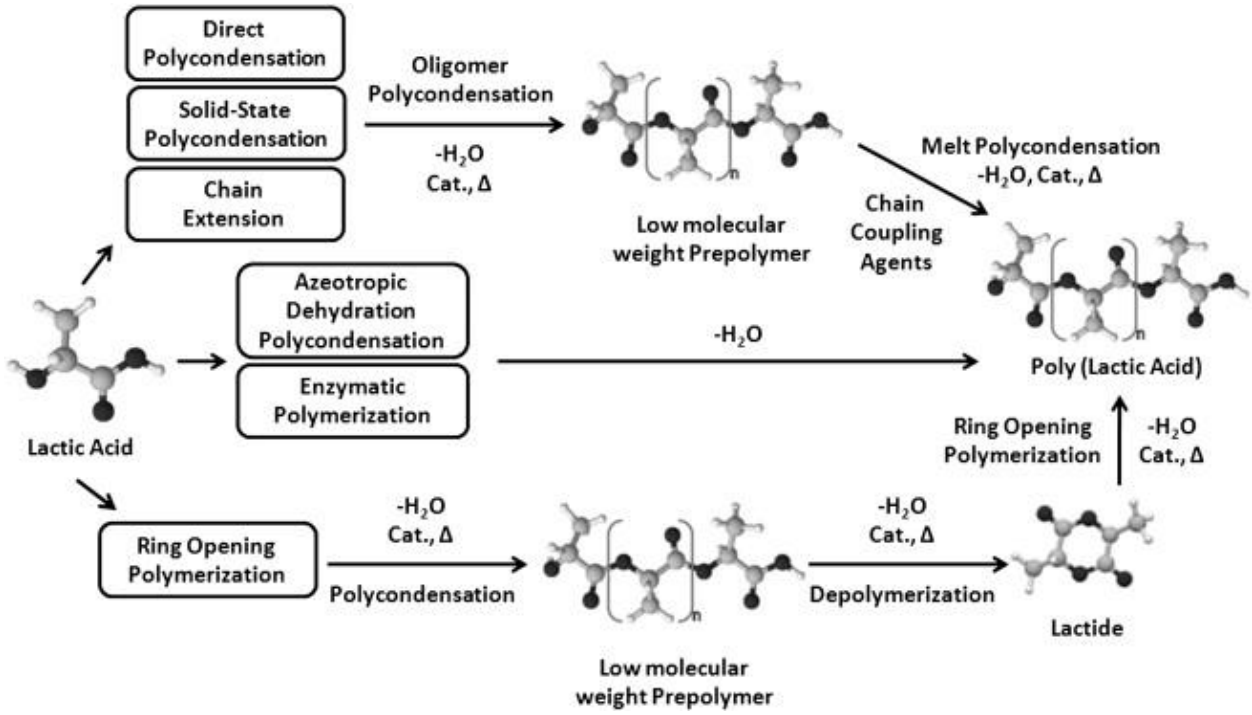

Figure 2-6: Summary of PLA synthesis from lactic acid. Adapted from Lasprilla et al. (2012)

2. 4 Hydrolysis of Lignocellulosic Materials from Agricultural Biomass

Agricultural biomass can be used as potential source of carbon and nitrogen in microbial fermentations. However, plant-based biomass is known for its rigid and compact structure that naturally acts as a barrier against microbial or enzymatic attacks (Zhao et al., 2012). The main contributors to the complex structure of plant biomass are polymers such

as lignin, cellulose and hemicellulose, as well as the crystallinity of cellulose itself. Lignin is a complex, cross-linked polymer of aromatic rings that renders the structure of lignocellulosic material highly robust and resistant against physical and chemical treatments or pathogen attack (Sticklen, 2010). It is linked to cellulose and hemicellulose through covalent and hydrogen bonds (Limayem & Ricke, 2012; Zhu et al., 2008). Cellulose is a linear chain polymer that links several hundreds to over ten thousand units of D-glucose via β-1,4 glycosidic bonds in 180^{o} orientation. It exists in the form of microfibrils with 30 nm diameter. Each microfibril consists of 36 hydrogen-bonded glucan chains (each glucan contains 10000 to 15000 linked glucose units) that are lined up parallel to each other (Mohnen et al., 2009; Sticklen, 2010; Zhao et al., 2012). These microfibrils can exist in amorphous (disorder) and crystalline (ordered) form. In crystalline region, cellulose chains are closely packed together by strong intra- and inter- molecular hydrogen bond linkages. This arrangement results in the strong crystal polymorphic structure of cellulose (Yildiz & Gümüşkaya, 2007; Zhou & Wu, 2012). Most cellulosic materials have more crystalline than amorphous structure. In contrast, hemicelluloses have an amorphous and variable branched structure compared to cellulose. Hemicelluloses are heteropolymers that are built of two or more different monomers such as pentoses (D-xylose, L-arabinose), hexoses (D-glucose, D-galactose and D-mannose) and/ or uronic acids (D-glucuronic, D-4-*O*-methylgalacturonic and D-galacturonic acid). The backbone structure of hemicellulose consists of xylose (approximately 90%) and arabinose (nearly 10%) that are linked via β-1,4 linkages (Gírio et al., 2010; Limayem & Ricke, 2012). Compared to cellulose, hemicellulose have a weak matrix structure and can easily break to release monomers at temperatures below 160 ^{o}C and less than 5% of acid concentration (Xu & Hanna, 2010).

Despite the complex structure of agricultural biomass, the need to investigate novel approaches to effectively convert lignocellulosic materials to valuable products is highly desired. The production of added value compounds such as fermentable sugars from such complex matrices requires physical, chemical or biological treatments or a combination of them. Examples of physical fractionation include milling, high temperature and high pressure processing treatment. On the other hand, chemical hydrolysis is usually conducted by concentrated acid such as sulphuric acid (H_2SO_4) or hydrochloric acid (HCl) (Binod et al., 2011). Biological fractionation or enzymatic hydrolysis is achieved by selection of suitable hydrolytic enzymes under specific conditions to produce simple sugars.

Among various treatments, chemical and enzymatic techniques have been shown to give optimal results. However, chemical hydrolysis is associated with several disadvantages. The concentrated acids that are used in chemical hydrolysis are toxic, corrosive and hazardous. Moreover, they require reactors that are resistant to corrosion. Also, further treatments to neutralise the produced sugars (end products) are needed (Binder & Raines, 2010; Binod et al., 2011). In the case of dilute acid hydrolysis which normally requires high temperatures (170 – 230 ^{o}C), toxic compounds such as furfural and 5-hydroxymethyl-furfural are produced together with the end hydrolysis products (Verardi et al., 2012). In contrast, enzymatic hydrolysis requires mild conditions (pH 4.8 to 6.5, depending on the enzymes and temperatures normally between 35– 50 ^{o}C) and does not lead to corrosion issues, therefore, no expensive corrosion-proof equipment is needed (Binod et al., 2011; Verardi et al., 2012). In addition, enzymatic reactions are more specific and can lead to the production of pure carbohydrate monomers such as glucose as well as minimize the release of undesired compounds. Due to these advantages, enzymatic treatment has been extensively

investigated for the fractionation of several lignocellulosic and hemicellulosic substrates such as in barley hull (Kim et al., 2008), turmeric waste (Nguyen et al., 2013b), rice straw (Vlasenko et al., 1997), rice bran (Tanaka et al., 2006), corn stover (Kim & Holtzapple, 2005), brewer's spent grain (Mussatto et al., 2008) and corn-based dried distillers' grains with solubles (DDGS) (Dien et al., 2008).

2.4.1 Enzymatic hydrolysis of hemicellulosic biomass

2.4.1.1 Cellulases

A complete bioconversion of cellulosic material to fermentable sugars usually requires the synergistic action of three classes of enzymes, namely endo-glucanases, exo-glucanases and β-glucosidases. Endo-glucanases (EC 3.2.1.4) attack the internal β-1,4-glycosidic linkages of cellulose fibre randomly, creating relatively short chain polysaccharides (oligosaccharides) with new free chain-ends. Exo-glucanases (EC 3.2.1.91), also known as cellobiohydrolases, cleave off cellobiose or glucose from cellulose or previously released short chain polysaccharides from non-reducing ends. The conversion of cellobiose to two units of glucose and the release of glucose from the cello-oligosaccharide ends are completed by the action of β-glucosidase (EC 3.2.1.21), also known as β-glucoside glucohydrolases or cellobiase. The action of cellulase can be divided into primary and a secondary hydrolysis as shown in Figure 2-7. Primary hydrolysis involves the action of endo-glucanases and exo-glucanases on the surface of the solid material to release soluble sugars

into the liquid phase. In the secondary hydrolysis, the reaction occurs in liquid media where the main reaction is the conversion of cellobiose to glucose by β-glucosidase (Binod et al., 2011; Philippidis & Smith, 1995; Verardi et al., 2012; Zhang et al., 2006).

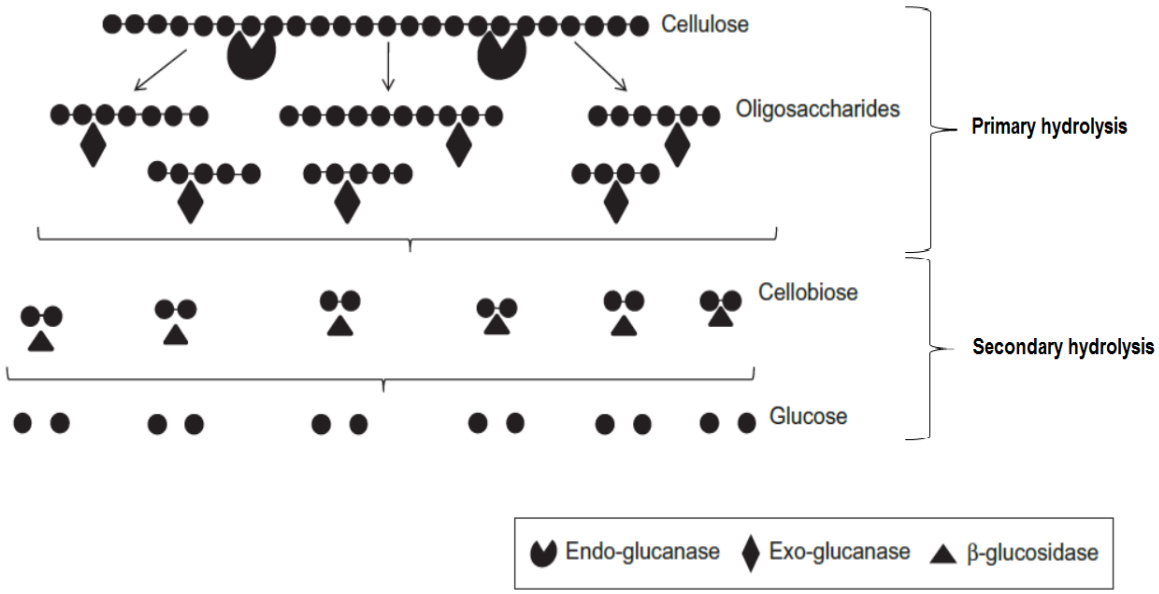

Figure 2-7: Cellulase (endo-glucanase, exo-glucanase and β-glucosidase) mechanism of action. Adapted from Binod et al. (2011)

2.4.1.2 Hemicellulases

The other compound that represents almost 30 - 40% of the total plant biomass is hemicellulose, which is primarily composed of xylose as the main carbohydrate, together with other compounds such as lignin and pectin. According to Binod et al. (2011), the enzymatic hydrolysis of xylan to xylose is usually completed by several enzymes, such as (i) endo-1,4-β-xylanase (EC 3.2.1.8), which cleaves the glycosidic bond of the xylan backbone to release xylo-oligosaccharides, (ii) β-xylosidase (EC 3.2.1.37) that acts on oligosaccharides

and xylobiose, releasing xylose, (iii) α-arabinofuranosidase (EC 3.2.1.55) and α-glucuronidase (EC 3.2.1.139) which cleave off arabinose and 4-O-methyl glucuronic acid, respectively, from the xylan backbone, and (iv) esterases that react on the ester linkages between xylose or arabinose with other compounds such as acetic acid, phenolic acid or p-coumaric acid. There are also other enzymes such as galactomannase and glucomannase that work synergistically on hemicellulose to release galactomannan and glucomannan, respectively.

2.4.2 Factors affecting enzymatic hydrolysis

Different approaches have been used by researchers when employing enzymes to hydrolyse lignocellulosic materials to end products (simple sugars). The process begins with some physical treatments such as, mechanical grinding or milling to reduce the particle size, followed by pre-treatment (when necessary), aiming to facilitate enzymatic hydrolysis of cellulosic materials. In addition to this, there are several factors that can be grouped into enzyme-related and substrate-related, which play significant roles in optimization of the yield of targeted end-products.

Enzyme-related factors include reaction conditions such as temperature, pH, enzyme loading concentration and inhibition by certain compounds such as tannic acid, cellobiose or glucose (Binod et al., 2011), and can be influenced by the nature of the enzyme itself. On the other hand, substrate-related factors are the characteristics of the substrate that have a significant effect on the enzymatic hydrolysis; these include the degree of polymerization,

accessibility of substrate by enzymes (adsorption) and biomass loading concentration. The enzymatic hydrolysis of lignocellulose is considered a heterogeneous reaction, where soluble enzyme reacts with insoluble substrate. Therefore, the adsorption of cellulase to the surface of cellulose plays an important role. Other than temperature, the rate of cellulase adsorption to the substrate depends also on the surface area of the substrate, the viscosity and agitation of system (Binod et al., 2011; Tolan & Foody, 1999). Moreover, from an economic point of view, high substrate loading has always been an important factor, as this would enable the production of high amounts of fermentable sugars on a commercial scale. However, high substrate concentrations can cause substrate inhibition phenomena and reduce the rate of hydrolysis by increasing the viscosities of the slurries, leading to reduced mass transfer rates (Binod et al., 2011).

2.4.3 Limitation in cellulase activity

The enzymatic hydrolysis can be one of the best approaches to hydrolyse cellulosic materials to fermentable sugars for microbial fermentations. However, cellulase activity is inhibited by certain compounds. Most of them are products that are released due to the pre-treatment of lignocellulosic materials at high temperatures, such as sugar degradation products (furfural and 5-hydroxymethyl furfural) and lignin degradation products (vanillin, syringaldehyde and 4-hydroxybenzaldehyde) (Binod et al., 2011; Chatzifragkou et al., 2015). In addition to these, cellulase activity is reduced by the release of its products, mainly cellobiose and glucose from cellulose itself. For cellobiose concentration of 6 g/L and

glucose concentration of 3 g/L, cellulase and β-glucosidase activities are reduced by 60% and 75%, respectively (Binod et al., 2011; Hahn-Hägerdal et al., 2006; Philippidis & Smith, 1995; Sun & Cheng, 2002; Xiao et al., 2004). Also, other compounds that are known to have an inhibitory effect to cellulase activity are aliphatic acids such as acetic acid, formic acid or levulinic acid. Acetic acid is released due to hydrolysis of the acetyl group of hemicellulose, while formic and levulinic acid is formed as a result of furan compounds degradation (Binod et al., 2011; Jönsson et al., 2013).

In addition, hemicellulose often acts as a physical barrier between cellulase and cellulose. Hu et al. (2011) and Oksanen et al. (1997) claimed that hemicellulose is usually found in the outer surface of cellulose fibre and diffuses into the pores between the interfibrillar structure of cellulose. Thus, the access of cellulase to cellulose is limited. Recent findings suggested that the oligomers of xylan (xylo-oligosaccharides) such as xylobiose and xylotriose are competitive inhibitors of cellobiohydrolase activity (Qing et al., 2010; Zhang et al., 2012; Zhang & Viikari, 2012). These oligomers are normally released during the pre-treatment stages. To minimise the inhibition effect, the authors suggested to supplement the hydrolysis medium with xylanase and β-xylosidase to convert those compounds into xylose (Qing & Wyman, 2011). In addition, lignin also prevents enzymes from accessing cellulose and hemicellulose in plant cell wall (Zhu et al., 2008). Lignin can irreversibly bind to hydrolytic enzyme and reduce the cellulase activity (Sun & Cheng, 2002).

2.4.4 Pretreatment of lignocellulosic materials prior to enzymatic hydrolysis

The main goal of pretreatment is to increase the accessibility of enzymes to plant cell wall polymers, by altering their chemical composition and structure. This will facilitate and improve the digestibility of polymeric carbohydrates to fermentable sugars during enzymatic hydrolysis as shown in Figure 2-8 (Silverstein et al., 2007). Besides, pretreatment also aims at reducing the crystallinity of cellulose and increase the porosity of lignocellulosic materials. A variety of physical (size reduction, hydrothermal), chemical (alkaline, acid, solvent, ozone), physico-chemical (steam explosion, ammonium fibre expansion) and biological (microbial fermentations) pretreatments have been developed to assist enzymatic hydrolysis of cellulosic fibre (Mosier et al., 2005; Wan & Li, 2013).

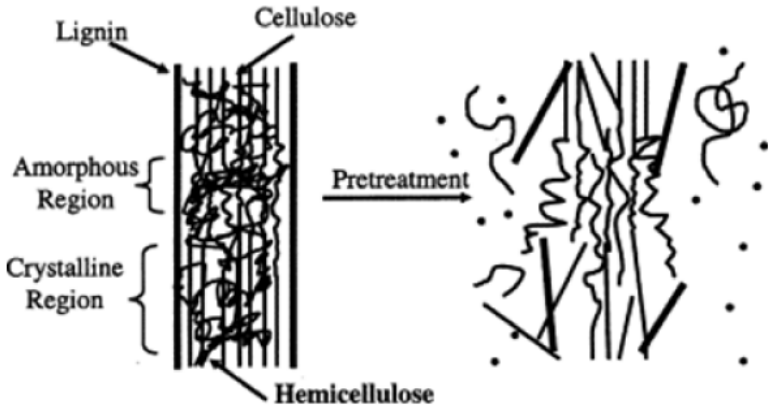

Figure 2-8: Goal of pretreatment on lignocellulosic material. Adapted from Zhang and Shahbazi (2011)

2.4.4.1 Alkaline pretreatment

Biomass pretreatment using alkaline solutions is a common approach used to facilitate enzymatic hydrolysis. It is relatively low in cost, effective on various agricultural residues and less energy demanding. Besides, alkaline pretreatment creates a clean substrate (free from lignin, hemicelluloses, protein and phenolics compounds) that is highly digestible and rich in cellulose (Chen et al., 2013). Low impurities of monosaccharide hydrolysates with high yields could be obtained after enzymatic hydrolysis of alkaline pretreated substrates. In addition to that, Kumar et al. (2009) claimed that alkaline pretreatment caused less sugar degradation, less corrosion problem and did not generate fermentation inhibitors such as furfural and hydroxymethyl furfural as in the case of acid pretreatment. Various alkaline reagents such as sodium hydroxide (NaOH), calcium hydroxide (CaOH), potassium hydroxide, ammonium hydroxide and hydrogen peroxide have been extensively studied. Among them, NaOH is of interest as it is associated with lower costs and effective pretreatment (Chen et al., 2013; Xu et al., 2010).

Alkaline reagents cleave the hydrolysable linkages in lignin and the glycosidic bonds of hemicellulose at high temperatures (> 93 ^{o}C) (Chen et al., 2013). Thus, the degree of polymerization and crystallinity of cellulose will be reduced due to disruption of the structural linkage of lignocellulose. Besides, alkaline pretreatment also causes swelling and increases the internal surface of biomass. Consequently, the chance of enzymatic saccharification of cellulose as a next processing step is increased (Alvira et al., 2010; Mosier et al., 2005).

2. 5 Dried Distiller's Grains with Solubles (DDGS)

In 2015, more than 235 bioethanol manufacturing plants were operational in the United States, the United Kingdom and Canada (Alberici & Toop, 2013; Renewable Fuels Association, 2015b; USDA Foreign Agricultural Services, 2014). The leading country in grain ethanol production is United States, which accounts approximately for 88% of the total world grain used (maize, wheat, sorghum), followed by Europe (6%), China (3.4%) and Canada (2.3%) (Food and Agriculture Organization, 2012). Figure 2-9 shows the global production of ethanol and its primary co-product, dried distillers' grains with solubles (DDGS) globally. The world production of ethanol increased between 2011 and 2014, when it reached the highest annual ethanol production (approximately 14000 million gallons). In accordance to this, DDGS production also increased from 2.7 million tonnes in 2000 to 39 million tonnes in 2014 (Renewable Fuels Association, 2015b).

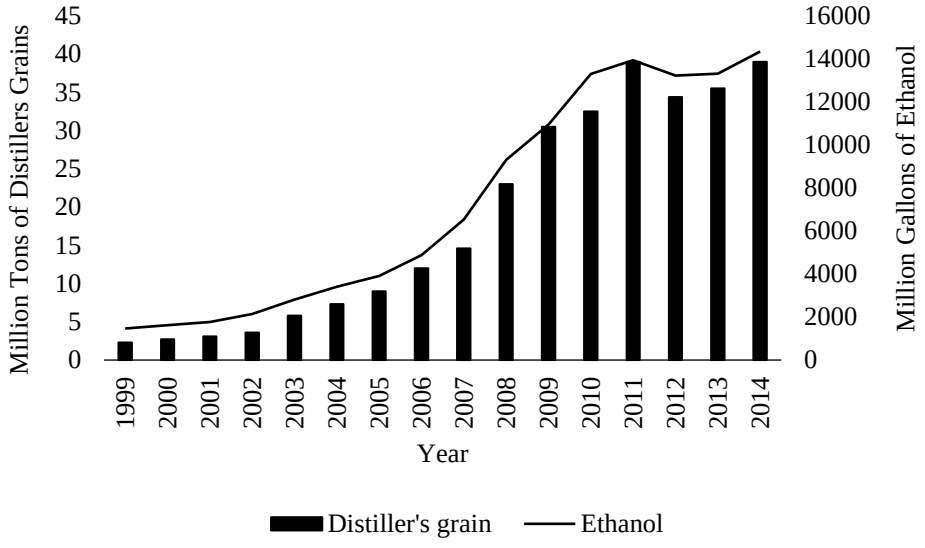

Figure 2-9: Global distillers grain production from 1999 to 2014 from ethanol refineries. Data from Renewable Fuels Association, 2015 (Renewable Fuels Association, 2015a; Renewable Fuels Association, 2015b)

The by-product of distillery or bioethanol industries exists either in its dry form, known as dried distillers' grains with solubles (DDGS) or in its wet form, distillers' wet grains (DWG). DDGS is the most prevalent in the market compared to DWG as animal feeds and increasingly exported overseas, due to its longer shelf-life (contains less than 10% moisture) (Rosentrater, 2012). The price of DDGS in the world market is within the range of 180 to 227 USD per metric tonne (Hazzledine et al., 2011; Jessen, 2011; U. S. Grains Council, 2015). DDGS can derive from various starchy-rich sources such as corn, wheat, sorghum and barley, which differ in their composition and physical properties. The main type of DDGS in United States is corn-based due to the predominant usage of corn as feedstock for bioethanol production (Moreau et al., 2012). On the other hand, in Canada, United Kingdom and most of European countries, DDGS is mainly produced from wheat (Burton et al., 2014).

The production of wheat DDGS in the United Kingdom has increased significantly (approximately four times) during the last few years due to the opening of two new bioethanol plants, Crop Energies AG (formerly known as Ensus) in 2010 and Vivergo, in 2013. Crop Energies AG produce over 316,000 tonnes of ethanol, 350,000 tonnes of DDGS and 300,000 tonnes of pure carbon dioxide per year. Vivergo produces up to 331,000 tonnes of ethanol and 500,000 tonnes of DDGS yearly. Before 2010, only 250,000 tonnes per annum of wheat DDGS were produced from UK distillery industries (Villegas-Torres et al., 2015).

2.5.1 DDGS production process

Industrially, bioethanol can be produced via the wet milling or dry-grinding process. By comparing these two methods, it can be deduced that the dry milling process is less complex and requires less capital investment as it uses the whole grain during the process. In contrast, during wet milling process, unwanted compounds such as germ, gluten and bran are removed prior to the process (Liu, 2011). Therefore, the majority of new bioethanol plants are established as dry milling facilities over the past few years (Mathews, 2010). A flow chart of the dry-grind processing of grains to ethanol is shown in Figure 2-10.

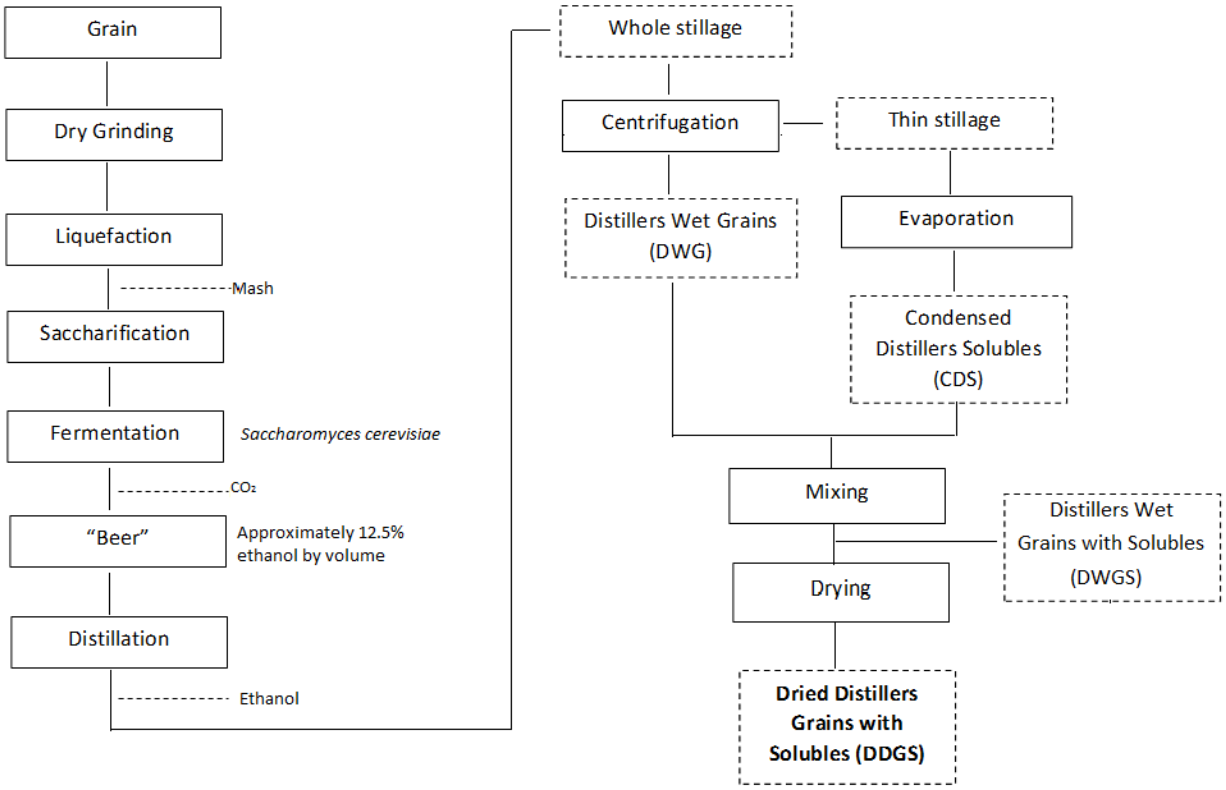

Figure 2-10: Schematic diagram of DDGS production from grains
Sources: Liu (2011) and Bruynooghe et al. (2013)

During production of alcohol in a dry-milling plant, starch is enzymatically converted into glucose by the action of α-amylase and glucoamylase (saccharification) and subsequently utilised by yeasts (*Saccharomyces cerevisiae*) to produce ethanol and carbon dioxide. According to Bruynooghe et al. (2013), approximately one third of every kilogram of wheat will be converted to ethanol, one third to DDGS and one third to carbon dioxide. The latter is usually recovered and sold to compressed gas markets such as beverages or dry ice manufacturers.

Once ethanol is distilled off after fermentation, the remaining material (slurry) is known as whole stillage. This slurry undergoes centrifugation to separate the liquid from the solid fraction. The liquid fraction is known as thin stillage (contains 5 - 10% solids) and the solid fraction is known as distillers wet grains, DWG (Liu, 2011). Normally, 10 to 50% of thin stillage is recycled back to the processing chain to minimise the amount of fresh water needed in the next processing cycle and to standardise the final product (DDGS). The remaining thin stillage is then concentrated through evaporation to produce condensed distillers solubles (CDS) or "syrup" containing 30 to 50% dry matter (Bruynooghe et al., 2013). CDS is then added back to DWG, aiming at recovering most of the nutritive components of the original feedstock and the added yeast from the whole stillage. This DWG with added "syrup" or distillers wet grains with solubles (DWGS) contains high amounts of protein, lipid, fibre, minerals and vitamins, and represents an excellent nutrient source for ruminents diet such as cattle. This is due to the ability of microorganisms that are present in the rumen to utilise feeds that is high in fibre and low in starch (Liu, 2011). However, the high moisture content of DWGS (65 - 69% moisture) limits its shelf life (5 to 7 days) (Jensen et al., 2013; Nuttelman et al., 2013; Schingoethe, 2007). Therefore, DWGS is often dried to

8 – 12% moisture level using rotary drum or ring dryers into DDGS (Bruynooghe et al., 2013; Food and Agriculture Organization, 2012; Lee & Shah, 2013).

2.5.2 Chemical and nutritional compositions of wheat DDGS

Almost all the starch that is present in the grain (wheat) will be converted into fermentable sugars that will be used by yeasts to produce ethanol and carbon dioxide. All other wheat grain components that are left unutilised (protein, lipids, fibre, minerals and vitamins) are approximately three times concentrated compared to the original grain within DDGS after the drying processing step (Bruynooghe et al., 2013). The nutrient composition of DDGS can vary due to several factors such as the type of parent grains, geographical growing locations, the amount of CDS added back to wet distillers grain and the time and temperature of the drying step (Spiehs et al., 2002).

Table 2-4 shows examples of proximate composition of wheat and corn DDGS that are found in literature. The average percentage of moisture, fat and protein in wheat DDGS is 7.6%, 5.1% and 35.8%, respectively. In contrast to corn DDGS, wheat DDGS contains higher amounts of protein and less oil and moisture content compared to corn DDGS. Due to its high protein content, DDGS is classified as an excellent source of protein for animal feed (Bruynooghe et al., 2013; Moreau et al., 2012). DDGS protein derives from the wheat itself as well as from the yeast (used during fermentation) that is recovered in the whole stillage (Villegas-Torres et al., 2015). In terms of minerals, Nuez Ortín and Yu (2009) reported that calcium (Ca), sulfur (S) and phosphorus (P) are the most important minerals

present in DDGS. However, wheat DDGS is lower in S (3.9 g/kg dry matter) but higher in Ca (1.8 g/kg dry matter) and P (9.1 g/kg dry matter) compared to corn DDGS. Other minor minerals that may be present in DDGS are zinc (Zn), manganese (Mn), copper (Cu), iron (Fe), aluminium (Al) and selenium (Se) with concentrations ranging from 6 ppm to 149 ppm (Adsul et al., 2007; Liu, 2011). In addition to that, DDGS is also contain high fiber (hemicelluloses and cellulose), but low in lignin content (Chatzifragkou et al., 2015). By employing enzymatic or chemical hydrolysis, these fibres can be hydrolysed to monosaccharides such as glucose, xylose or arabinose.

Table 2-4: Proximate composition of wheat and corn DDGS

Composition (%)	**Wheat DDGS**						**Corn DDGS**
	University of Saskatchewan (2015)	Nuez Ortín and Yu (2009)		Thacker and Widyaratne (2007)	Slominski et al. (2011)	**Average**	Nuez Ortín and Yu (2009)
		Feed source	Bioethanol plant				
Moisture	8.2	6.24	7.57	7.35	8.0	**7.47**	8.56
Crude protein	38.9	39.32	39.99	35.67	37.5	**38.27**	32.01
Crude Fat (Ether extract)	5.1	4.98	6.18	5.38	4.1	**5.15**	16.5
Ash	5.3	5.12	4.98	4.61	4.6	**4.92**	4.32
Crude fibre (ADF)	15.0	10.99	10.82	NA	NA	**12.27**	14.68
Neutral detergent fibre (NDF)	42.5	48.07	52.76	33.16	24.5	**40.20**	49.46

NA- Data Not Available ADF – Acid Detergent Fibre

2.5.3 Current uses, issues and challenges

The fast expansion of bioethanol industry worldwide has led to a dramatic increase in the availability of DDGS. Currently, DDGS is widely used as animal feed due to its high content of digestible protein and the selling price is low (~ 211 USD / tonne) compared to traditional animal feeds such as soybean meal (~ 333 USD / tonne) (U. S. Grains Council, 2018). In UK, the use of DDGS as animal feed has increased from 160,700 tonnes in 1999 to 402,600 tonnes in 2015 (Oplatowska-Stachowiak et al., 2015). Additionally, research on improving the quality of DDGS as animal feed for swine, poultry and aquaculture is still ongoing (Liu & Rosentrater, 2012). However, DDGS has to compete with other protein sources such as soybean meal and rapeseed meal within the animal feed market which are considered better quality (high protein) (Hoffman & Baker, 2011; Villegas-Torres et al., 2015). Additionally, the possible high levels of mycotoxins (3-fold over original sources), heavy metals and sulfur concentration in DDGS have become a concern for the farming industry (Haughey et al., 2017; Oplatowska-Stachowiak et al., 2015; Zhang & Caupert, 2012). Thus, there is a need to develop alternative ways of utilising DDGS in other areas, apart from its current exploitation route as feed.

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Chapter 3 - Alkaline fractionation and enzymatic saccharification of wheat Dried Distiller's Grains with Solubles (DDGS)

Abstract

The complete utilisation of Dried Distiller's Grains with Soluble (DDGS) requires effective pretreatment strategies aiming to increase the enzymatic digestibility of cellulose and improve its conversion to fermentable sugars. To this end, the effect of different NaOH concentrations (0 – 5%, w/v) and temperature (30 – 121 ^{o}C) on the fractionation of DDGS to its main components (cellulose, hemicellulose, proteins) was evaluated. As the NaOH increased from 0 to 5% and the temperature from 30 to 121 ^{o}C (16 psi) the total sugar content of the pretreated DDGS solids progressively increased, from around ~45% to a maximum of ~88% in the case of 121 ^{o}C with 5% NaOH. However, the glucose recovery decreased from around ~73% to ~ 45% most likely due to the degradation of cellulose under alkaline conditions. At 121 ^{o}C and 5% NaOH, the DDGS solid residue consisted primarily of glucose (~53%), a 5-fold increase compared to the original DDGS, reflecting the presence of cellulose, and to a lesser extent by xylose (~25%) and arabinose (~10%) reflecting the presence of hemicellulose. Approximately 83% of the initial hemicellulose and 79% of the protein contents were removed into the liquid fraction during alkaline pretreatment. The enzymatic digestibility of the pretreated DDGS solids by the Accellerase® 1500 cellulase enzyme was significantly improved,

resulting in a 3.6 fold increase in glucose yield compared to untreated DDGS, which can be attributed to morphological and structural changes which were observed by Environmental Scanning Electron Microscopy, Fourier Transform Infrared Spectroscopy and X-Ray Diffraction analyses. Mass balance analysis demonstrated that the proposed process scheme recovers the majority of the key DDGS components (cellulose, hemicellulose, proteins) in an efficient manner with relatively low losses, and provides a viable approach for the valorisation of DDGS.

Keywords: DDGS, alkaline pretreatment, cellulose, hemicellulose, cellulase

3.1 Introduction

The production of biofuels and platform chemicals such as bioethanol and organic acids from renewable bioresources is attracting significant attention. Dried Distillers Grains with Solubles (DDGS), is a bioresource that is produced in large amounts worldwide as a by-product from the bioethanol and distillery industries. With regards to bioethanol production, approximately one third of every kilogram of wheat or corn is converted into ethanol, one third into carbon dioxide and one third into DDGS (Bruynooghe et al., 2013; Chatzifragkou et al., 2015). In the USA, DDGS from bioethanol plants is corn-based (Moreau et al., 2012), whereas, in the United Kingdom, Canada, and most of European countries, it is wheat based (Burton et al., 2014). DDGS is used as animal feed and is an important source of energy, protein, water-soluble vitamins and minerals for livestock. However, the addition of DDGS to livestock feed can only account for up to 30% (on a dry matter basis) of the diet, as higher levels may cause palatability and excessive protein consumption issues (Schingoethe et al., 2009). Moreover, the variation in the nutritional composition of DDGS, particularly its protein content, depends on the source and the production processes, , and is an obstacle for the extensive utilisation of DDGS as animal feed supplement for ruminants (Belyea et al., 2010). Considering the above, as well as the increased bioethanol-derived DDGS availability in future years, it is important to identify higher value alternative ways for the valorisation of DDGS, which support the circular bioeconomy concept.

The main components of DDGS include protein, fibre, lipids, minerals and vitamins; compared to the original grain composition, they are concentrated approximately 3-fold in DDGS, as a result of the DDGS production process which

consists of a series of concentration steps and a final drying step (Bruynooghe et al., 2013). In the case of wheat DDGS, the protein derives from wheat (gluten, globulins and albumins) and the yeast cells, as the latter are not separated during the DDGS production process (Villegas-Torres et al., 2015). According to Han and Liu (2010), around 20% of the total protein in corn DDGS are contributed by yeast. From a valorisation perspective, the protein in DDGS can be recovered using chemical extraction methods and could be potentially utilised in food, feed and agricultural applications (Chatzifragkou et al., 2015; Chatzifragkou et al., 2016). Cellulose and hemicellulose are the main carbohydrates in DDGS, although β-glucan might also present, albeit at very low composition (~1 - 2%) (Gibreel et al., 2011). These carbohydrates can be potentially hydrolysed to monomeric sugars, namely glucose, xylose and arabinose and used as fermentation feedstocks (Bals et al., 2006; Xu & Hanna, 2010). However, plant-derived biomass residues including DDGS are known for their rigid structure and reluctance to enzymatic breakdown, which renders the release of fermentable monomeric sugars from such matrices difficult (Zhao et al., 2012). The main contributors towards the complex and rigid structure of plant biomass are the interactions between lignin, cellulose and hemicellulose as well as the crystallinity of cellulose. Cellulose is tightly packed into microfibrils via strong hydrogen bonding and is surrounded by lignin and hemicellulose (Vishtal & Kraslawski, 2013). Lignin is a complex, cross-linked polymer of aromatic rings that is linked to cellulose and hemicellulose through covalent and hydrogen bonds (Limayem & Ricke, 2012; Zhu et al., 2008), whereas hemicellulose is a heteropolymer, consisting of a linear xylose backbone (approximately 90%) linked with arabinose (nearly 10%) via β-1,4 linkages (Gírio et al., 2010; Limayem & Ricke, 2012). Because of the complexity of biomass structure, the hydrolysis of lignocellulosic material into fermentable sugars constitutes a major bottleneck in biorefining industries. DDGS has the advantage over other agri-food

biomass residues and by-products that it contains little amount of lignin (~ 5% on a dry basis), which renders the fractionation process considerably easier (Chatzifragkou et al., 2016).

A significant amount of work has demonstrated that for the complete hydrolysis of various agri-food materials, efficient, scalable and cost-effective pretreatment strategies are required to enhance the enzymatic digestibility of the carbohydrates and thus increase their conversion to fermentable sugars (Kim et al., 2016). A number of pretreatment strategies have been investigated for a variety of feedstocks with the focus being on lignocellulosic materials, including dilute acid hydrolysis (Hsu et al., 2010), ammonium fibre expansion (AFEX) (Dien et al., 2008; Kim et al., 2008b), hot water extraction (Kim et al., 2009; Yang et al., 2011), steam explosion (Yang et al., 2011) and alkaline treatment (Asghar et al., 2015; Kim et al., 2016; Kim et al., 2008a; McIntosh & Vancov, 2011; Subhedar & Gogate, 2014). Among these pretreatments, the use of alkaline regents is promising as it can alter the degree of polymerization of lignocellulosic components and increase the porosity and surface area of the biomass by solubilising hemicellulose, thus swelling its structure and potentially rendering it more susceptible to subsequent enzymatic saccharification (Chatzifragkou et al., 2015; Wan et al., 2011). Moreover, alkaline pretreatment is relatively simple and scalable, while the method normally uses chemicals such as ammonia, sodium hydroxide (NaOH), sodium carbonate ($NaCO_3$), and calcium hydroxide ($CaOH_2$). NaOH has attracted more attention as it is one of the strongest base catalysts and has a long history of being used as a reagent to digest cellulose (Kim et al., 2016). The aim of this study was to investigate the effect of alkaline (NaOH) pretreatment of DDGS on the enzymatic digestibility of its cellulosic and hemicellulosic components, and monitor the chemical and physical changes taking place during DDGS alkaline pretreatment, fractionation and subsequent enzymatic hydrolysis.

This knowledge is important in order to design effective strategies for the fractionation and hydrolysis of DDGS targeting the production of nutrient-rich fermentation feedstocks.

3.2 Materials and Methods

3.2.1 Raw materials and enzymes

Dried Distillers Grains with Solubles (DDGS) was supplied from a UK bioethanol plant (Vivergo, Yorkshire, UK). DDGS was ground into fine powder using a coffee grinder (DeLonghi, Australia), sieved through sieve mesh No. 20 (particle size smaller than 0.85 mm) and stored at room temperature (20 ^{o}C) prior to analysis. Commercial enzyme, Accellerase® 1500 was kindly provided by Danisco US Inc. (Genencor, Leiden, Netherlands) and was stored at 4 ^{o}C until further use. According to the manufacturer's specifications, Accellerase® 1500 exerted of endo-glucananase (2200 – 2800 CMC U/g), exoglucanase, hemicellulase and β-glucosidase (450 – 775 *p*NPG U/g) activities.

3.2.2 Composition of DDGS

The proximate composition (moisture, crude protein, crude fat and ash) of DDGS was determined according to the official methods of the Association of Analytical Communities (1996) (AOAC). The moisture content was determined using the oven-dry method at 105 ^{o}C (overnight) and was expressed as percentage by weight of sample. The

dried DDGS was then heated for ash content determination in a muffle furnace at 530 ^{o}C for 4 hours. The protein content was determined using the Kjeldahl method, where DDGS was digested in concentrated sulfuric acid, H_2SO_4, followed by distillation and titration with 0.1N H_2SO_4. The nitrogen content of DDGS was then multiplied by a factor of 5.7 (nitrogen conversion factor for wheat) to get the percentage of crude protein. For fat content analysis, petroleum ether was used as the extraction solvent. The extraction was carried out in a Soxhlet apparatus for 4 hours. The solvent was removed from the extracted fat by evaporation using a rotary evaporator at 60 ^{o}C, followed by oven drying at 105 ^{o}C for approximately 1 hour. The percentage fat was calculated on a mass basis compared to the initial sample. The total starch content was quantified using the Megazyme determination kit (K-TSTA 09/14, Megazyme, Ireland).

The carbohydrate (cellulose and hemicellulose) content and lignin content of DDGS were determined according to the method by the National Renewable Energy Laboratory (NREL/TP-510-42618), which involves a two-step acid hydrolysis process to hydrolyse polysaccharides to monosaccharides (glucose derived from cellulose, and xylose and arabinose derived from hemicellulose). Acid-soluble lignin was quantified by ultraviolet spectroscopy at 320 nm wavelength, while acid-insoluble lignin was determined gravimetrically after acid hydrolysis (Sluiter et al., 2011). The sugar composition of the acid hydrolysed DDGS samples was determined by high performance liquid chromatography (HPLC) following the protocol described in Section 3.2.6.

3.2.3 DDGS alkaline pretreatment process

DDGS was treated with sodium hydroxide (NaOH) at different concentrations (0 – 5%, w/v), temperatures (30 – 121 ^{o}C) and time (0.25, 0.5 and 6 h) in 250 ml screw cap

glass bottles, using a 1:10 solid to liquid ratio (100 mL of solution). The trials at 30, 50 and 70 ^{o}C were conducted in a water-bath, with the stirring set at 200 rpm, whereas the trial at 121 ^{o}C was carried out in an autoclave (pressure ~16 psi). After the pretreatments, the material was cooled down to room temperature and centrifuged at 17,105 x g (Heraeus Multifuge X3R, Thermo Fisher, USA) for 20 minutes at 4 ^{o}C. The obtained solids were extensively washed with distilled water until the pH reached around 8; the pH was then adjusted between 5 - 5.5 using 6 M HCl. Both insoluble and soluble fractions were frozen (-20 ^{o}C), freeze-dried (VisTis Sentry 2.0, Warminster, PA) for approximately 5 days, and stored in a closed container at room temperature until further analysis.

3.2.4 Enzymatic hydrolysis of DDGS

Enzymatic hydrolysis of untreated and pretreated DDGS solids was carried out in 250 ml Duran bottles at 50 ^{o}C and 300 rpm for 48 hours. Different ratio of Accellerase® 1500 to cellulose (1 ml: 3.3, 0.66, 0.33 and 0.22 g) were used to hydrolyse untreated DDGS (30%, w/v). Samples were collected at several times intervals, heat inactivated at 95 ^{o}C for 10 minutes and centrifuged at 17,105 x g 20 minutes (15 ^{o}C). The supernatant was kept for sugar analysis, which was conducted by HPLC (see Section 3.2.6). The enzyme loading concentration that gave the highest amount of glucose released was selected and tested against alkaline pretreated DDGS.

3.2.5 Physicochemical characterisation of DDGS

3.2.5.1 Fourier transform infrared (FTIR) analysis

FTIR analysis of untreated and pretreated DDGS solid samples was performed to determine the changes in functional groups caused as a result of the pretreatment process.

One gram of dried sample (particle size < 0.85 mm) was uniformly spread on the crystal surface area and covered by a flat probe tip. The spectra (10 scans per sample) of both DDGS samples were collected from 4000 to 500 cm^{-1} at a 4 cm^{-1} resolution using a benchtop FTIR Spectrometer (Perkin-Elmer Spectrum 100, USA), equipped with a universal attenuated total reflection (ATR) accessory and the Atmospheric Vapor Compensation (AVC) software, which was used to remove spectral interferences caused by water and carbon dioxide.

3.2.5.2 X-ray diffraction (XRD) analysis

The X-ray diffraction pattern and crystallinity of untreated and pretreated DDGS solid samples was determined by a powdered X-ray diffractometer (Bruker D8 Advance, Germany) at 40 kV and 40 mA using Cu Kα radiation (λ = 1.54 Å). The scan range was between 2θ = 5 and 65^o with a step size of 0.02° and the scattered ray beam was detected using a Lynxeye XE detector. The degree of crystallinity (X_c) was calculated as suggested by Zhou et al. (2005) and Binod et al. (2012) as:

$$X_c\ (\%) = F_c / (F_c + F_a)\ \text{X}\ 100,$$

Where F_c and F_a are the areas of the crystalline and non-crystalline region, respectively.

3.2.5.3 Environmental scanning electron microscopy (ESEM) analysis

The surface of untreated and pretreated DDGS solid samples was analysed by Quanta FEG 600 Environmental Scanning Electron Microscopy (FEI Co. Inc., Hillsboro, Oregon). Samples were mounted onto SEM stubs using carbon tape and then sputter coated with a thin layer of gold to prevent charging during imaging. The parameters used

for imaging were: 20 kV of accelerating voltages, 4.0 spot size and a working distance approximately 10 – 12 mm. Images were recorded under vacuum at 200X magnification.

3.2.6 Sugar analysis

The sugar composition of DDGS solid samples (untreated, alkaline treated, enzymatically treated) following acid hydrolysis (according to the NREL/TP-510-42618 protocol) was determined by HPLC. An Agilent Infinity 1260 system (Agilent Technologies, USA) was used with an Aminex HPX-87H column (Bio-rad, Hercules, CA). Analysis was performed at 0.6 ml/min flow rate, with 5 mM H_2SO_4 as mobile phase. The temperature of the column was set at 65 ^oC and sugars were detected using a refractive index detector (RID). Quantification of compounds was performed according to external calibration curves using glucose, arabinose, xylose (Sigma Aldrich), cellobiose, xylobiose and xylotriose (Megazyme) as standards.

Mass recoveries were calculated as the mass of insoluble solids or mass of soluble material (dried liquid fraction) recovered, and expressed as a percentage of the initial mass. Total mass recovery was calculated by adding up the masses of insoluble solids and soluble material together as described by Wan et al. (2011). Sugar recovery was calculated by expressing the amount of glucose, xylose or arabinose released in the hydrolysates as a percentage of the amount of each respective sugar in the original DDGS (da Silva et al., 2016).

3.2.7 Statistical Analysis

Statistical analysis was conducted using the Minitab®16 statistical analysis software. One-way analysis of variance (ANOVA) with a Tukey's multiple comparison

test was used to determine significant differences between treatments, at a confidence level of 95% ($P < 0.05$). Results are presented as mean ± standard deviation.

3.3 Results and Discussion

3.3.1 Chemical composition of DDGS

The composition of wheat DDGS is presented in Table 3-1. DDGS contained high amounts of protein and fibre (total of cellulose and hemicellulose) equal to approximately 28.3 and 30.4% (w/w), respectively, whereas the amounts of lignin, lipids and starch were approximately 2.9, 3.4 and 0.8 % (w/w), respectively. The low starch content was expected as starch is hydrolysed during the saccharification step during the bioethanol production process. The protein, fibre, fat and mineral contents were broadly in line with other works reporting the composition of wheat DDGS. In terms of the protein and fat contents, the results obtained in this study are similar with the data reported by Chatzifragkou et al. (2016), who reported 29% for protein and 3.4% for fat. However, higher protein and fat contents were reported by Cozannet et al. (2010) (36% and 4.6%, respectively) and Pedersen et al. (2014) (33.4% and 5.25%, respectively). In terms of the ash content, this study found similar result (5.6%, w/w) with Cozannet et al. (2010) (5.2%) and Widyaratne and Zijlstra (2007) (5.3%), whereas other studies reported varied ash contents, i.e. 3.9% (Chatzifragkou et al., 2016) and 9.1% (w/w) (Pedersen et al., 2014). The cellulose (including β-glucan) and hemicellulose contents of DDGS were approximately 11.1 and 20.3% (w/w), respectively. These values are lower than those

reported by Chatzifragkou et al. (2016) (15% cellulose and 25% hemicellulose). However, lower cellulose (7%) and higher hemicellulose (42%) was reported for wheat DDGS derived from a bioethanol plant in western Canada (Nuez Ortín & Yu, 2009). The variation in the nutritional composition of DDGS might be associated with differences in the production processes used by different plants, the type of wheat cultivar, seasonal variation of harvest and different in N-to- protein conversion factor (6.25 over 5.7) (Chatzifragkou et al., 2016).

Table 3-1: Chemical composition of wheat DDGS

Component	**Composition (%, w/w dry basis)**
Moisture	10.8 ± 0.1
Dry matter	89.2 ± 0.1
Crude protein	28.3 ± 0.5
Crude fat	3.40 ± 0.04
Starch	0.8 ± 0.2
*Cellulose (glucose)	11.1 ± 0.4
Hemicellulose	20.3 ± 1.7
Xylose	13.7 ± 1.6
Arabinose	6.6 ± 1.9
Lignin	2.0 ± 0.1
Acid Soluble Lignin	2.9 ± 0.1
Acid Insoluble Lignin	n.d.
Ash	5.64 ± 0.13

*It is assumed that all glucose comes from cellulose, though β-glucan might be also present at small amounts
n.d.: not detected

3.3.2 Enzymatic hydrolysis of untreated DDGS

Figure 3-1 shows the effect of enzyme loading concentration on glucose yield from untreated DDGS. The highest glucose concentration was achieved when DDGS was hydrolysed at a ratio of 1 : 0.22 (Accellerase® 1500, ml : g cellulose) for 24 h, corresponding to a yield of ~ 26 % (w/w), although there was no significant difference ($P < 0.05$) with 1 : 0.33 (Accellerase® 1500, ml : g cellulose) ratio. Since cellulose exists in both amorphous and crystalline states, the enzymatically produced glucose was most likely derived from the amorphous state. At this amorphous state cellulose exists in a disordered arrangement, and Accellerase® 1500 through its endoglucanase and β-glucosidase activities was most likely able to selectively hydrolyse it into glucose and cellulo-oligosaccharides, as also shown previously (Gao et al., 2013). In the case of the crystalline state, the cellulose chains are closely packed together by strong intra- and inter- molecular hydrogen bond linkages. Because of these structural characteristics, cellulose digestibility has been reported to be around 20 % or less without any pretreatment steps for cellulosic biomass residues, such as wheat straw and sugarcane bagasse (Bensah & Mensah, 2013; Mosier et al., 2005; Rabelo et al., 2011). Besides glucose, xylose, arabinose and xylo-oligosaccharides (xylobiose and xylotriose) were also detected (data not shown) at the end of the hydrolysis which are due to the hemicellulase activities of Accellerase® 1500. As hemicelluloses exist in amorphous state due to their branched structure, their hydrolysis was more efficient compared to cellulose. The results obtained with DDGS in this work are in line with previous observations with lignocellulosic materials, indicating that glucose recovery from DDGS by an enzymatic process is limited by the structural characteristics of DDGS.

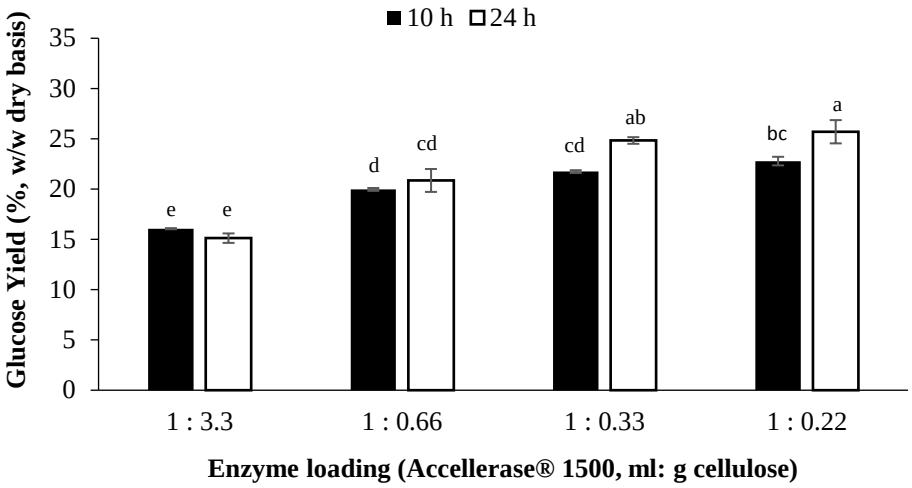


Figure 3-1: Effect of different concentrations of Accellerase ® 1500 enzyme on glucose production from DDGS at 50 ºC. Different letters [abcd] indicated significant differences ($P < 0.05$) of glucose yield (%).

3.3.3 Alkaline pretreatment of DDGS

The enzymatic digestibility of cellulose present in lignocellulosic biomass can be improved by employing appropriate pretreatment strategies, such as steam explosion, AFEX, ultrasonication, liquid hot water and alkaline treatment. This has been previously demonstrated for a variety of biomass including soybean straw (Wan et al., 2011), sugarcane bagasse (da Silva et al., 2016; Zhao et al., 2009), switchgrass (Xu et al., 2010), wheat straw (Asghar et al., 2015; Han et al., 2012; McIntosh & Vancov, 2011), barley hull (Kim et al., 2008a), corn stover (Yang et al., 2011) and corn-based DDGS (Dien et al., 2008; Kim et al., 2008b). In this study, the influence of NaOH concentration, residence time and extraction temperature on the recovery of carbohydrates from wheat DDGS was investigated. Table 3-2 presents the monomeric sugar composition of DDGS solids following pretreatment at various temperatures (30, 50, 70 and 121 ºC) and NaOH concentrations (0, 1, 3 and 5%, w/v) and the recoveries of glucose, xylose and arabinose compared to the original DDGS sample. When DDGS was treated with water at 30, 50,

70 and 121 ºC the total concentrations of glucose, xylose and arabinose in the residual solids ranged from 38 - 42% (w/w) with the recoveries (compared to untreated DDGS) being 70 - 90% (w/w). This finding suggests that the majority of hemicellulose was not extracted from the DDGS solids (reflected by the high recovery of xylose and arabinose in the DDGS solids) and that if water was to be used for the pretreatment of DDGS, higher temperatures than 121 ºC would most likely be needed in order to breakdown the cellulose bonds and solubilise hemicellulose. Pisupati and Tchapda (2015) reported that hemicellulose bonds breakdown at 150 – 230 ºC, while significantly higher temperatures are required for cellulose breakdown (300 to 350 ºC).

As the NaOH increased from 0.5 to 5% and the temperature from 30 to 121ºC (16 psi) the total sugar content of the pretreated DDGS solids progressively increased, from around ~45% to a maximum of ~88% in the case of 121 ºC (~16 psi), with 5% NaOH for 15 minutes. Glucose was in all cases the main sugar component, reflecting the significant presence of cellulose in the pretreated DDGS solids, as opposed to arabinose and xylose, reflecting hemicellulose. In the case of the treatment at 121 ºC with 5% NaOH, the glucose content of the DDGS solids was ~53%, a 5-fold increase compared to the glucose content of the original DDGS, whereas the xylose content was ~25 % and the arabinose ~10%. Alkaline pretreatment causes the solubilisation of hemicelluloses by disrupting the ester and ether bond of hemicellulose with lignin and cellulose microfibrils (Kim et al., 2016; McIntosh & Vancov, 2011). For this reason, and in line with the results in this study, the glucose concentration in the recovered solids has been shown to significantly increase in the case of alkaline pretreatment of lignocellulosic materials such as wheat straw and sugarcane bagasse (Barman et al., 2012; da Silva et al., 2016; McIntosh & Vancov, 2011). Moreover, the increase in cellulose and decrease in the hemicellulose of the pretreated DDGS solids was accompanied by a significant decrease

in their protein content, which were solubilised and extracted in the liquid fraction (data not shown). It has been previously shown that under ethanol-alkali conditions (0.1 M), approximately 39.5 and 49.1% of the protein (primarily gliadin and glutenin) was extracted from wheat DDGS and wet distiller's grains, respectively (Chatzifragkou et al., 2016). Similarly, approximately 40% protein was extracted with 1M NaOH from corn wet distillers grains at 70 ^{o}C (Bals et al., 2009).

Taking into account the sugar recovery data presented in Table 3-2, it can be deduced that alkaline pretreatment selectively removed hemicelluloses over cellulose from DDGS solids. To this end, as the NaOH concentration increased from 0.5% to 5%, in all treatment temperatures, the recovery of xylose and arabinose in the pretreated solids progressively decreased; the lowest hemicellulose recovery was obtained after treatment with 5% NaOH at the highest temperature, 121 ^{o}C, with recoveries of ~17% for xylose and ~15% for arabinose. This is due to the fact that hemicellulose exists in an amorphous state, as heterogeneous and branched polysaccharides, which makes them more susceptible to alkaline action. The alkali reagent solubilises hemicellulose by disrupting the ester linkage of hydroxycinnamic acid and the arabinose units present in the arabinoxylan molecules as well as and the linkage of the hydroxyl groups in lignin (Dodd & Cann, 2009; Xu et al., 2010). Interestingly, in terms of glucose recovery in the DDGS pretreatment solids, which reflects cellulose recovery, it was observed that as the NaOH increased from 0 to 5% the glucose gradually decreased at all temperatures. More specifically, at 70 ^{o}C, glucose recovery decreased from ~73% (no NaOH) to ~47% (5% NaOH), whereas at 121 ^{o}C, glucose recovery decreased from ~68% (no NaOH) to ~45% (5% NaOH), a phenomenon that will be discussed later on.

Table 3-2: Sugar composition and recovery in pretreated DDGS solids *

Temperature (°C)	Time (h)	NaOH (%, w/v)	Sugar content in pretreated solids (%, w/w dry basis)				Sugar recovery in pretreated solids (%, w/w dry basis) **		
			Glucose	Xylose	Arabinose	Total	**Glucose**	Xylose	Arabinose
30	6	0	**13.4 ± 0.8^{gh}**	19.0 ± 1.4	9.9 ± 0.3	42.3	**73.6 ± 4.2^{a}**	83.9 ± 6.0	90.2 ± 3.0
		1	**14.7 ± 0.2^{fgh}**	18.8 ± 0.1	9.6 ± 0.4	43.1	**63.9 ± 2.6^{b}**	65.9 ± 1.9	69.6 ± 5.1
		3	**17.4 ± 0.4^{fgh}**	18.5 ± 0.1	9.5 ± 0.8	45.4	**53.7 ± 1.3^{c}**	45.6 ± 0.2	48.9 ± 4.3
		5	**20.0 ± 1.4^{f}**	14.4 ± 1.0	9.6 ± 0.2	44.0	**47.7 ± 1.1^{cd}**	27.6 ± 0.7	38.2 ± 2.8
50	6	0	**13.3 ± 0.0^{gh}**	18.7 ± 0.0	9.1 ± 0.0	41.1	**73.3 ± 0.3^{a}**	82.8 ± 0.4	83.3 ± 0.4
		1	**18.0 ± 0.1^{fgh}**	19.4 ± 0.1	8.2 ± 0.1	45.6	**62.5 ± 1.2^{b}**	54.3 ± 0.6	47.7 ± 0.5
		3	**27.6 ± 0.9^{e}**	25.2 ± 0.2	8.9 ± 0.0	61.7	**50.8 ±1.8^{cd}**	37.2 ± 0.4	27.4 ± 0.1
		5	**34.0 ± 0.5^{cd}**	18.9 ± 0.6	9.7 ± 0.6	62.6	**45.6 ± 0.02^{d}**	20.4 ± 0.3	21.7 ± 0.9
70	6	0	**13.2 ± 0.2^{gh}**	17.9 ± 0.4	9.3 ± 0.5	40.4	**72.5 ± 0.04^{a}**	78.7 ± 0.6	84.7 ± 3.1
		1	**28.3 ± 1.3^{de}**	25.2 ± 0.6	9.6 ± 0.5	63.2	**64.9 ± 3.4^{b}**	46.4 ± 0.9	36.6 ± 1.7
		3	**35.3 ± 0.8^{c}**	29.9 ± 0.2	10.2 ± 0.6	75.4	**48.6 ± 1.^{cd}**	33.2 ± 04	23.3 ± 1.4
		5	**44.7 ± 2.2^{b}**	21.8 ± 0.8	10.8 ± 0.3	77.2	**47.1 ± 1.1^{cd}**	18.4 ± 0.2	18.9 ± 0.0
121	0.25	0	**13.5 ± 0.2^{gh}**	18.3 ± 0.3	10.6 ± 0.1	42.4	**68.4 ± 0.5^{ab}**	74.3 ± 0.8	89.2 ± 0.7
(16 psi)		0.5	**18.6 ± 0.7^{fg}**	21.2 ± 0.2	10.1 ± 0.02	49.9	**62.9 ± 2.2^{b}**	57.4 ± 0.3	56.9 ± 0.4
		1	**29.5 ± 0.4^{cde}**	29.7 ± 0.3	9.7 ± 0.2	69.0	**50.7 ± 0.04^{cd}**	40.9 ± 1.1	28.2 ± 0.1
		3	**43.8 ± 0.8^{b}**	31.5 ± 2.4	10.59 ± 0.3	85.9	**46.8 ± 0.5^{cd}**	27.0 ± 1.9	18.9 ± 0.4
		5	**52.6 ± 0.7^{a}**	25.0 ± 0.2	10.34 ± 0.1	87.9	**44.7 ± 1.1^{d}**	17.1 ± 0.8	14.7 ± 0.7
121	0.5	0	**12.7 ± 0.2^{h}**	17.0 ± 0.5	8.17 ± 0.04	37.8	**64.1 ± 0.6^{b}**	69.3 ± 2.2	70.0 ± 0.1
(16 psi)		0.5	**17.4 ± 1.6^{fgh}**	19.9 ± 1.4	8.40 ± 0.4	45.7	**62.3 ± 2.6^{b}**	57.4 ± 1.2	50.4 ± 5.1
		1	**27.9 ± 1.0^{e}**	28.7 ± 1.4	7.99 ± 0.5	64.7	**49.0 ± 1.4^{cd}**	40.5 ± 1.6	23.4 ± 1.3
		3	**41.1 ± 1.0^{b}**	30.0 ± 1.8	10.01 ± 0.0	81.2	**47.0 ± 1.5^{cd}**	27.6 ± 1.3	19.1 ± 1.0
		5	**45.4 ± 5.2^{b}**	21.8 ± 1.6	9.47 ± 0.3	76.6	**44.4 ± 1.0^{d}**	17.1 ± 1.1	15.5 ± 1.7

* Data reported as an average of two replicates

** Sugar recovery is calculated as the % of a particular sugar compared to its content in untreated DDGS

Means within each vertical line with different alphabet ^{abcdefgh} are significantly ($P < 0.05$) different

In order to investigate in more detail the effect of the process on the recoveries of cellulose and hemicellulose, a total mass and sugar recovery balance was conducted, as shown in Table 3-3. Even with no NaOH being added, the extraction process carried out at temperatures from 30 to 121 ^{o}C extracted between 23 to 30% of soluble material from DDGS. This probably denotes the solubilisation of readily water soluble compounds that are found in DDGS, which are derived from the condensed distillers soluble (CDS) fraction that is commonly mixed with wet solids prior to drum drying during DDGS production process, and may include soluble proteins, organic acids and minerals (Bruynooghe et al., 2013; Liu, 2011). As the NaOH concentration increased, the mass of the liquid fraction increased due to the solubilisation of hemicelluloses and protein. However, the total mass balance, for both solid and liquid fractions, was not 100%, which could be attributed partly to differences in the moisture content of the dried solid (~ 2%) and dried liquid fractions (~2%), compared to the initial moisture content of untreated DDGS (~11%). Moreover, there is also a possibility that some organic compounds such as hydroxymethyl furfural (HMF), aldehydes, glycerol or short chain fatty acids such as lactic acid, which were originally present in DDGS, decomposed to their gas state during alkaline pretreatment at high temperatures (Yin & Tan, 2012).

The total xylose and arabinose recovery in both solid and liquid fractions was in most cases very close to 100% (with discrepancy in the arabinose was found), indicating no obvious losses for these compounds during the alkaline pretreatment process. The inconsistency in arabinose recovery (> 100%) might due to the variations in moisture content after drying process. In contrast, considerable glucose losses were noted, especially as the temperature progressively increased from 30 ^{o}C to 121 ^{o}C. The highest losses were detected when DDGS was treated with 5% NaOH at 121 ^{o}C and ranged from 30% to 40%. A comprehensive review by Knill and Kennedy (2003) suggested that the degradation of

glucose during exposure to alkaline reagents such as NaOH, $CaCO_3$ and KOH leads to the production of various compounds including formic acid, acetic acid, hydroxyactic acid, 2-hydroxy-propanoic acid, butyric acid, 2-hydroxybutanoic acid, 3-deoxy-D-pentonic acid, β-D-glucoisaccharinic acid, D-gluconic acid and D-mannoic acid. According to Knill and Kennedy (2003), end-wise degradation and alkaline scissions are the main mechanisms contributing to glucose losses during alkaline pretreatment. In end-wise degradation (or peeling), glucose loses are due to the dissolution of short chain material, which detaches from the reducing end of cellulose, and results in the formation of 3-deoxy-2-*C*-(hydroxymethyl)-erythro and thereo-pentonic acids (D-glucoisosaccharinic acids). This mechanism normally occurs at temperatures less than 170 ^{o}C. On the other hand, alkaline scission (or hydrolysis) normally occurs at higher temperatures (> 170 ^{o}C), where random hydrolysis of the glycosidic linkages takes place, and results in significant weight losses and decreases in the degree of polymerisation of cellulose (Knill & Kennedy, 2003). These reactions have also been suggested in other works, where it was shown that during alkaline pretreatment of lignocellulosic biomass at high temperatures, cellulose was converted into dissolved organic compounds such as dihydroxy and dicarboxylic acids, aldehydes, furfural or 1,2,3-benzenetriol (Argun & Onaran, 2016; Jönsson & Martín, 2016; Yin & Tan, 2012).

Table 3-3: Overall mass recovery and total sugar in pretreated DDGS solids *

Temperature (ºC)	Time (h)	NaOH (%, w/v)	Mass recovery of pretreated DDGS (% of initial weight)			Total sugar recovery (%)**		
			Solid	Liquid	Total	Glucose	Xylose	Arabinose
30	6	0	60.4 ± 0.1	23.6 ± 0.1	83.9	86.8 ± 3.8^{ab}	104.3 ± 5.5	113.4 ± 2.9
		1	48.0 ± 1.5	33.7 ± 0.3	81.7	82.2 ± 2.4^{abc}	101.9 ± 1.2	112.9 ± 5.7
		3	33.9 ± 0.1	39.1 ± 0.7	73.0	80.4 ± 2.6^{bcd}	98.5 ± 0.2	107.6 ± 5.7
		5	26.4 ± 2.5	49.4 ± 1.5	75.7	82.0 ± 2.1^{abc}	99.4 ± 2.4	115.9 ± 7.1
50	6	0	60.6 ± 0.3	26.5 ± 0.1	87.1	87.3 ± 0.02^{a}	101.9 ± 0.3	109.1 ± 1.2
		1	38.3 ± 0.6	46.2 ± 2.1	84.4	77.0 ± 1.3^{cd}	97.2 ± 1.1	110.5 ± 1.9
		3	20.3 ±0.1	58.0 ± 0.1	78.2	75.2 ± 0.1^{d}	99.8 ± 2.5	119.8 ± 3.9
		5	14.8 ± 0.2	67.9 ± 0.1	82.7	78.8 ± 1.8^{cd}	103.4 ± 0.9	132.7 ± 6.2
70	6	0	60.3 ±0.9	26.9 ± 0.6	87.2	87.6 ± 0.4^{a}	99.1 ± 1.0	109.7 ± 2.6
		1	25.2 ± 0.1	56.9 ± 0.1	82.1	76.6 ± 2.8^{cd}	91.3 ± 0.6	105.5 ± 3.9
		3	15.2 ± 0.1	67.3 ± 0.0	82.5	66.5 ± 2.3^{ef}	91.4 ± 3.5	99.4 ± 5.2
		5	11.6 ± 0.3	77.7 ± 0.5	89.3	67.1 ± 1.2^{e}	88.7 ± 4.8	100.9 ± 5.0
121	0.25	0	55.7 ± 0.2	27.0 ± 0.1	82.6	87.1 ± 0.7^{a}	102.4 ± 1.2	117.9 ± 1.5
(16 psi)		0.5	37.1 ± 2.1	43.9 ± 0.2	81.0	77.0 ± 1.8^{cd}	101.1 ± 0.2	114.6 ± 0.7
		1	18.9 ± 0.3	56.3 ± 1.7	75.2	61.5 ± 0.1^{ef}	90.0 ± 2.4	110.3 ± 1.6
		3	11.8 ± 0.1	75.9 ± 1.0	87.6	66.1 ± 0.5^{ef}	96.3 ± 0.04	114.7 ± 1.2
		5	9.4 ± 0.4	82.0 ± 0.1	91.3	66.2 ± 1.2^{ef}	94.0 ± 6.8	116.6 ± 2.4
121	0.5	0	55.8 ± 0.2	30.6 ± 0.4	86.4	87.1 ± 0.6^{a}	104.9 ± 3.9	110.6 ± 6.1
(16 psi)		0.5	39.6 ± 2.1	41.8 ± 1.7	81.4	77.4 ± 1.1^{cd}	97.4 ± 2.5	104.2 ± 2.8
		1	19.3 ± 0.1	59.3 ± 0.3	78.6	60.0 ± 1.0^{f}	88.4 ± 1.0	99.4 ± 0.1
		3	12.6 ± 0.7	72.6 ± 3.1	85.1	64.1 ± 0.9^{ef}	90.3 ± 5.9	104.9 ± 9.3
		5	10.9 ± 1.5	72.6 ± 0.3	83.5	65.8 ± 0.1^{ef}	91.6 ± 1.5	110.1 ± 3.0

* Data reported as an average of two replicate

** Total sugar recovery is calculated as the % of a particular sugar in both the solid and liquid fractions compared to its content in untreated DDGS

Means within each vertical line with different alphabet ^{abcdef} are significantly ($P < 0.05$) different

3.3.4 Enzymatic hydrolysis of pretreated DDGS solids

The DDGS solid residues obtained after alkaline pretreatment which demonstrated the highest total sugar content (5% NaOH, 121 ^{o}C, 15 min) were then subjected to enzymatic hydrolysis using Accellerase® 1500 (Table 3-4). It was noted that glucose release was significantly higher when the pretreated DDGS solids were hydrolysed with the enzyme compared to untreated DDGS (86% vs 25%, respectively). This study demonstrated that pretreatment is an important step in improving cellulose digestibility of DDGS. Similar findings were also reported by Xu et al. (2010) who compared the effect of alkaline pretreatment on switchgrass and showed that the yield of total reducing sugars increased 3.78 times compared to untreated switchgrass. Alkaline pretreatment is known to cause a swelling effect due to the solvation and saponification of hemicelluloses, thus it results in increased porosity and loosening of the structure of DDGS. Therefore, the surface area of cellulose is increased and is more exposed to enzymatic hydrolysis (Han et al., 2012; Kim & Han, 2012).

Accellerase® 1500 has side activities, including hemicellulase and β-glucosidase activities, as demonstrated by the considerable amounts of xylose and arabinose released into the hydrolysate. The concentration of xylose and arabinose in the hydrolysate after alkaline pretreatment was lower than in the case of untreated DDGS solids due to the significant removal of hemicelluloses during alkaline treatment. However, the pretreated DDGS solids still contained ~25% of xylose and ~10% of arabinose (Table 3-2), which are important to be released during enzyme hydrolysis through the action of hemicellulases in order to enhance glucose release. Enzyme cocktails that have in addition to cellulase activity hemicellulase activity, can facilitate glucose release from DDGS and lignocellulosic biomass, as shown for pretreated corn stover (Hu et al. 2011).

Hemicellulases remove the xylan coat at the surface of the pretreated fibre and thus increase the accessibility of cellulase to the cellulosic fibre (Kumar & Wyman, 2009). Interestingly, hemicellulases such as xylanases, were also been shown to significantly improve the cellulose hydrolysis of steam pretreated softwood by cellulases, regardless of the fact that this material did not contain xylan; this was due to the synergistic interaction between cellulase and xylanase, which changed the gross fibre characteristics of softwood (Hu et al., 2011).

Table 3-4: Enzymatic hydrolysis of untreated and pretreated DDGS solids at 1 : 0.33 (Accellerase® 1500, ml : g cellulose) ratio

Conditions	**Monosaccharides (g/l) in the hydrolysate**			
	Glucose	Xylose	Arabinose	**Glucose Yield (%)**
No pretreatment	8.2 ± 0.1	10.8 ± 0.0	1.7 ± 0.0	24.8
5% NaOH (121ºC, ~16 psi, 15 min)	28.5 ± 0.6	6.2 ± 0.2	0.6 ± 0.0	86.5

*Enzymatic reaction was conducted at 50 ºC for 24 h

Figure 3-2 shows the time course of the enzymatic hydrolysis of the alkaline pretreated DDGS solids, at 50 ºC. In addition to glucose, xylose and arabinose, some oligosaccharides were also detected, including xylobiose and xylotriose. Interestingly, cellobiose was not detected. The absence of cellobiose suggests that the activity of exo-glucanase and/or β-glucosidase in Accellerase® 1500 was high, which resulted in the conversion of cellobiose and possibly other gluco-oligosaccharides to glucose. Borges et al. (2014) reported a value of 228 U/ml of β-glucosidase activity in Accellerase® 1500 when cellobiose was used as a substrate. As shown in Figure 3-2, within 6 hours of hydrolysis, approximately 25g/l of glucose, 6 g/l of xylobiose, 5 g/l of xylose, 1.3 g/l of

xylotriose and ~0.6 g/l of arabinose were produced. Further increase in hydrolysis time resulted in a slow increase in glucose concertation, reaching a maximum of ~29 g/l after 30 h of hydrolysis. During the same period, the concentration of xylotriose and xylobiose decreased with a concomitant increase in xylose, which reached 7.5 g/l after 30 h. It is most likely that xylobiose and xylotriose were produced through the activities of endo-xylanases present in Accelerase® 1500, whereas xylose, through the activity of beta-xylosidase, which hydrolyses xylobiose to xylose (Badhan et al., 2014).

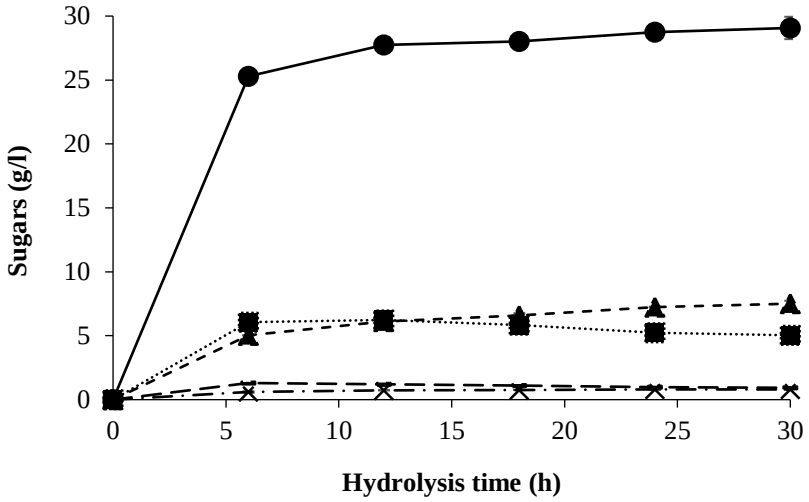


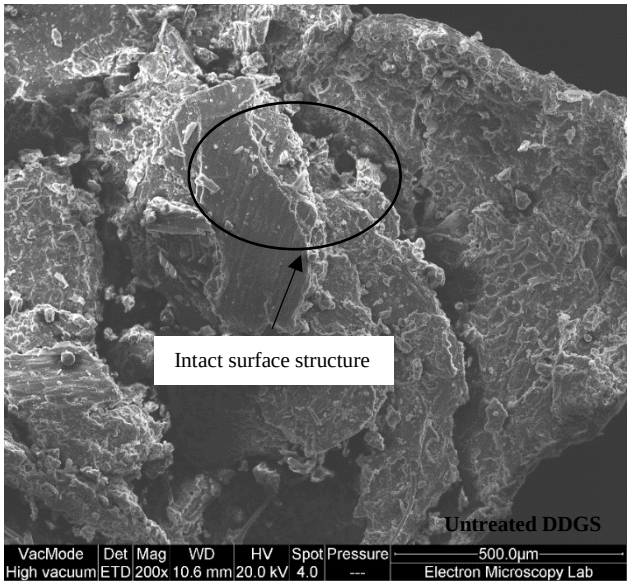
Figure 3-2: Time course of sugars released during enzymatic hydrolysis of pretreated DDGS solids (121 ºC, ~16 psi, 15 minutes , 5% NaOH) at 1 : 0.33 (Accellerase® 1500, ml : g cellulose) ratio, (–■– –) xylotriose, (···■···) xylobiose, (—●—) glucose, (· –▲– ·) xylose and (–×—) arabinose. No cellobiose was detected.

3.3.5 Physicochemical characterisation of untreated and pretreated DDGS

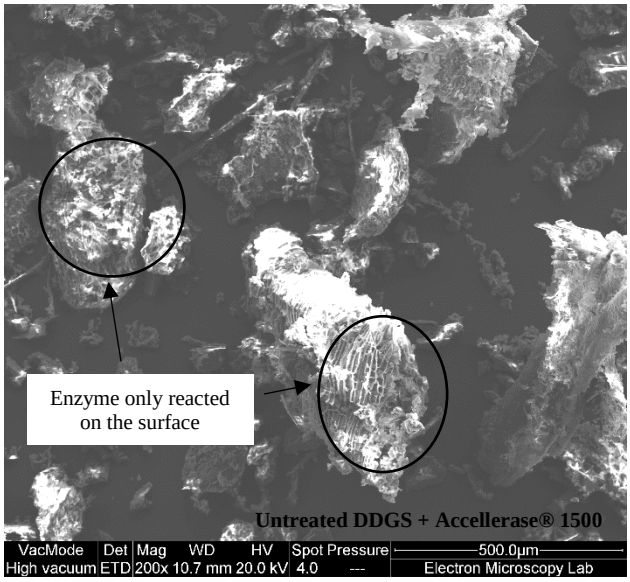
3.3.5.1 Morphological surface of DDGS

The surface of untreated and alkali treated DDGS was observed using ESEM to identify possible structural changes. The intact, compact and rigid surface structure of untreated DDGS (Figure 3-3a) changed into a fully exposed, separated and peeled-off surface in the alkali treated DDGS (Figure 3-3c). According to Bensah and Mensah (2013) alkaline pretreatment loosens the structure of biomass, hydrolyses lignin and carbohydrate bonds and decreases the degree of polymerisation and crystallinity of cellulose. Moreover, it also causes the enlargement of the internal surface area of biomass, thus it increases the access of cellulase to the cellulose present in the biomass (Chen et al., 2013; Xu et al., 2010). Previous studies with sugarcane bagasse (Binod et al., 2012) and wheat straw (Asghar et al., 2015) reported that alkaline pretreatment caused the formation of pores on the biomass surface, an effect that was not observed in our study though.

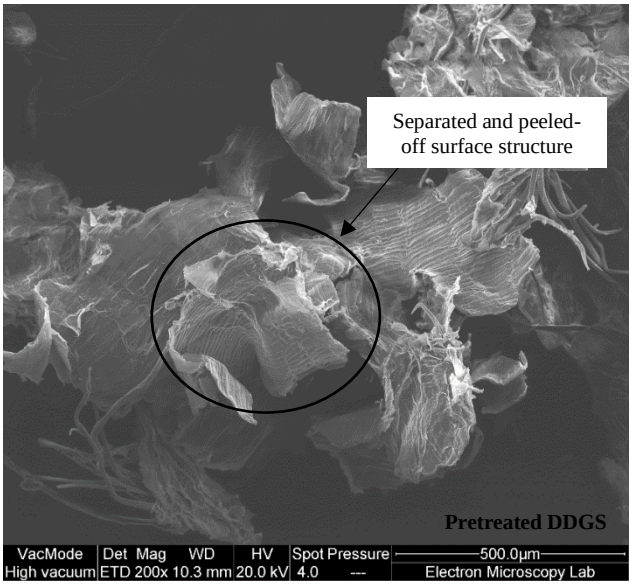
When untreated DDGS solids were hydrolysed with Accellerase® 1500, the changes to the structure were not profound (Figure 3-3b). It seems that the enzyme was not able to penetrate the rigid structure of the untreated DDGS cell wall, a fact that can further explain the low enzymatic release of glucose. In contrast, the Accellerase® 1500 pretreated (with alkali) DDGS solids demonstrated a less rigid structure that was broken into small pieces, indicating the disruption of biomass (Figure 3-3d).



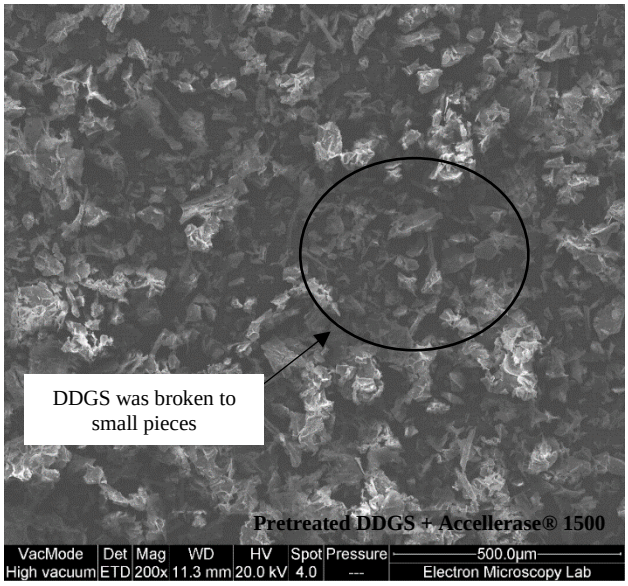
(a)



(b)



(c)



(d)

Figure 3-3: SEM images of: (a) untreated DDGS, (b) untreated DDGS hydrolysed with Accellerase® 1500, (c) alkaline pretreated (121ºC ~16 psi, 15 minutes, 5% NaOH) DDGS and (d) alkaline pretreated DDGS hydrolysed with Accellerase® 1500

3.3.5.2 Spectral characterisation

The changes in functional groups caused by alkaline pretreatment on DDGS were evaluated by Fourier Transform Infrared (FTIR) spectroscopy (Figure 3-4). Table 3-5 provides details on the assignment of the different peaks in untreated and pretreated DDGS solids. A prominent broad peak was observed at a wavelength of 1034 cm^{-1} in the case of pretreated DDGS solids as opposed to untreated DDGS. A numbers of previous research works assigned this peak to C-O, C=C and C-C-O stretching of β(1,4) glycoside bonds in cellulose, hemicellulose and lignin (Maryana et al., 2014; Morales-Ortega et al., 2013; Schwanninger et al., 2004; Sills & Gossett, 2012; Xu et al., 2013). This suggests that the increased peak intensity was due to the higher content of cellulose and hemicellulose in pretreated DDGS solids (~88% total cellulose and hemicellulose), compared to untreated DDGS (~31% total cellulose and hemicellulose). Similar FTIR data were reported by Asghar et al. (2015) for alkaline treated wheat straw and by Zhang et al. (2013) for sugarcane bagasse. Moreover, the broad peak centred at 3332 cm^{-1} in pretreated DDGS solids, which is absent in untreated DDGS, most likely reflects an increased intermolecular hydrogen bonding between the β-1,4-glucan chains of cellulose in the sample (Hishikawa et al., 2017); this is line with the higher cellulose content of pretrated DDGS (~53%) (Table 3-2) compared to untreated DDGS (Table 3-1). In addition, the very small peaks that were present in untreated DDGS at 1598 and 1744 cm^{-1} is assigned to C=O stretching and most likely corresponds to the hemicellulose present in DDGS. However, these bands could also be related to the uronic esters and acetyl groups of the ferulic and p-coumaric acids present in lignin (Barman et al., 2012; Schwanninger et al., 2004). The peaks disappeared after pretreatment suggesting that some of hemicellulose or lignin-related compounds were removed during the

pretreatment; this coincides with the results in Table 3-2, where only 17% xylose and 15% arabinose were recovered in pretreated DDGS solids.

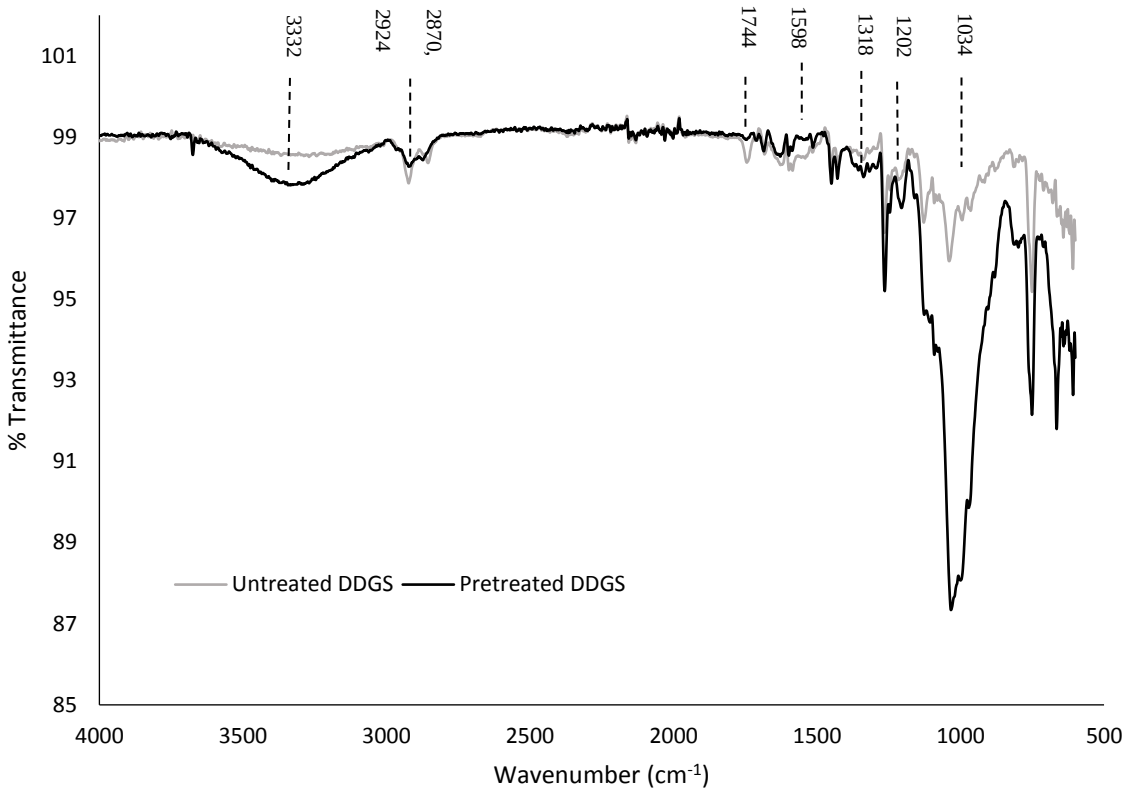


Figure 3-4: FTIR spectra of untreated DDGS and pretreated DDGS solids (121 ºC, ~16 psi, 15 minutes, 5% NaOH)

Table 3-5: Assignment of the peaks observed by FTIR analysis of untreated and pretreated (121 ^{o}C, ~16 psi, 15 minutes, 5% NaOH) DDGS

Wavenumber (cm^{-1})	Assignment / Functional group	Polymer	References
1034	C-O, C=C, and C-C-O stretching of glycosidic bond	Cellulose, Hemicellulose, Lignin	Maryana et al. (2014); Morales-Ortega et al. (2013); Schwanninger et al. (2004); Sills and Gossett (2012); Xu et al. (2013)
1202	O-H bending	Cellulose, Hemicellulose	Sills and Gossett (2012); Xu et al. (2013)
1318	CH_2 wagging / vibration	Hemicellulose, Lignin	Asghar et al. (2015); Schwanninger et al. (2004)
1598	Aromatic ring vibration + C=O stretching	Lignin	Schwanninger et al. (2004); Sills and Gossett (2012)
1744	Free esters and acids C=O stretching	Hemicellulose	Sills and Gossett (2012); Xu et al. (2013)
2870, 2924	C-H stretching	Lignin	Maryana et al. (2014); Xu et al. (2013)
3332	O-H stretching (intermolecular)	Cellulose	Schwanninger et al. (2004); Subhedar and Gogate (2014); Xu et al. (2013)

3.3.5.3 X-ray diffraction (XRD)

X-ray diffraction analysis was conducted to assess the effect of crystallinity on the digestibility of DDGS. The XRD pattern of untreated and alkaline pretreated DDGS solids is shown in Figure 3-5, whereas the obtained spectra were analysed and the results are presented in Table 3-6. The degrees of crystallinity (X_c) for untreated and pretreated DDGS were different. For untreated DDGS solids, the X_c was estimated at 39.7%, while

pretreated DDGS solids had 3 times lower degree of crystallinity, estimated at 13.1%. This was most likely due to the structural changes caused by alkaline pretreatment, more specifically the fact that NaOH cleaves the ester linkages between lignin and hemicellulose, which reduces the degree of polymerisation of cellulose and consequently the crystallinity of DDGS (Barman et al., 2012). This was shown in Figure 3-3c, where the expansion of internal surface area of pretreated DDGS structure was observed.

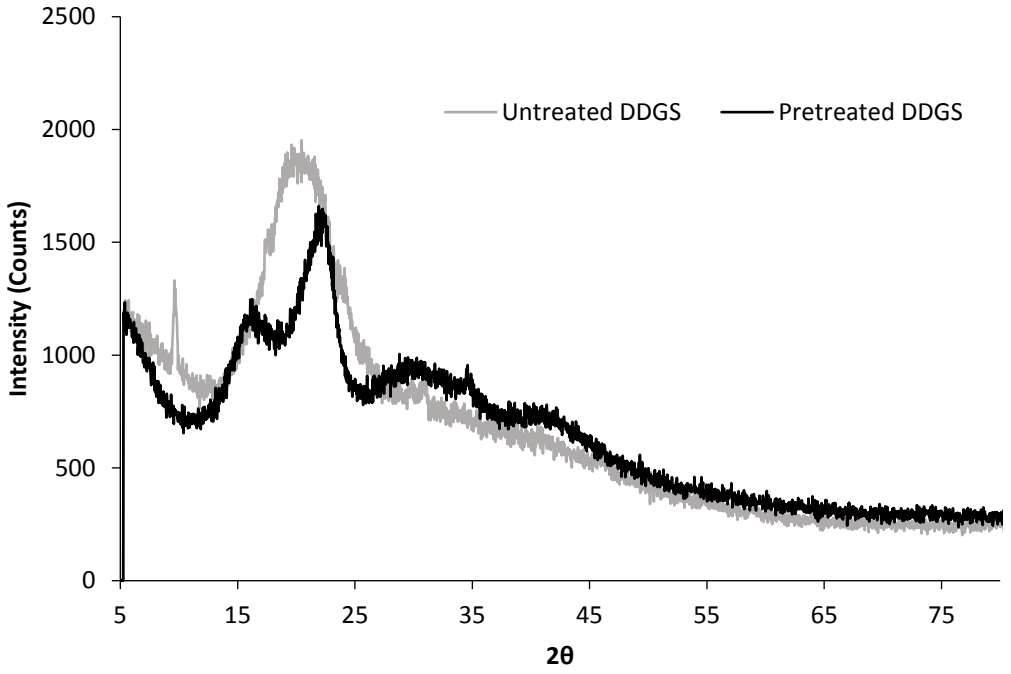


Figure 3-5: XRD pattern of untreated and pretreated (121 ºC, ~16 psi, 15 minutes, 5% NaOH) DDGS

Table 3-6: Crystalline and amorphous area of untreated and pretreated solids (121 ºC, ~16 psi, 15 minutes, 5% NaOH) DDGS*

Substrate	Untreated DDGS	Pretreated DDGS
Crystalline Area	9409.63	1338.27
Amorphous Area	14272.53	8846.83
Degree of crystallinity (X_c), %	**39.73**	**13.14**

* Data generated from TOPAS 2.1 software (Rietveld refinement method)

3.3.6 Overall mass balance of valorisation process scheme

Figure 3-6 shows the overall process design based on a alkaline pretreatment process of DDGS with 5% NaOH at 121^{o}C (~16 psi) for 15 minutes (the best pretreatment condition for obtaining maximum carbohydrate content in the solid fraction), followed by enzymatic hydrolysis of the residual solids using Accellerase® 1500, and the mass balances of the key components (sugar monomers reflecting cellulose and hemicellulose, proteins) in the solid and liquid fractions. Untreated DDGS contained ~11% glucose reflecting the presence of cellulose (the assumption is that the level of β-glucan is zero), and ~13.7% xylose and ~6.6% from arabinose, reflecting the presence of ~20.3% hemicellulose. DDGS was then subjected to alkaline pretreatment after which ~45% of glucose, ~17% of xylose and ~15% of arabinose remained in the recovered solids compared to the starting DDGS material. The majority hemicellulose, i.e. ~77% of xylose and ~100% of arabinose (although the discrepancy in the arabinose mass balance is noted) and the majority of the protein (~79%) were extracted into the liquid fraction. The enzymatic hydrolysis (using Accellerase® 1500) of the pretreated DDGS solids led to the recovery of ~87% of glucose and ~92% of xylose, leading to the production of a glucose-rich medium. Such medium can be used as a fermentation medium for the production of a range of platform or speciality chemicals with high market potential, such as lactic acid and succinic acid, in various industrial sectors (e.g. food, plastics, packaging and chemical sectors). The hemicellulose and protein rich liquid fraction could be further explored. For instance, the liquid fraction can be subjected to ultrafiltration and the isolated protein can be used as starting material for biodegradable films and bioplastics production or as precursor for chemical synthesis (Brehmer et al., 2008; Chatzifragkou et al., 2016; Jones et al., 2015). On the other hand, research on the use of hemicelluloses

as materials for edible coating, films or food packaging applications has been reported (Hansen & Plackett, 2008; Xiang et al., 2014) and has led to commercial applications. For example, Xylophane AB have successfully marketed their xylan-based packaging material (Chatzifragkou et al., 2015). Overall, the proposed process scheme recovers the majority of the key DDGS components (cellulose, hemicellulose, proteins) in an efficient manner with relatively low losses and provides a viable approach for the valorisation of DDGS.

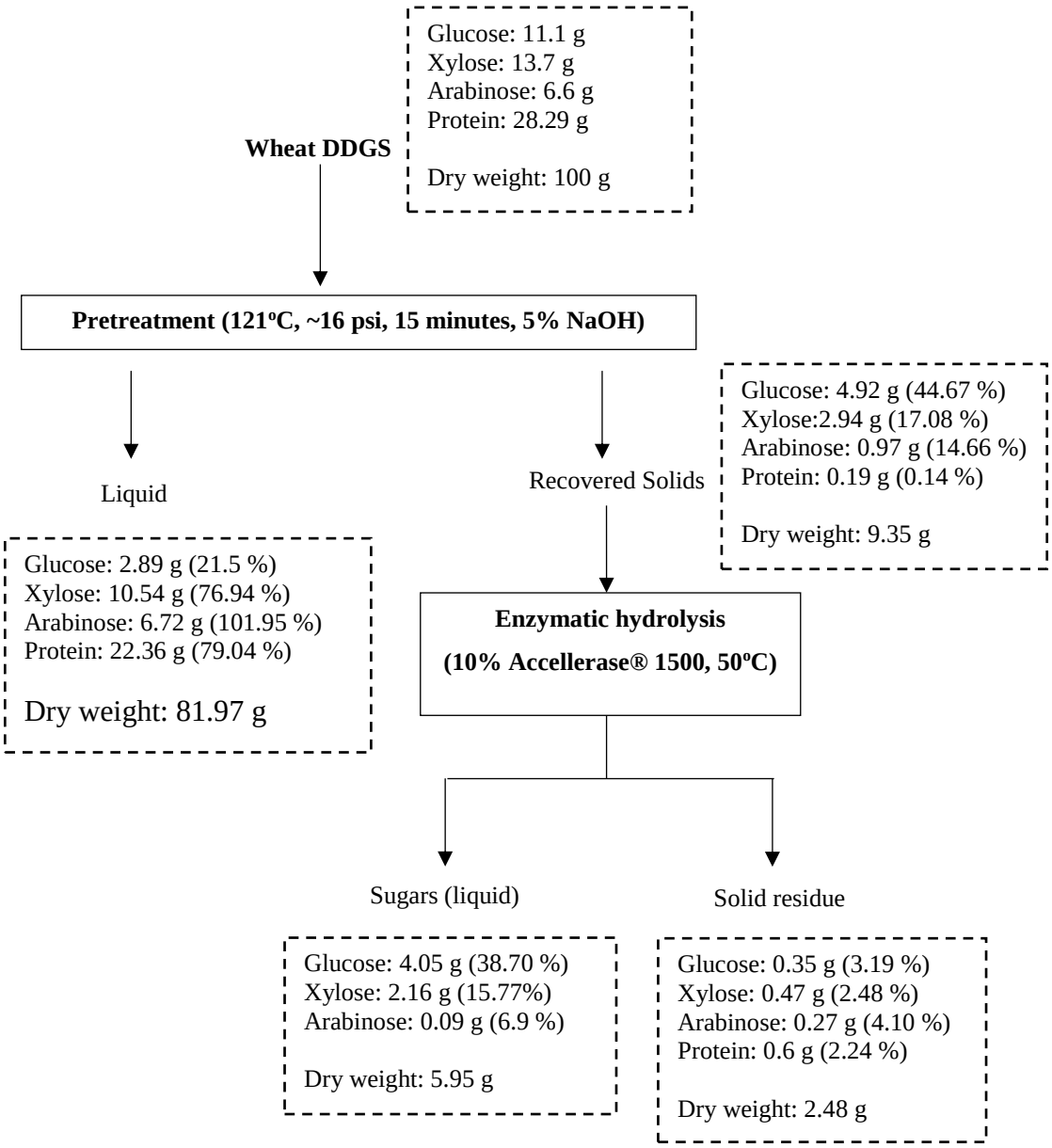


Figure 3-6: Process scheme and mass balances for the valorisation of DDGS.

3.4 Conclusions

A cellulose-rich solid material was generated from DDGS by alkaline treatment at high temperature, containing ~88% (w/w) of total sugars with the majority attributed to the presence of cellulose (~53%). The pretreated DDGS solids exhibited significant digestibility by a commercial cellulose enzyme, leading to 3.6 fold higher glucose concentration compared to untreated DDGS, which can be attributed to morphological and structural changes. Approximately 83% of the hemicellulose and 79% of the protein present in untreated DDGS were removed during alkaline pretreatment into the liquid fraction. Mass balance analysis of the proposed DDGS valorisation scheme demonstrated that the major DDGS components (cellulose, hemicellulose, proteins) were recovered in the solid and liquid process fractions in an efficient manner.

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Chapter 4 - Microbial production of lactic acid from wheat Dried Distiller's Grains with Solubles

Abstract

D-Lactic acid production is gaining increasing attention due to the thermostable properties of its polymer, poly-D-lactic acid (PDLA). In this study, three different lactic acid producers, namely *Lactobacillus coryniformis* subsp. *torquens*, *Lactobacillus pentosus* and *Lactobacillus brevis* were evaluated for their ability to produce lactic acid using Dried Distiller's Grains with Solubles (DDGS) hydrolysate as carbon source. DDGS was first subjected to alkaline pretreatment with sodium hydroxide to remove hemicellulose that act as a barrier for cellulase activity and the generated carbohydrate-rich solids were then subjected to enzymatic hydrolysis with Accellerase® 1500. Among the three strains, *L. coryniformis* and *L. pentosus* exhibited better cell growth with a maximum lactic acid production of 24.0 and 25.0 g/l, respectively, whereas only small amount of lactic acid (1.9 g/l) was produced by *L. brevis*. Co-fermentation of *L. coryniformis* and *L. pentosus* resulted in higher lactic acid production (28.5 g/l when simultaneously added) compared to single strain fermentation, however, lower optical purity of D-lactic acid was obtained (51.1%). When comparing separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) using *L. coryniformis* on its own, the later method demonstrated higher lactic acid production (27.9 g/l, 99.9% optical purity of D-lactic acid), with a very high conversion yield

(84.5%). In addition, the effect of increasing the DDGS loading concentration was investigated via a “fed-batch” SSF approach. Although lactic acid production increased to 38.1 g/l, the conversion yield decreased to 70%. In conclusion, the SSF approach proved to be an efficient strategy for the production of D-lactic acid from DDGS as it reduced the overall processing time and yielding high D-lactic acid concentrations.

Keywords: D-lactic acid, DDGS, Simultaneous Saccharification and Fermentation (SSF), Separate Hydrolysis and Fermentation (SHF), co-fermentation

4.1 Introduction

Lactic acid ($C_3H_6O_3$) is considered one of the most useful chemical products and has attracted a great attention worldwide due to its widespread applications in food, chemical, cosmetic, textile and pharmaceutical sectors. It has also emerged into the bioplastics industry, where lactic acid serves as the building block for polylactic acid (PLA) synthesis. PLA is a biodegradable polymer that holds great potential in replacing petroleum-based polymers. Because of its degradability and biocompatibility, PLA is extensively used in biomedical fields as a surgical suture, drug-delivery material and bone fixation material (Conn et al., 1995; Nampoothiri et al., 2010). In addition, PLA received a Generally Recognized as Safe (GRAS) status from the US Food and Drug Administration (FDA) in 2002, which allowed the expansion of its applications within the food industry. PLA can be utilised as a food contact-material, e.g. for the production of cutlery, cups, plates and containers, or as food packaging material (Conn et al., 1995; Jamshidian et al., 2010). At the moment, the PLA market demand accounts for 11.4% of the total bioplastic production worldwide, and is equal to approximately 180,000 metric tonnes per year. In addition, the PLA demand is estimated to grow significantly, by 28% per year until 2025, as a result of the expansion of the bioplastics market (Aeschelmann & Carus, 2015; Cellulac Ltd., 2015).

PLA can be manufactured by utilising either the D- or L- forms of lactic acid, or its racemic mixture (Yañez et al., 2003). Poly-L-lactic acid (PLLA) polymer has a low melting point (180 ^{o}C) and low crystallisation ability (Xu et al., 2006). On the other hand, polymer blends of purified PLLA and purified poly-D-lactic acid (PDLA) produce racemic crystals called stereo-complexes which have higher melting point (230 ^{o}C) and

distortion temperatures, and as such, offer significant advantages for a number of applications such as high heat packaging materials (Ikada et al., 1987; Ishida et al., 2006; Kim et al., 2014; Nguyen et al., 2013a). The optical purity of lactic acid is one of the crucial factors towards the production of highly crystalline PLA. To this end, the microbial lactic acid production route offers advantages compared to the chemical production route, as specific isomers of D- or L- lactic acid can be produced depending on the selected bacterial strain.

Over 90% of the commercially produced lactic acid is derived from microbial fermentation utilising glucose, sucrose or corn starch as carbon sources (Buyondo & Liu, 2011). However, the relatively high cost of pure sugars has driven research on industrial fermentation towards the use of alternative resources, which can be obtained through the valorisation of cheap, renewable agricultural biomass (Abdel-Rahman et al., 2013; Buyondo & Liu, 2011; Dusselier et al., 2013; Van Wouwe et al., 2016). Specifically, agricultural residues such as corn stover (Bai et al., 2015; Zhang & Vadlani, 2013), rice bran (Tanaka et al., 2006), peanut meal (Wang et al., 2011), broken rice (Nakano et al., 2012) and unpolished rice (Lu et al., 2009) have been studied as potential carbon sources for lactic acid production. However, the hydrolysis of biomass materials that are rich in cellulose/hemicellulose produces a mixture of sugar monomers such as glucose, xylose, mannose, arabinose and galactose. Most of the homofermentative D-lactic acid producers (i.e. *Lactobacillus* sp. and *Sporolactobacillus* sp.) are unable to ferment sugars other than glucose (Bai et al., 2015; Zhang & Vadlani, 2013). A potential approach to maximise the utilisation of pentose sugars within a fermentation feedstock for lactic acid production is the simultaneous use of pentose-fermenting microorganisms together with glucose-fermenting ones (Taniguchi et al., 2004).

Agricultural biomass needs to be pretreated either chemically (acid or alkaline) or be enzymatically hydrolysed in order to be converted into fermentable sugars. The enzymatic approach is preferred to chemical hydrolysis, as the reactions are more specific and less hazardous (Binod et al., 2011). The overall production process can consist either of two steps operated sequentially, i.e. Separate Hydrolysis and Fermentation (SHF) or concurrently, i.e. Simultaneous Saccharification and Fermentation (SSF). In SHF, enzymatic hydrolysis and fermentation take place separately, and each process is conducted at its optimal conditions. The major disadvantage of SHF is that the accumulation of sugars after hydrolysis can reduce the activity of enzymes, particularly cellulase and β-glucosidase, by 60 – 75% (Binod et al., 2011; Jönsson et al., 2013; Limayem & Ricke, 2012). In contrast, in SSF, enzymatic hydrolysis and fermentation process are carried out simultaneously, allowing for the direct assimilation of monomeric sugars by the microbial cells, thus reducing the risk of sugar accumulation in the medium. Additional advantages of SSF include shorter production times for the targeted product, higher production yields (% g product / g of substrate) and lower production costs due to the lower amount of energy and labor required (Binod et al., 2011; Marques et al., 2008; Zhang et al., 2007).

Dried Distillers Grains with Solubles (DDGS) is a by-product of bioethanol production from wheat or corn, as well as of the distillery industry, and is currently used as animal feed due to its high protein (29 - 38%) and fibre content (40 - 46%) (Chatzifragkou et al., 2016; Villegas-Torres et al., 2015; Widyaratne & Zijlstra, 2007). However, DDGS has to compete with other protein sources such as soybean meal and rapeseed meal within the animal feed market which are considered of a better quality (Villegas-Torres et al., 2015). Moreover, the possible high levels of mycotoxins (3-fold compared to the original sources, i.e. wheat or corn grains) in DDGS have become a

concern for the farming industry (Zhang & Caupert, 2012). In term of fibre composition, DDGS is primarily composed of cellulose and hemicellulose, which mainly consist of the monosaccharides glucose, xylose and arabinose as the main sugars. As DDGS is also characterised by a high fibre content, it could potentially be used as an alternative carbon source for lactic acid fermentation (Chatzifragkou et al., 2015). In commercial lactic acid production, glucose and corn starches have been widely used as substrates for fermentation. However, this is economically unfavourable as pure sugars have a higher economic value than the lactic acid produced (Buyondo & Liu, 2011).

The main objective of this study was to develop a fermentation process for the production of optically pure D-lactic acid from wheat DDGS hydrolysates using *Lactobacillus coryniformis* subsp. *torquens*. Two fermentation approaches, SHF and SSF, were evaluated in terms of lactic acid yield, productivity and purity. In addition, a mixed culture fermentation which included pentose-fermenting microorganisms, namely *Lactobacillus pentosus* and *Lactobacillus brevis*, was also investigated, aiming at maximising the utilisation of the available pentose sugars present in the DDGS hydrolysate.

4.2 Materials and Methods

4.2.1 Microorganisms and culture conditions

Lactobacillus coryniformis subsp. *torquens* (DSM 20004), *Lactobacillus pentosus* (DSM 20314) and *Lactobacillus brevis* (DSM 20054) were used in this study and were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The stock of bacterial cultures was kept in mixtures of commercial MRS broth and glycerol and stored at -80 ^{o}C. Bacterial strains were cultivated in 250 ml Erlenmeyer flasks containing 50 ml MRS broth at 37 ^{o}C and 150 rpm agitation speed for either 18 h (*L. coryniformis* and *L. brevis*) or 15 h (*L. pentosus*) and were subsequently used as inoculum. The cell growth of each bacterial strain was monitored by optical density (OD) using a Biomate 3 UV/VIS Spectrophotometer (Thermo Spectronic, Rochester, NY) at 600 nm wavelength.

4.2.2 Raw materials and enzymes

Dried Distillers Grains with Solubles (DDGS) was supplied from a bioethanol plant (Vivergo, Yorkshire, UK). It was ground using a coffee grinder (DeLonghi, Australia) into a fine powder (particle smaller than 0.85 mm) and stored at room temperature (25 ^{o}C) prior to alkaline pretreatment. The commercial cellulase mixture Accellerase® 1500 was kindly provided by Danisco US Inc. (Genencor, Leiden, Netherlands); it consisted of multiple enzyme activities including endoglucanase (2200 – 2800 CMC U/g), β- glucosidase (450 - 775 pNPG U/g), exoglucanase and hemicellulase. The enzymes were kept at 4 ^{o}C before use.

4.2.3 Growth of *Lactobacillus* sp. in semi-defined media

The microbial growth of *L. coryniformis*, *L. pentosus* and *L. brevis* was initially studied in 100 ml fermentation vessel using containing 50 ml of deMan Rogosa Sharpe (MRS) as the basal medium [casein peptone, 10.0 g/l; meat extract, 10.0 g/l; yeast extract, 5.0 g/l; tween 80, 1.0 g/l; potassium phosphate dibasic (K_2HPO_4), 2.0 g/l; sodium acetate (CH_3COONa) 5.0 g/l; di-ammonium hydrogen citrate ($C_6H_6O_7$), 2.0 g/l; magnesium sulphate heptahydrate ($MgSO_4$ x $7H_2O$), 0.2 g/l; and manganese (ll) sulphate monohydrate (MnSO4 x H2O), 0.05 g/l]. The fermentation system consisted of a 100 ml glass vessel connected to a temperature controlled water bath (GD 120, Grant, Cambridge) set at 37 ^oC, a FerMac 260 pH controller (Electrolab, Hertfordshire) and a Stuart stirrer; no nitrogen or air addition was included in the system. Each strain was inoculated at a similar starting OD (calculated to be OD ~ 0.05) in the fermentation media. Three fermentation runs were conducted: (i) with the pH controlled pH at 5, (ii) with the pH controlled pH at 6, and (iii) with uncontrolled pH. The maximum specific growth rate (μ_{max}) of each strain at these conditions was calculated from the slope of the plot depicting the natural logarithm (ln) of the OD against time. Based on the best pH for each *Lactobacillus* sp. (obtained above), the strains were then cultivated at 37 ^oC in MRS basal medium supplemented with a single carbon source (20 g/l of glucose, xylose or arabinose) as well as mixed sugars (10 g/l glucose and 10 g/l xylose). For all fermentations, samples were taken at regular time intervals and analysed for cell growth by OD measurement, lactic acid and acetic acid concertation, residual sugar, nitrogen content and optical purity of D-lactic acid (%), as described in Section 4.2.8.

4.2.4 Alkaline pretreatment of DDGS

DDGS was pretreated with 5% (w/v) NaOH at 121 ^{o}C (~16 psi) for 15 minutes at 10% (w/v) DDGS/NaOH loading as described in **Chapter 3**. After pretreatment, the mixture was cooled down to room temperature and centrifuged at 17,105 *x g* (Heraeus Multifuge X3R, Thermo Fisher, USA) for 20 minutes at 4 ^{o}C. The obtained solids were extensively washed with distilled water until the pH reached around 8, and the pH neutralised with HCl (6 M) to a final pH between 5 - 5.5. The solids were frozen (-20 ^{o}C), freeze-dried (VisTis Sentry 2.0, Warminster, PA) and stored in a closed container at room temperature (25 ^{o}C) until further use.

4.2.5 Separate hydrolysis and fermentation (SHF) of DDGS hydrolysates

Alkaline pretreated DDGS solids (3.3 g) were hydrolysed with Accellerase® 1500 (5 ml) at a ratio of 1 ml enzyme : 0.33 g cellulose; the cellulose content of DDGS pretreated solids was calculated according to the method described in **Chapter 3** and was approximately 50% w/w. The enzymatic hydrolysis was conducted at 50 ^{o}C for 24 h with 300 rpm speed in a shaking incubator (SciQuip, Shropshire, UK), followed by heat inactivation at 95 ^{o}C for 10 minutes. The mixture was centrifuged at 17,105 *x g* for 20 minutes (4 ^{o}C) and the supernatant was collected and filter sterilised using 0.22 µm sterile vacuum filter (EMD Milipore Stericup^{TM}). 5 ml of sterile concentrated yeast extract (200 g/l) were then added aseptically into the 100 ml fermentation vessel (final concentration of 20 g/l). Each strain (*L. coryniformis*, *L. brevis* and *L. pentosus*) was inoculated into 50 ml of DDGS hydrolysate at a similar starting OD (~0.05). Lactic acid fermentation was carried out at 37 ^{o}C for 54 h; no nitrogen or air passed through the fermentation medium. The pH of the cultures was maintained at 6 for *L. coryniformis* and *L. pentosus* and pH 5

for *L. brevis* through the addition of NaOH (2 M). Samples were taken at regular time intervals and kept at -20 ^{o}C until further analysis.

4.2.6 Co-fermentation of DDGS hydrolysate by *L. coryniformis* and *L. pentosus*

In the co-fermentation experiments, *L. coryniformis* and *L. pentosus* were both added together into 50 ml of DDGS hydrolysate (supplemented with yeast extract at a final concentration of 20 g/l) in a 100 ml fermentation vessel, using the fermentation system described in Section 4.2.3. The fermentations were carried out at 37 ^{o}C, with the pH set at 6. Appropriate volumes of the pre-cultures were added so that the initial OD of each strain was ~0.05. In the sequential co-fermentation experiments, *L. coryniformis* was firstly grown and *L. pentosus* was inoculated after 24 h of fermentation. Samples were taken at regular time intervals and stored at -20 ^{o}C for further analysis. The cell growth of *L. coryniformis* and *L. pentosus* was monitored by spectrophotometric method ($OD_{600\ nm}$) and by viable cell counts, expressed as colony-forming units per millilitre of culture (CFU/ml). In the case of the latter, collected samples were serially diluted in phosphate buffer saline (PBS) and were spread onto: (i) MRS agar medium containing 20 g/l glucose for the quantification of *L. coryniformis*, and (ii) modified MRS agar medium, containing 20 g/l xylose for the quantification of *L. pentosus*.

4.2.7 Simultaneous saccharification and fermentation (SSF) of DDGS hydrolysate

The SSF experiments were conducted in a 100 ml fermentation vessel using the fermentation system described in Section 4.2.3; the pH was controlled at pH 5 at the temperature at 37 ^{o}C. 3.3 g of alkaline pretreated DDGS were steam-sterilised inside the fermentation vessel by autoclaving the vessel at 121 ^{o}C for 15 minutes. After cooling,

sterile distilled water and yeast extract (20 g/l) were added into the fermentation vessel. The SSF process was initiated by the addition of Accellerase® 1500 into the DDGS hydrolysate at a loading rate of 1 : 0.33 (ml enzyme : g cellulose), followed by inoculation with *L. coryniformis* at a starting OD of approximately 0.05. In some cases, 1.1 g (11 g/l glucose) and 2.2 g (22 g/l glucose) of pretreated DDGS were aseptically added, with the aid of a portable Bunsen burner, when the glucose in the fermentation medium was depleted ("fed-batch" SSF); this was approximately after 24 h of fermentation. For all SSF experiments, samples were taken at regular time intervals and kept at -20 ^{o}C for further analysis.

4.2.7.1 2L Bioreactor studies

The SSF process was also studied in a 2L stirred tank bioreactor (Biostat B, Sartorious, Germany) with 1.5 L working volume. 99 g of alkaline pretreated DDGS solids were added into the bioreactor and steam-sterilised by autoclaving at 121 ^{o}C for 30 minutes. After cooling the bioreactor, 1500 ml of sterile distilled water were added plus yeast extract (final concentration 20 g/l). Accellerase® 1500 was added at a loading rate of 1 : 0.33 (ml enzyme : g cellulose), followed by the addition of *L. coryniformis* inoculum at a starting OD of ~0.05. The fermentation was carried out at 37 ^{o}C with an initial agitation speed of 250 rpm. The minimum dissolved oxygen (DO) level was kept at 20% (through a cascade with the stirrer speed) and the pH was maintained at 5 with 5M NaOH and HCl. Antifoam 204 (10%, v/v, Sigma) was added to prevent foaming during fermentation.

4.2.8 Analytical methods

Sugars (xylotriose, xylobiose, glucose, xylose and arabinose) and organic acids (lactic acid and acetic acid) were analysed by high performance liquid chromatography (HPLC) in an Agilent Infinity 1260 system (Agilent Technologies, USA) equipped with an Aminex HPX-87H column (Bio-rad, Hercules, CA) at 0.6 ml/min flow rate, with 5 mM H_2SO_4 as mobile phase. The temperature of the column was set at 65 ^oC and the sugars and organic acids were detected using a refractive index detector. The presence of D- and L-lactic acid in the fermentation broth was determined by using the D- and L-lactate dehydrogenase enzyme kit (K-DLATE 07/14, Megazyme, Ireland). The optical purity (%) of D-lactic acid was calculated as follows (Prasad et al., 2014; Wang et al., 2011):

$$Optical\ purity\ (\%) = \frac{D - lactic\ acid\ \left(\frac{g}{l}\right)}{D - lactic\ acid\ \left(\frac{g}{l}\right) + L - lactic\ acid\ \left(\frac{g}{l}\right)} \times 100\ \%$$

The nitrogen consumption during fermentation was determined by the Free Amino Nitrogen (FAN) method as described by Lie (1973) with some modifications. 0.5 ml of diluted sample was mixed with 0.25 ml of colour reagent (49.71 g of $Na_2HPO_4 \cdot 2H_2O$, 5 g of ninhydrin, 3 g of fructose and ~ 40 g of KH_2PO_4 dissolved in 1 l of distilled water; pH 6.6 – 6.8) in a 2 ml Eppendorf tube. The mixture was heated at 100 ^oC in a thermal block (Grant, Cambridge) for exactly 16 minutes and immediately cooled in an ice bath. 1.5 ml of dilution reagent (2 g potassium iodate, KIO_3, in 616 ml distilled water and 384 ml 96% ethanol) was added and the free amino nitrogen content was measured at 570

nm. A calibration curve was constructed using glycine as standard at several concentrations (0.25 – 2 mg/l).

4.3 Results and Discussion

4.3.1 Effect of growth conditions on *Lactobacillus* sp. fermentation

The results from the growth experiments of *L. coryniformis*, *L. pentosus* and *L. brevis* in MRS medium with glucose as the carbon source under pH controlled (pH 5 and 6) and non pH controlled conditions are shown in Table 4-1. The optimum pH value for the cell growth of *Lactobacillus* sp., including the species used in this work is normally between 5.0 and 7.0 (Garde et al., 2002; Kim et al., 2009; Slavica et al., 2015). The results are in line with the above as good cell growth was obtained in the pH values tested. In the uncontrolled pH culture, the pH dropped to ~ 4.1 (*L. corynifromis* and *L. brevis*) and 3.8 (*L. pentosus*) after 24 h of growth, and the maximum specific growth rate (μ_{max}) for *L. coryniformis*, *L. pentosus* and *L. brevis* were 0.30, 0.31 and 0.29 h^{-1}, respectively. When the pH of the medium was controlled, the μ_{max} increased to 0.36 h^{-1} for *L. coryniformis* and 0.38 h^{-1} for *L. pentosus* at both pH 5 and 6, indicating improved microbial growth. For *L. brevis*, the μ_{max} was significantly higher at pH 5 than at pH 6 (0.37 h^{-1} and 0.22 h^{-1}, respectively).

Another factor that plays an important role for lactic acid fermentation is the type of carbon source used. Glucose, xylose and arabinose (at 20 g/l) were tested as the carbon source in a MRS based medium, as these sugars are most likely present in DDGS

hydrolysates. Among the sugars, *L. coryniformis* only consumed glucose, *L. pentosus* and *L. brevis* were able to consume glucose and xylose efficiently, whereas a small amount of arabinose was utilised only by *L. pentosus* (< 1 g/l arabinose). *L. coryniformis* is a homo-lactic acid producer which utilises the Embden-Meyerhof pathway (EMP) to convert one molecule of glucose into two molecules of lactic acid (Nguyen et al., 2012; Slavica et al., 2015). *L. pentosus* utilized the EMP pathway when glucose was used as the carbon source, with a conversion yield ($Y_{Lac/S}$, w/w) of glucose to lactic acid of 99.8%. However, when xylose was used as the carbon source the conversion yield of xylose to lactic acid was reduced to 59.0% with acetic acid being produced as a by-product. This finding indicates that *L. pentosus* utilised the Phosphoketolase pathway (PKP) in the presence of xylose, where from a stoichiometric point of view, one molecule of lactic acid is produced from xylose along with acetic acid, ethanol, carbon dioxide or formic acid. However, the proportion of each product depends on several factors such as aeration, initial sugar concentration and the presence of other proton and electron acceptors (i.e. oxygen) (Bustos et al., 2005; Buyondo & Liu, 2011).

In the case of *L. brevis*, the yields of lactic acid from glucose and xylose were 62.1% and 54.1%, respectively, with acetic acid and ethanol were also produced as co-products. This suggests that *L. brevis* employed the PKP pathway in the presence of both glucose and xylose. According to Abdel-Rahman et al. (2011), the maximum theoretical yield of lactic acid from the PKP pathway is 1 mol / mol of sugar, which is in line with the findings in the present study. When *L. pentosus* and *L. brevis* were grown in mixtures of glucose and xylose, the strains exhibited different consumption patterns. For *L. pentosus*, the utilisation of xylose only started after glucose depletion (data not shown) as two both pathways, i.e. EMP and PKP were most likely used, whereas simultaneous

glucose and xylose utilisation occurred for *L. brevis*, indicating that the PKP pathway was used for both glucose and xylose (Kim et al., 2009; Spector, 2009).

Lactobacillus species can produce L-or D- lactic acid, depending on the type of lactate dehydrogenase (nLDH) present in their cluster (*ldhL* or *ldhD*, respectively) (Garvie, 1980; Gu et al., 2014; Zheng et al., 2012). In the case where both D- and L-lactic acid are produced from a single strain, this is likely due to the presence of both *ldhD* and *ldhL* (Salminen et al., 2004; Wright & Axelsson, 2012). In this study, only *L. coryniformis* produced D-lactic acid exclusively, whereas *L. pentosus* and *L. brevis* produced a mixture of D- and L- lactic acid with 62.3 and 43.3% optical purity, respectively, which are in line with the reported previous studies (Garvie, 1967; Nguyen et al., 2013a; Zanoni et al., 1987) .

Table 4-1: Growth parameters of three different lactic acid producers in semi-defined media

Parameter		*L. coryniformis*	*L. brevis*	*L. pentosus*
Optical density ($OD_{600\ nm}$)*				
	Uncontrolled pH	4.5 ± 0.06	4.7 ± 0.25	5.9 ± 0.13
	pH 6	6.4 ± 0.16	2.2 ± 0.30	6.6 ± 0.47
	pH 5	7.0 ± 0.50	7.0 ± 0.58	7.7 ± 0.63
Lactic acid (g/l)*				
	Uncontrolled pH	12.5 ± 0.10	9.1 ± 0.09	14.3 ± 0.02
	pH 6	15.6 ± 1.85	9.5 ± 0.08	15.6 ± 0.01
	pH 5	15.7 ± 0.47	11.4 ± 0.01	15.6 ± 0.31
Maximum specific growth rate (μ_{max}, h^{-1})*				
	Uncontrolled pH	0.30	0.29	0.31
	pH 6	0.36	0.22	0.38
	pH 5	0.36	0.37	0.38
Lactic acid (g/l) **				
	Glucose (20 g/l)	19.7 ± 0.02	12.8 ± 0.06	18.87 ± 0.16
	Xylose (20 g/l	-	11.4 ± 0.02	11.8 ± 0.00
	Arabinose (20 g/l)	-	-	-
	Glucose and xylose (10 g/l each)	10.5 ± 0.97	12.7 ± 0.16	14.11 ± 0.02
[a]$Y_{Lac/S}$ (%, w/w)**				
	Glucose (20 g/l)	99.9	62.1	99.8
	Xylose (20 g/l	-	54.1	59.0
	Arabinose (20 g/l)	-	-	-
	Glucose and xylose (10 g/l each)	-	59.9	67.9
Co-products		Acetic Acid	Ethanol, Acetic Acid	Acetic Acid
Optical purity of D-lactic acid (%)		99.9	43.3	62.3

*Experiment was performed using a MRS based medium at 37 ºC (~ 16 g/l glucose initial concentration) at 37 ºC for 24 h

**Experiment was performed using a MRS based medium at 37 ºC for 24 h, at pH 6: *L. coryniformis* and *L. pentosus*, pH 5: *L. brevis*

[a] $Y_{Lac/S}$ (%, w/w) = (g lactic acid produced/ g sugar consumed) x 100

4.3.2 Single strain fermentation by *Lactobacillus* strains of DDGS hydrolysates

Alkaline pretreated DDGS consisted of 52.6 g glucose, 25.0 g xylose, 10.3 g arabinose and 0.04 g protein per 100 g of dried material, as described in **Chapter 3**. In the first part of the work, alkaline treated DDGS solids were hydrolysed to simple sugars using the Accellerase® 1500 enzyme at 50 ^{o}C for 24 h. The sugar composition in the DDSG hydrolysate was glucose ~27.0 g/l, xylose ~6.1 g/l, xylobiose ~5.9 g/l, and arabinose ~0.8 g/l. 86.5% cellulose was successfully hydrolysed to glucose during hydrolysis. The hydrolysate was then used as fermentation medium for lactic acid production by *L. coryniformis*, *L. brevis* and *L. pentosus*. The fermentation pH for each strain was set based on the results presented in Table 4-1.

Figure 4-1 shows the fermentation characteristics (cell growth, nitrogen and sugar consumption, lactic acid production) of the three strains in the DDGS hydrolysate as a function of time. Among the three strains, *L. coryniformis* and *L. pentosus* grew very well, with highest OD values of 8.7 and 10.2, respectively, after 18 hours. The highest lactic acid production by *L. coryniformis* (24.0 g/l) was obtained after 18 hours, which was the time point at which almost all of the available glucose was depleted. The concentration of xylobiose, xylose and arabinose remained unchanged throughout the fermentation, which is in line with the results obtained in the semi-defined media (Table 4-1). An increase in acetic acid production was observed after glucose depletion, reaching 2.0 g/l after 30 hours fermentation. According to Yáñez et al. (2005) and Hofvendahl and Hahn–Hägerdal (2000), under glucose limitation, homofermentative bacteria tend to produce other by-products such as formic or acetic acid through alternative pyruvate catabolic pathways, whereas Slavica et al. (2015) also reported an increase in acetic acid when glucose was depleted from a MRS

fermentation medium. Interestingly, between 18 and 30 hours, the concentration of lactic acid slightly decreased to 22.9 g/l, most likely due to degradation of lactic acid to acetic acid. Although lactic acid bacteria, especially lactobacilli, are classified to homofermentative or heterofermentative according to their ability to produce lactic acid through the EMP pathway, many are able to degrade lactic acid, especially if oxygen is available as an electron acceptor. The lactic acid that is initially formed from the EMP pathway can be converted to acetic acid after glucose depletion under aerobic conditions. This has been reported for *L. brevis* (Guo et al., 2017) and *L. plantarum* (Quatravaux et al., 2006), although no works have been conducted with *L. coryniformis*.

In the case of *L. pentosus*, the highest concentration of lactic acid, i.e. 24.9 g/l, was also observed after 18 hours fermentation. The cell growth of *L. pentosus* was faster compared to *L. coryniformis*, as demonstrated by the higher OD (9.5) and lactic acid (24.4 g/l) after 12 hours of fermentation, compared to an OD of 4.9 and a lactic acid concentration of 15.6 g/l for the latter. At the end of the fermentation, the optical purity of the D- lactic acid produced was 51.01%, as *L. pentosus* produced both D- and L- lactic acid, which is in line with the literature (Zanoni et al., 1987). In terms of sugar consumption by *L. pentosus*, all the available glucose was utilised after 12 hours of fermentation. Both xylose and arabinose started to be consumed after around 8 hours and until the end of the fermentation (30 h), they were exhausted (~5.8 g/l of xylose and 0.8 g/l of arabinose). A very low increase in lactic acid production (~0.5 g/l) was observed after glucose was exhausted (12 - 30 h), and during this period acetic acid was the main product (3.2 g/l). The reduction in FAN concentration was clearly observed from 8 to 12 hours indicating the utilisation of the available nitrogen for cell multiplication.

L. brevis did not grow well in the DDGS hydrolysate, as at the end of the fermentation the OD was ~1.1, and the lactic and acetic acid concentrations were 1.9 g/l and 1.1 g/l respectively. Glucose was the only sugar utilised, at a small amount (3.5 g/l), while xylobiose, xylose and arabinose remained unused during the fermentation. The FAN concentration did not decrease substantially demonstrating also the lack of good cell growth. Several *Lactobacillus* species and strains are fastidious and require additional nutrients such as minerals, vitamins, fatty acids and emulsifiers to support their growth (Hayek & Ibrahim, 2013). This has been demonstrated for wheat straw hydrolysate, where the hydrolysate was supplemented with many of the components present in a typical MRS medium to support the growth of *L. brevis* (Garde et al., 2002).

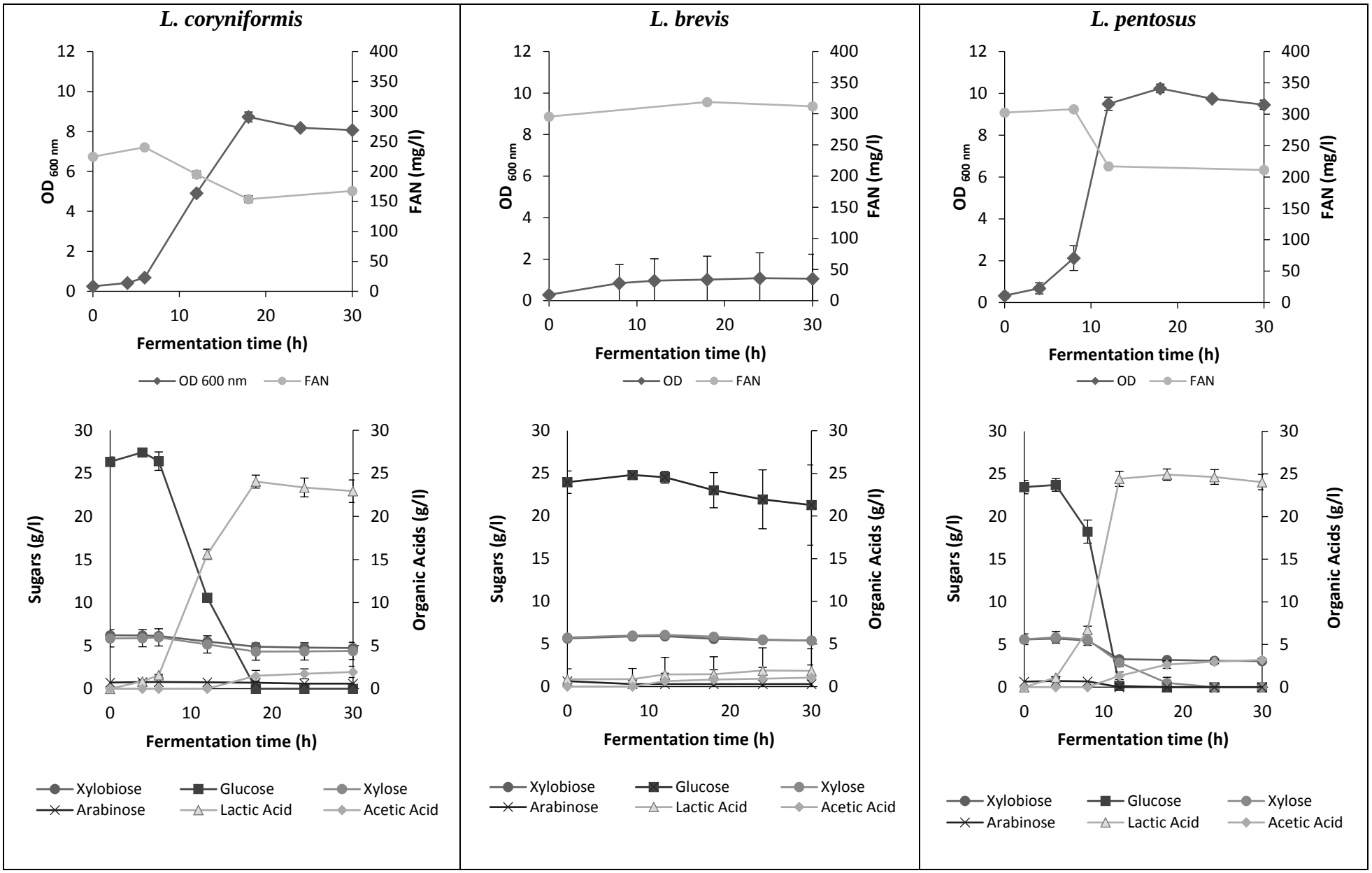

Figure 4-1: Fermentation profiles of *L. coryniformis*, *L. pentosus* and *L. brevis* in alkaline pretreated DDGS hydrolysate in a 100 ml bioreactor (37 ^{o}C, pH 6; *L. coryniformis* and *L, pentosus*, 5; *L. brevis*)

4.3.3 Co-fermentation by *Lactobacillus* strains of DDGS hydrolysates

The DDGS hydrolysate contained mixtures of hexose and pentose sugars. Most of lactobacilli selectively utilise hexose (glucose) over pentose (xylose and arabinose) sugars during growth. To overcome this barrier, co-fermentation of two *Lactobacillus* species that consume hexose and pentose sugars, respectively, is often an approach taken. More specifically, the co-fermentation of *L. pentosus* and *L. brevis* in wheat straw hemicellulose (Garde et al., 2002), *L. rhamnosus* and *L. brevis* in corn stover hydrolysate, and *L. plantarum* and *L. brevis* in poplar hydrolysate (Zhang & Vadlani, 2015), have been investigated to increase lactic acid production from biomass hydrolysates. In all cases, higher lactic acid production (~ 35% higher) was observed when co-fermentation was conducted compared to single-strain fermentations.

In this study, the co-fermentation of *L. coryniformis* and *L. pentosus* in the DDGS hydrolysate was investigated, as *L. brevis* did not grow well in the DDGS hydrolysate (as shown in Section 4.3.2). Two different cultivation approaches were studied, including the simultaneous and sequential inoculation of the strains, and the fermentation results are shown in Figure 4-2. Both strains showed similar growth patterns in the simultaneous co-fermentation run. All sugars (glucose, xylose and arabinose) except xylobiose (3.2 g/l remained) were depleted after 18 hours. The highest lactic acid concentration was observed after 18 hours (28.5 g/l, 51.1% optical purity of D-lactic acid), corresponding to a 83.3% conversion yield, based on the total amount of the consumed sugars in the DDGS hydrolysate. In addition to lactic acid, acetic acid was also produced gradually, reaching 3.6 g/l after 30 hours of fermentation.

In the case of the sequential co-fermentation of *L. coryniformis* and *L. pentosus* in the DDGS hydrolysate, the highest lactic acid production (24.7 g/l, 99.9% optical purity of D- lactic acid) was observed before the addition of *L. pentosus* (24 hours), corresponding to a 91% conversion yield of glucose to D-lactic acid; 1.2 g/l of acetic acid were produced during this period. After addition of *L. pentosus*, a decrease in xylose and arabinose were observed (4.5 and 0.9 g/l, respectively) until the end of the fermentations (48 hours). During this period, the lactic acid concentration decreased to 21.9 g/l (58.4% optical purity of D-lactic acid) (at 48 hours), whereas the acetic acid concentration increased from 1.2 g/l (at 24 hours) to 5.8 g/l (at 48 hours). The reduction of optical purity of D- lactic acid might be due to: i) the slower production rate of lactic acid from pentose sugars compared to the degradation rate of lactic acid to acetic acid, or ii) the possibility of xylose being converted to acetic acid and carbon dioxide instead of lactic acid under nutrient limitation (Zhang et al., 2015). Similar results were reported for the sequential fermentation of *L. rhamnosus* and *L. brevis* (Cui et al., 2011) and *L. casei* and *Enterococcus casseliflavus* (Taniguchi et al., 2004) in mixed glucose and xylose media, where no increase in lactic acid concentration was observed during xylose consumption. When comparing simultaneous and sequential co-fermentation of *L. coryniformis* and *L. pentosus*, the former approach showed better lactic acid production and higher conversion yield from the alkaline pretreated DDGS hydrolysate.

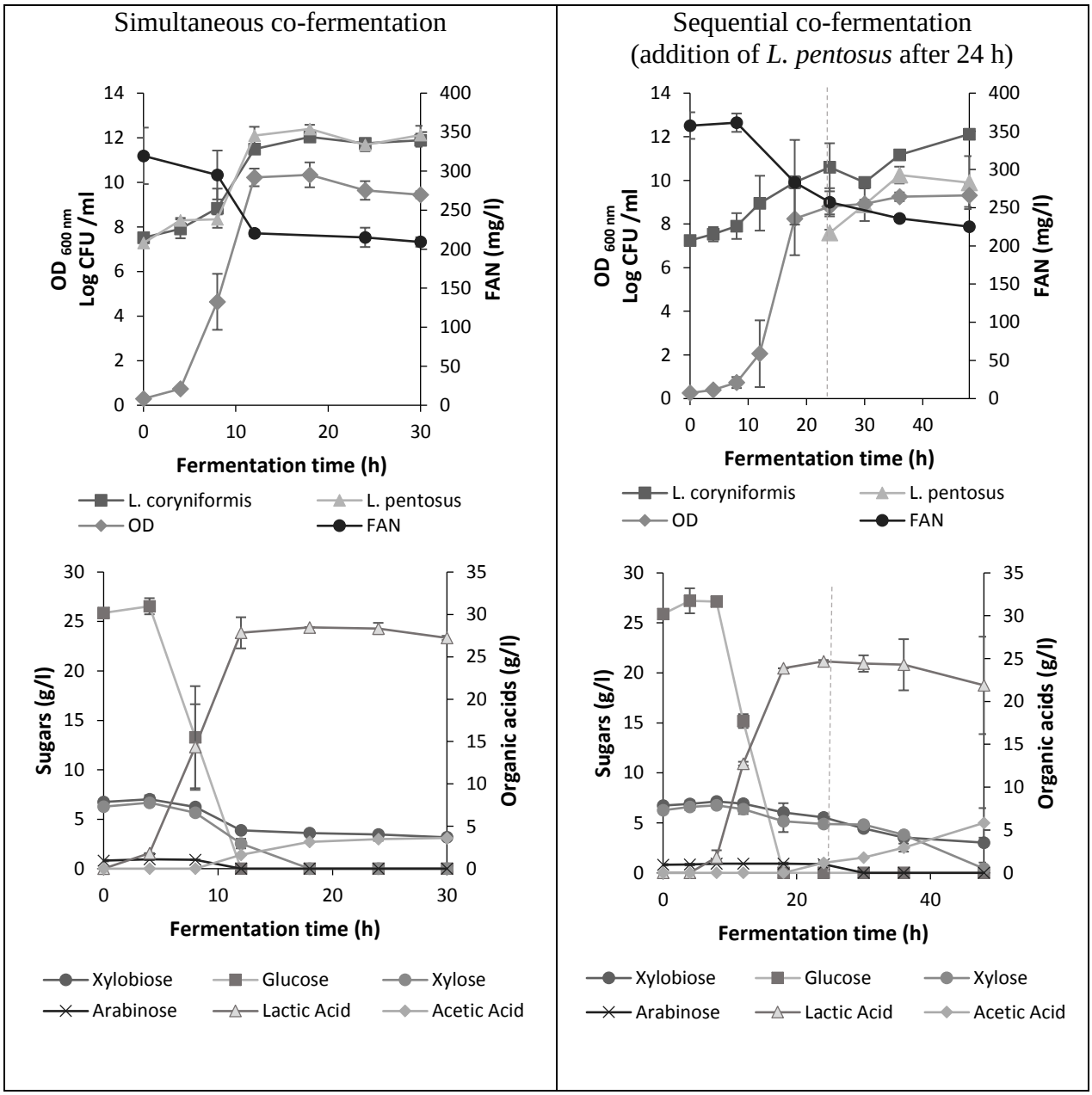

Figure 4-2: Co-fermentation of *L. coryniformis* and *L. pentosus* in DDGS hydrolysate in a 100 ml bioreactor (37 ^{o}C, pH 6)

4.3.4 Simultaneous saccharification and fermentation (SSF) of DDGS hydrolysate at 100 ml scale

In order for lactic acid to be used as a monomer for PLA synthesis, optical purity is one of the most important factors to be considered. The optical purity of D- or L- lactic acid has to be more than 90% in order to be used for the synthesis of crystalline PLA (Nguyen et al., 2013a; Niaounakis, 2015). In the work described previously, although the mixed fermentations of *L. coryniformis* and *L. pentosus* resulted in higher total lactic production compared to single cultures, the optical purity of D-lactic acid was only 51.1% as both D- and L- lactic acid were produced, the latter by *L. pentosus.* Therefore, subsequent experiments focused on the *L. coryniformis* strain, which is a D-lactic acid producer.

Figure 4-3 depicts the fermentation data for the SSF of *L. coryniformis* in alkaline pretreated DDGS medium. Accellerase® 1500 was used to hydrolyse DDGS during the SSF process. SSF offers several advantages compared to the sequential hydrolysis and fermentation (SHF), performed previously, as it combines enzymatic hydrolysis and fermentation in a single step process, resulting in reduced overall processing times and capital costs (Zhang et al., 2015). Moreover, SSF also reduces the potential of cellulase inhibition due to the high concentration of glucose in the hydrolysate (Nguyen et al., 2013a). However, compatible operating temperatures and pH for both processes (hydrolysis and fermentation) should be carefully selected to ensure high lactic acid production. Previous research works have reported that a temperature range from 37 to 40 ^{o}C and a pH around 5 is appropriate for the production of lactic acid production from biomass by lactobacilli. For example, lactic acid was produced via a SSF approach from cassava bagasse (John et al.,

2006) and from rice bran by *L. delbrueckii* (Tanaka et al., 2006), and from *curcuma longa* (tumeric) biomass of by *L. paracasei* and *L. coryniformis* (Nguyen et al., 2013b).

In this study, the SSF process was carried out in 100 ml bioreactor containing 33 g/l of glucose from alkaline pretreated DDGS at 37 ^{o}C, with the pH being controlled at 5 throughout the process. During the first 6 hours ~ 68% of the cellulose present in the alkaline pretreated DDGS (22 g/l) was converted to glucose and was not utilised by *L. coryniformis*, as the strain was still in the lag phase; as a result, low production of lactic acid (0.6 g/l) was detected during this period. *L. coryniformis* started to consume glucose after 12 hours, with the highest lactic acid and viable cell concentrations obtained after 24 hours, i.e. 28 g/l of lactic acid (99.9% optical purity of D- lactic acid) and 9.8 CFU/ml, respectively after 24 hours (Figure 4-3a). Unlike SHF, in SSF no reduction in the lactic acid concentration was observed after glucose depletion (24 to 48 hours). This might be due to the action of the enzyme that was still actively converting the remaining traces of cellulose in the DDGS to glucose, albeit in very small amounts, which was most likely rapidly consumed by *L. coryniformis*. At the end, around 84.5% of the cellulose present in the pretreated DDGS was converted to lactic acid, demonstrating the efficient utilisation of DDGS during the SSF process.

In an attempt to increase lactic acid production, the effect of increasing the amount of pre-treated DDGS loading in the SFF process was investigated. However, increasing the substrate loading results in highly viscous suspensions, which reduces the efficiency of enzymatic hydrolysis (Triwahyuni et al., 2015). One way to overcome this problem is via multi-step feeding or "fed-batch" SSF. In this approach, additional cellulosic biomass substrate is added at a particular point during the process; as a result free water is liberated,

which reduces the viscosity and stiffness of the suspension (Elliston et al., 2013; Moldes et al., 2000). In this study, when the glucose concentration reached less than 0.5 g/l (at 24 hours), alkaline pretreated DDGS was added at two levels, 11 g/l glucose (Figure 4-3b) and 22 g/l glucose (Figure 4-3c); 34.0 g/l and 38.1 g/l of lactic acid (99.9% optical purity of D-lactic acid) were produced respectively, after 48 h of fermentation. However, a reduction in the conversion yield of glucose to lactic acid (76 % and 70%, respectively) was observed with the higher substrate loading. This might be due to inadequate stirring at the higher solid content, which resulted in insufficient mass transfer and thus reduced the adsorption capacity of cellulase to cellulose and the efficiency of the enzymatic digestion of DDGS solids (Triwahyuni et al., 2015; Varga et al., 2004).

A comparison of the fermentation characteristics obtained by employing different fermentation approaches is shown in Table 4-2. Overall, SSF demonstrated better fermentation characteristics compared to SHF, more specifically high D-lactic acid concentration (27.9 g/l), productivity (1.46 g/l/h), glucose conversion yield (84.5%) and D-lactic acid yield (42.3%) were observed when SSF process was employed. When DDGS solids were added using the SSF "fed-batch" approach, the D-lactic acid concentration increased up to ~38 g/l (when adding 22 g/l glucose from alkaline pretreated DDGS), but in this case the other fermentation characteristics decreased. On the other hand, the fermentation characteristics in the case of adding 11 g/l glucose during the SSF "fed-batch" approach were deemed overall more optimal and demonstrate the potential of using this approach at a commercial large scale operation.

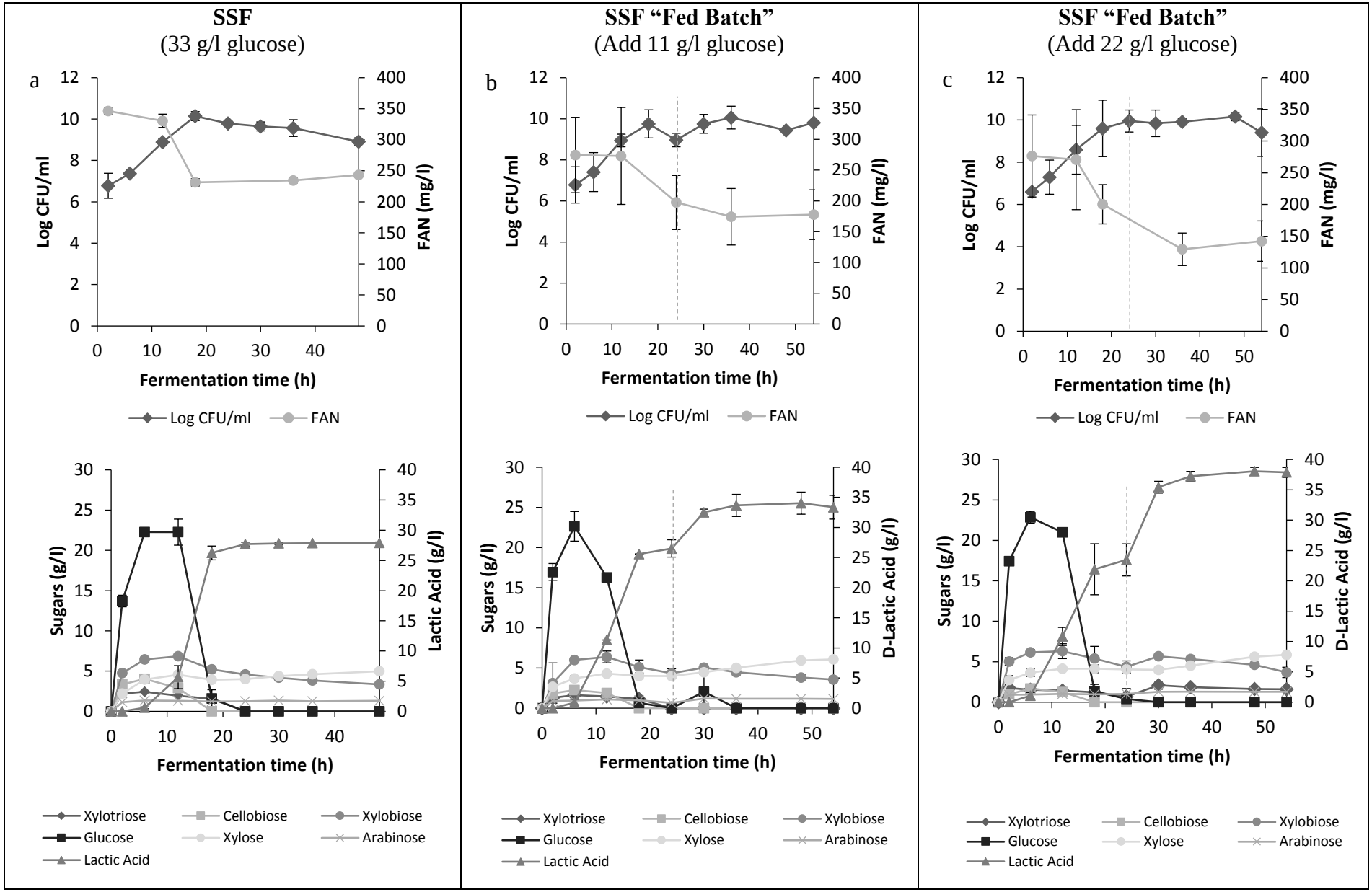

Figure 4-3: Simultaneous saccharification and fermentation (SSF) of alkaline pretreated DDGS by *L. coryniformis* in a 100 ml bioreactor (37^{o}C, pH 5) under different fermentation regimes: 3a) DDGS concentration: 33 g/l; 3b) 11g/l added after 24 h; 3c) 22 g/l added after 24 h.

Table 4-2: Overall fermentation characteristics of different fermentation processes for the production of D-lactic by *L. coryniformis* cultivation

Parameter	**SHF 33 g/l glucose***	**SSF 33 g/l glucose***	**'Fed batch' SSF**	
			Addition of 11 g/l glucose*	Addition of 22g/l glucose*
Lactic acid production (g/l)	24.1	27.9	34.0	38.1
Lactic acid productivity (g/l/h)	1.3	1.5	0.7	0.8
Glucose conversion (%)[a]	72.9	84.5	76.1	70.0
Lactic acid yield (%)[b]	32.1	42.3	40.1	35.0

*from alkaline treated DDGS solid
[a]Lactic acid (mg) / glucose in alkaline pretreated DDGS (mg) x 100
[b]Lactic acid (mg) / pretreated DDGS (mg) x 100

4.3.4.1 Simultaneous saccharification and fermentation (SSF) of DDGS hydrolysate in 2L bioreactors

The feasibility of the SSF process was evaluated in a 2-L stirred tank bioreactor with 1.5L working volume (Figure 4-4). The data obtained were very similar to those obtained for the SSF in the 100 ml bioreactor, i.e. the maximum D-lactic acid concentration was 26.4 g/l (produced after 18 hours), the glucose conversion yield was ~ 80%, the productivity was 1.47 g/l/h, the lactic acid yield was ~ 40%, and the D-lactic acid optical purity was 99.9%. The key difference compared to the smaller scale SSF process was the fact that after glucose depletion (18 hours), the lactic acid concentration decreased (from 26.4 g/l at 18 hours to 22.6 g/l at 30 hours) and acetic acid was produced (from 1.3 g/l at 18 hours to 4.0 g/l after 30 hours). This phenomenon could be due the ability of *L. coryniformis* to convert lactic acid to acetic acid under aerobic conditions, once glucose was depleted as shown in fermentation of *L. plantarum* (Quatravaux et al., 2006). In order to avoid the accumulation of acetic acid in the fermentation medium, which may interfere with the purification process, it is important

that the optimum time for stopping the fermentation and the optimal aeration conditions are identified.

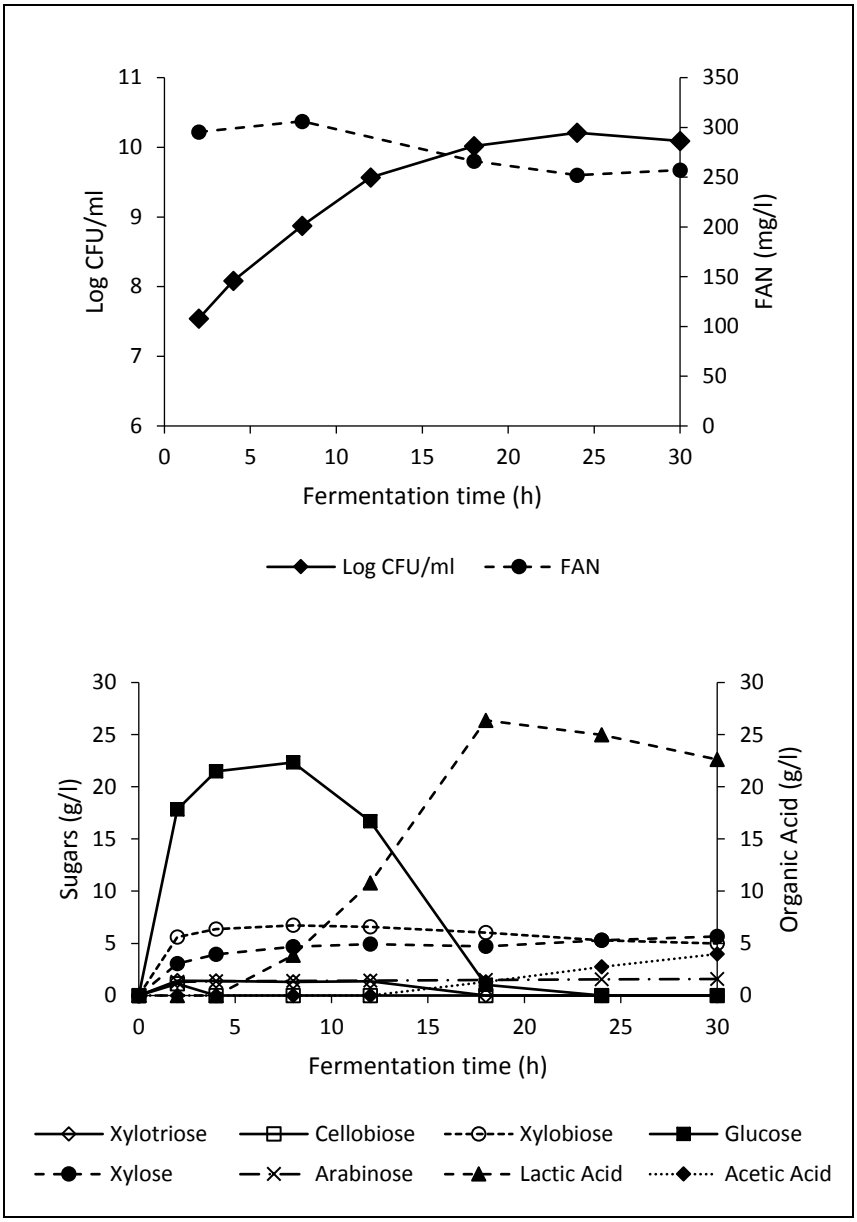

Figure 4-4: Simultaneous saccharification and fermentation (SSF) of alkaline pretreated DDGS by *L. coryniformis* in a 2 L bioreactor (temperature 37 ^{o}C, pH 5)

4.4 Conclusions

This study highlights the potential of producing D-lactic acid with high optical purity from alkaline pretreated DDGS, which is originally produced in large amount by the bioethanol industry. Simultaneous co-fermentation by *L. coryniformis* and *L. pentosus* showed better sugar utilisation compared to single strain fermentation and higher total lactic acid production, however, low purity of the D- lactic acid isomer was obtained. The SSF approach using *L. coryniformis* resulted in faster and higher production of optically pure D-lactic acid (99.9%), with a higher conversion yield for glucose to lactic acid (84.5%), compared to conventional SHF (72.9%). The D-lactic production could be further enhanced by employing a "fed-batch" SSF process, however, further work is needed to identify the operating conditions that result in high substrate conversion yields. The SSF process demonstrated good scalability as similar fermentation characteristics were obtained between the small (100 ml) and larger scale (2L) fermentation vessels.

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Chapter 5 - Purification of D-lactic acid after fermentation of wheat Dried Distiller's Grains with Soluble (DDGS) hydrolysate

Abstract

The aim of this study was to develop a multi-step process based on adsorption chromatography for the purification of D-lactic acid obtained from microbial cultures of dried distiller's grains with solubles (DDGS) hydrolysates. Initially, several anion exchange resins were screened for their binding capacity at different pH and temperatures using model lactic acid solutions. The weak base anion exchange resin, Amberlite® IRA67 showed the highest adsorption towards lactic acid, with a maximum adsorption capacity, q_{max}, estimated as 136.11 mg lactic acid / g of resin, based on the Langmuir-Freundlich model, and was further selected to purify D-lactic acid from the DDGS fermentation broth through a 3-step process. In the first step, the fermentation broth was treated with 7% w/v activated carbon to reduce its dark colour and led to a 95.9% recovery of D- lactic acid. In the second step, the cation exchange resin Amberlite® IRA120 was used in order to: acidify the fermentation broth to below the pKa value of lactic acid (pKa 3.86) by converting lactate salt formed during the fermentation to undissociated lactic acid; 92.5% of the D-lactic acid initially present in the fermentation broth was recovered at this stage. In the third step, the acidified fermentation broth was passed through the anion exchange resin Amberlite® IRA67 and D- lactic acid was eluted

using 0.5 M HCl. At the end of the overall purification process, approximately 80.4% D-lactic acid was recovered with 91.8% purity (99.9% optical purity of D- lactic acid), indicating the effectiveness of the developed multi-step downstream process.

Keywords: purification, D-lactic acid, activated carbon, cation exchange resin, anion exchange resin

5.1 Introduction

The world demand for lactic acid has increased significantly over the past few years, as the application of lactic acid based polymers, polylactic acid (PLA), offers distinctive advantages over petroleum-based polymers. PLA is a biodegradable plastic that can be used in biomedical and pharmaceutical industries as surgical suture, tissue engineering scaffolds or as drug delivery tool (Hans et al., 2009). In the early stages of commercialisation, PLA was only produced for biomedical device applications due to its high cost (Sin et al., 2013). Nowadays, the application of PLA has expanded to the electric and electronic industries for the production of casings and circuit boards, as well as in food industry for the production of food packaging and cutlery materials (Ingrao et al., 2015; NatureWorks LLC, 2017; Siracusa & Ingrao, 2016). Currently, the main producers of PLA are NatureWorks® LLC under the trade name Ingeo™, Cereplast, Inc. (United States), Corbion Purac (Netherlands), Toray Industries (Japan) and Zhejiang Hisun Biomaterial Co., Ltd (China) (Jamshidian et al., 2010; Sin et al., 2013). A recent report by IHS Markit (2015), predicted that PLA will be the leading application of lactic acid by 2020.

The production of PLA from agricultural biomass represents a promising route for production, as such biomass is available at low cost, is accessible throughout the year and does not compete with food crops. According to Buyondo and Liu (2011), over 90% of the commercial production of lactic acid comes from microbial fermentation using glucose, sucrose and corn starch as carbon sources. Depending on the type of lactic acid bacteria used for fermentation, L (levorotatory)-lactic acid, D (dextrorotatory)-lactic acid or mixture of LD-lactic acid can be produced (Ghaffar et al., 2014). Research on D-lactic

acid has intensified after Ikada et al. (1987) reported that higher thermostability of PLA (melting point of 230 °C) can be achieved by mixing poly-L-lactic acid and poly-D-lactic acid. In terms of D-lactic acid production from renewable sources, corn stover (Bai et al., 2015; Zhang & Vadlani, 2013), rice bran (Tanaka et al., 2006), peanut meal (Wang et al., 2011), broken rice (Nakano et al., 2012) and unpolished rice (Lu et al., 2009) have been studied as potential fermentation substrates. However, the fermentation broths derived from renewable sources contain a mixture of compounds, including a variety of sugars and proteins, polyphenols and organic acids, and thus require an effective downstream processing for the successful recovery of the targeted compound (Binder & Raines, 2010).

Several downstream processing techniques such as ion exchange chromatography, precipitation, solvent extraction, distillation, nanofiltration, membrane extraction and electrodialysis have been investigated for the recovery and purification of lactic acid from fermentation broths (Inkinen et al., 2011; Khunnonkwao et al., 2012; Kim & Moon, 2001; Kislik, 2012; Li et al., 2016; Min et al., 2011; Pradhan et al., 2017). Among these, adsorption by ion exchange offers a distinct advantage as it is a simple and relatively cheap process that offers product specificity, which leads to high purification yields (Bishai et al., 2015; Li et al., 2016; Wasewar, 2005). In organic acid separation, anion exchange resins are widely used. However, no specific conclusions on the optimum conditions for lactic acid binding have been drawn so far for anion exchange resins. For example, some researchers reported that a solution pH above the pKa of lactic acid (pKa lactic acid, 3.86) give the highest binding of lactic acid to Amberlite® IRA67 (John et al., 2008; Moldes et al., 2003; Nair et al., 2016), Amberlite® IRA96 (Bishai et al., 2015) and Amberlite® IRA92 (Tong et al., 2004). On the other hand, other studies have found that a pH below the pKa value give the highest adsorption of lactic acid and other carboxylic

acids to Amberlite® IRA67 (Murali et al., 2017), Amberlite® IRA35 (Evangelista & Nikolov, 1996; Kulprathipanja & Oroskar, 1991) and Lewatit S3428 resins (González et al., 2006). To promote lactic acid binding on an anionic resin below the pKa value, the fermentation broth was acidified by treatment with strong acid or by passing the broth through a strong acidic cation exchange resin, i.e. Duolite C-464, to convert lactate salt to lactic acid (Evangelista & Nikolov, 1996).

The aim of the study was to develop a multi-step process, consisting of activated carbon treatment, cation exchange chromatography and anion exchange chromatography for the purification of D-lactic acid from a fermentation broth obtained through the lactic acid fermentation of a dried distiller's grains with solubles (DDGS) hydrolysate. Initially, the adsorption behaviour and maximum binding capacity of selected anion exchange resins, was investigated using model lactic acid solutions. Subsequently, the resin with the highest binding capacity was selected and employed for the recovery of D-lactic acid from the fermentation broth, which was pre-treated by activated carbon and passed through a cation exchange resin. As the purification of D- lactic acid from fermentation broth hydrolysates is rarely reported, this study provides novel information on D- lactic acid separation employing a multiple purification step.

5.2 Materials and Methods

5.2.1 Materials

Dried Distillers Grains with Solubles (DDGS) was supplied from a bioethanol plant (Vivergo, Yorkshire, UK). DDGS was pretreated with 5% NaOH (w/v) at 121ºC for 15 minutes, washed with distilled water and freeze dried (VisTis Sentry 2.0, Warminster, PA). Powdered pretreated DDGS was stored at room temperature in a closed container before use. The resins (Amberlite® IRA67, Diaion® WA30, Amberlite® IRA400, Dowex® Marathon™ MSA and Amberlite® IRA120) and activated carbon used in this study were purchased from Sigma-Aldrich (US).

5.2.2 D-lactic acid production

D-lactic acid was produced by *L. coryniformis* subsp. *torquens* (DSM 20004) using a Simultaneous Saccharification and Fermentation (SSF) process, as described in **Chapter 4**. The process was initiated by the simultaneous addition of Accellerase® 1500 (1 ml enzyme : 0.33 g cellulose) and *L. coryniformis* inoculum and was carried out for 30 hours (1.5 L fermentation medium, 37 ºC, pH 5). The culture containing the enzyme was inactivated by heat treatment at 95 ºC for 10 minutes, followed by centrifugation at 17,105 *x g* for 20 minutes (4 ºC). Supernatants containing D-lactic acid solutions were collected and kept at -20 ºC for purification.

5.2.3 Resin preparation

Weak anion exchange resins (Amberlite® IRA67 and Diaion® WA30) and strong anion exchange resins (Amberlite® IRA400 and Dowex® Marathon™ MSA) were selected for this study. Before utilisation, the resins in free base form, were first converted to Cl^- form as described by Moldes et al. (2003). Resins that were purchased in Cl^- form were only washed with distilled water (Pradhan et al., 2017; The Dow Chemical Company, 2017). For the acidification of the fermentation broth, a cation exchange resin, Amberlite® IRA120, a strongly acidic resin in H^+ form, was used. The resin was washed with distilled water three times to remove any contaminants. All resins were then oven dried at 50 °C overnight and stored at room temperature in closed containers before use. The properties of the ion exchange resins that were used in this study are presented in Table 5-1.

Table 5-1: Properties of ion exchange resins (information provided by Sigma-Aldrich, US)

Ion exchange type	Resin	Strength	Particle size (µm)	Matrix	Active functional group	pH
Anion	Amberlite® IRA67	Weak basicity	500-750	Acrylic (gel)	Tertiary amine	0 - 7
Anion	Diaion® WA30	Weak basicity	680	Styrene-divinylbenzene (highly porous)	Tertiary amine	0 - 9
Anion	Amberlite® IRA400	Strong basicity	600-750	Styrene-divinylbenzene (gel)	Quaternary ammonium	0 - 14
Anion	Dowex® Marathon™ MSA	Strong basicity	640	Styrene-divinylbenzene (macroporous)	Quaternary ammonium	0 - 14
Cation	Amberlite® IRA120	Strong acidity	620-830	Styrene-divinylbenzene (gel)	Sulfonic acid	0 - 14

5.2.4 Screening and optimisation of anion exchange resins binding and recovery

For the screening experiments of the anion exchange resins, model lactic acid solutions were prepared using commercial lactic acid (85%, Food Chemical Codex, FCC, Sigma-Aldrich).

5.2.4.1 Effect of pH on lactic acid binding

The effect of pH on lactic acid binding was determined through batch experiments according to Bishai et al. (2015) with slight modifications. 1 g of dried resin (Amberlite® IRA67, Diaion® WA30, Amberlite® IRA400 or Dowex® Marathon™ MSA) was mixed with 5 ml of lactic acid (50 g/l) at different initial pH conditions (2, 3, 4, 5, 6, 7 and 8) at 25 ^{o}C. The pH of the solutions was adjusted with 5 M NaOH. The mixtures were shaken at 200 rpm for 8 hours. The liquid fractions from each mixture were collected by filtration and analysed for lactic acid concentration by HPLC.

5.2.4.2 Effect of temperature on lactic acid binding

In order to investigate the effect of temperature on lactic acid binding, 1 g of each dried resin (Amberlite® IRA67, Diaion® WA30, Amberlite® IRA400 or Dowex® Marathon™ MSA) was mixed with 5 ml lactic acid (50 g/l), prepared at the optimum pH (obtained in Section 5.2.4.1) and incubated at temperatures of 25, 30, 40, 50 and 60 °C at 200 rpm. The mixtures were shaken at 200 rpm for 8 hours. The liquid fractions from each mixture were collected by filtration and analysed for lactic acid concentration by HPLC.

The binding capacity, *q*, and adsorption efficiency, *E*, of the resin at different pH and temperatures were calculated as follows, as described by Pradhan et al. (2017) and Gao et al. (2010),

$$q = \frac{(C_i - C_f) * V}{R} \quad (1)$$

$$E = \frac{(C_i - C_f)}{C_i} * 100 \quad (2)$$

where, q is the amount of lactic acid adsorbed to the resin (mg/g), E is the efficiency of lactic acid binding (%), C_i is the initial concentration of lactic acid (g/l), C_f is the concentration of lactic acid after being mixed with the resin (g/l), V is the volume of lactic acid solution (l) and R is the weight of the resin (g).

5.2.4.3 Adsorption capacity of lactic acid by resins

Amberlite® IRA67 and Diaion® WA30 were selected to carry out adsorption isotherm analysis in batch operation as described by Bernardo et al. (2016) and John et al. (2008), with slight modifications. 1 g of each resin was mixed with 5 ml of lactic acid at various concentrations (4 - 650 mg lactic acid per 5 ml). The initial pH of the lactic acid solutions was set taking into account their optimum binding pH (obtained in Section 5.2.4.1) for the respective resin. The reaction took place at optimum temperature (obtained in Section 5.2.4.2) at 200 rpm for 8 hours. Liquid samples from each mixture were filtered and collected for further analysis.

Three different nonlinear models, the Langmuir, Freundlich and Langmuir - Freundlich models, were then fitted to the data using Origin Pro 8.0 software (OriginLab,

USA) (Arcanjo et al., 2015; Bayazit et al., 2011; Dethe et al., 2006; Sala et al., 2014) using the following equations:

Langmuir model $$q = \frac{q_{max} * K * C}{K * C + 1} \quad (3)$$

Freundlich model $$q = K_f * C^{\frac{1}{n}} \quad (4)$$

Langmuir – Freundlich model $$\frac{q}{q_{max}} = \frac{K_{LF} * C^{nLF}}{1 + K_{LF} * C^{nLF}} \quad (5)$$

where, q is the amount of lactic acid adsorbed to the resin (mg/g), q_{max} is the maximum amount of lactic acid adsorbed to the resin (mg/g), K is the Langmuir adsorption constant, K_f is the Freundlich adsorption constant, K_{LF} is the affinity constant for adsorption, C is the amount of lactic acid (mg), n is the Freundlich adsorption constant and nLF is the Langmuir-Freundlich coefficient.

2.4.4 Preparative column separation: Effect of ionic strength of eluent (HCl) on lactic acid recovery

The resin that exhibited the highest binding capacity, q, (Amberlite® IRA67) was selected for further recovery experiments using HCl with different ionic strengths as eluent. 4 g of Amberlite® IRA67 were packed into a 100 mm (length) column (Fisher Scientific, Leicester, UK) and saturated with 20 ml of 30 g/l lactic acid solution. The resin was then washed with distilled water to remove any unbound lactic acid. Different concentrations of HCl were used as eluent (0.05, 0.1, 0.5 and 1.0 M), which were passed

down the packed column by gravity and fractions of effluents were collected (2 ml per fraction) at regular time intervals (~ every 1 minutes). All fractions were analysed for lactic acid concentration.

5.2.5 Purification of D-lactic acid from fermentation broth

5.2.5.1 Colour removal by activated carbon

The effect of activated carbon on the removal of the colour from the fermentation broth was determined. Powdered activated carbon was mixed with 5 ml of clarified fermentation broth at different loading concentrations (0, 1, 5, 7 and 10%, w/v), for 1.5 hours at 150 (25 $^{\circ}$C). The mixture was then separated by centrifugation at 17,105 *x g* for 10 minutes (4 $^{\circ}$C). The pellet was washed twice with distilled water and the supernatants collected for sugar and lactic acid analysis.

5.2.5.2 Acidification of fermentation broth by cation exchange resin

After treatment with activated carbon, the fermentation broth was subjected to Amberlite® IRA 120, H^+ resin aiming to convert sodium lactate into lactic acid (Beitel et al., 2016; Liang et al., 2015). 10 g of dried resin were packed into a 30 cm length Econo-glass column (i.d. 1 cm) which was first filled with distilled water and the height of the resin was fixed with a flow adaptor (i.d. 1 cm) (Biorad, California, US). Distilled water was allowed to pass through the column until the pH of effluent was around 6.5. Then, a constant flow of 3 ml/min using a peristaltic pump was applied. The fermentation broth containing sodium lactate with a pH around 5.5 was then pumped into the column at the same flow rate. When the pH of the effluent started to increase, the resin was considered

saturated. Effluent fractions (4 ml each) were collected for lactic acid and sugar analysis. Fractions containing D-lactic acid were pooled for subsequent purification. The resin was washed with distilled water to remove the remaining solution in interstitial spaces, regenerated with 1 M HCl and thoroughly rinsed with distilled water, before a new cycle (Bishai et al., 2015).

5.2.5.3 Adsorption by anion exchange resin

Pooled supernatant fractions containing D-lactic acid were passed through an anion exchange resin, (Amberlite® IRA67) in fixed-bed column operation. 25 g of dried resin were packed into a 30 cm length Econo-glass column (i.d. 2.5 cm) which was first filled with distilled water and the upper side of resin was fixed with a flow adaptor (i.d. 2.5 cm). The system was washed with distilled water until the pH of the effluent was around 6.5. Then, the acidified broth obtained from Amberlite® IRA 120, H^+ resin was pumped into the column at 3 ml/min. D=lactic acid was recovered with 0.5 M HCl. Fractions of effluents at each stage were collected (4 ml each) for lactic acid and sugar analysis.

5.2.6 Analytical methods

The nitrogen content was determined using the Free Amino Nitrogen (FAN) method as described by Lie (1973) with some modifications. 0.5 ml of diluted sample was mixed with 0.25 ml of colour reagent (49.71 g of $Na_2HPO_4 \cdot 2H_2O$, 5 g of ninhydrin, 3 g of fructose and ~ 40 g of KH_2PO_4 dissolved in 1 l of distilled water; pH 6.6 – 6.8) in 2 ml Eppendorf tube. The mixture was heated at 100 ^oC in a thermal block (Grant,

Cambridge) for exactly 16 minutes and immediately cooled in an ice bath. 1.5 ml of dilution reagent (2 g potassium iodate, KIO_3, in 616 ml distilled water and 384 ml 96% ethanol) was added and the free amino nitrogen content was measured at 570 nm. A calibration curve was constructed using glycine at different concentrations (0.25 – 2 mg/l) as standard.

Sugar and lactic acid concentrations were analysed by high performance liquid chromatography (HPLC) in an Agilent Infinity 1260 system (Agilent Technologies, USA) equipped with an Aminex HPX-87H column (Bio-rad, Hercules, CA) at a 0.6 ml/min flow rate with 5 mM H_2SO_4 as mobile phase. The temperature of the column was set at 65 $^{\circ}$C and sugars and lactic acid were detected using a refractive index detector. The D-lactic acid recovery and purity were calculated using the following equations (Bishai et al., 2015):

$$Recovery\ (\%) = \frac{mg\ LA\ in\ each\ purification\ stage}{mg\ LA\ in\ fermentation\ broth} * 100 \quad (6)$$

$$Purity\ (\%) = \frac{HPLC\ peak\ area\ of\ LA\ in\ each\ purification\ stage}{Total\ HPLC\ peak\ areas\ in\ each\ purification\ stage} * 100 \quad (7)$$

5.3 Results and Discussion

5.3.1 Selection of anion exchange resin

When considering ion exchange chromatography, the efficiency of the product adsorption by the resin determines the success of the purification process. Therefore, factors such as pH, temperature and lactic acid concentration were initially investigated in this study to select the most appropriate anion exchange resin. The effect of the ionic strength of the eluent (HCl solution) on product recovery was also investigated. Before use, the resins that existed in free base were converted to Cl^- form. The Cl^- form was selected as previous studies have shown that in this form the resins exhibit the highest adsorption capacity for lactic acid (Bernardo et al., 2016; John et al., 2008). Moreover, the simultaneous lactic acid recovery and resin regeneration for subsequent adsorption cycles by HCl, reduces the number of steps involved in the purification process (Moldes et al., 2003). Four different resins, categorised into weak base anion exchange (Amberlite® IRA67 and Diaion® WA30) and strong base anion exchange (Amberlite® IRA400 and Dowex® Marathon™ MSA) were tested against different initial pH values, ranging from 2 to 8, as shown in Figure 5-1.

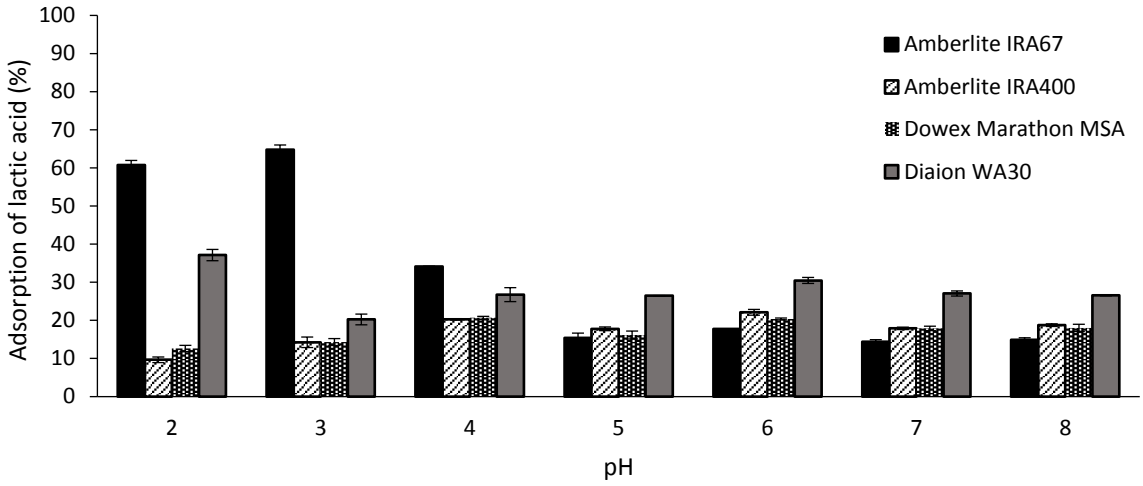


Figure 5-1: Effect of pH on lactic acid binding by four anionic resins at 25°C

Among the tested resins, the weak base anion exchange resins showed capability for lactic acid binding at low pH, with the highest adsorption being 65% with Amberlite® IRA67 at pH 3, which corresponded to a maximum binding capacity (q_{max}) of 155 mg lactic acid/g of resin. For strong base anion exchange resins, less than 22% of lactic acid was adsorbed by both Amberlite® IRA400 and Dowex® Marathon™ MSA, with the highest binding at pH 6 and pH 4, respectively, suggesting that the pH did not influence the adsorption of lactic acid to the strong base anion exchange resins. On the other hand, the adsorption of weak base anion exchange resins was strongly influenced by the pH of the feed solution. In the case of Amberlite® IRA67 and Diaion® WA30, the best pH for lactic acid adsorption was below its pKa (3.86), where lactic acid exists in its undissociated form (Evangelista & Nikolov, 1996; González et al., 2006; Yousuf et al., 2016). This can be associated with the charge of the tertiary amine (the functional group in both reeins) which is cross-linked to the polymeric matrix (acrylic or styrene) in Amberlite® IRA67 and Diaion® WA30. It is assumed that the lone pair electron of the nitrogen atom in the tertiary amine is likely to hydrogen bond to lactic acid through the

chloride ion, as shown in Figure 5-2 (Kulprathipanja & Oroskar, 1991; Syzova et al., 2004). This likely mechanisms is also supported by works from Yousuf et al. (2016), Kislik (2012) and Kulprathipanja and Oroskar (1991), who reported that the possible interactions between amine-based extractants and carboxylic acids are through hydrogen bonding, acid-base interaction, hydrophobic interaction, ion-ion pair formation or solvation.

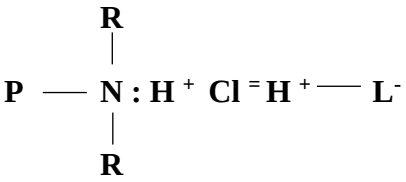


Figure 5-2: Possible mechanism of lactic acid adsorption to Amberlite® IRA67 and Diaion® WA30; P= polymer matrix, R = alkyl group, L= lactate ion

Subsequently, the effect of temperature (25 ^{o}C to 60 ^{o}C) on lactic acid adsorption was investigated at pH values that were previously shown to give the highest lactic acid binding for each resin (Figure 5-3). No differences were observed between the different temperatures, for each resin. As shown previously, the highest lactic acid adsorption (~73%) was exhibited by Amberlite® IRA67. According to Niazi and Brown (2016), the effect of temperature on ion exchange resins is mainly attributed to pKa changes of the targeted compound as a result of the temperature change. In the case of lactic acid, as the temperature increased from 25 to 30, 40 and 50^{o}C, the pKa value also increased to 3.896, 3.942 and 4.028, respectively (Saeeduddin & Khanzada, 2004). As lactic acid adsorption to weak base anion exchange resins occurs below its pKa value, the increase in temperature from 25 to 60 $^{\circ}$C did not have any significant effect on lactic acid adsorption.

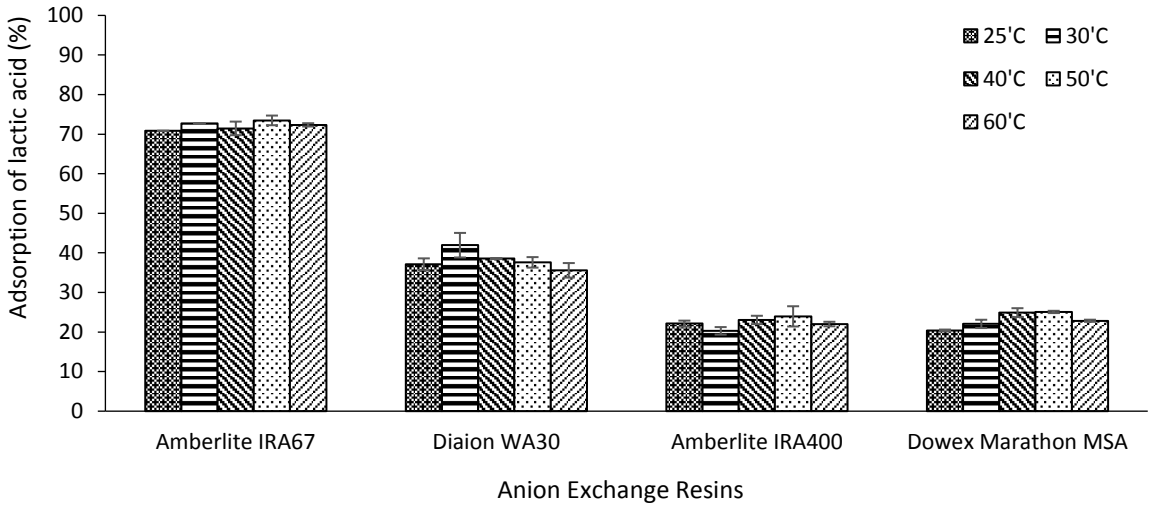
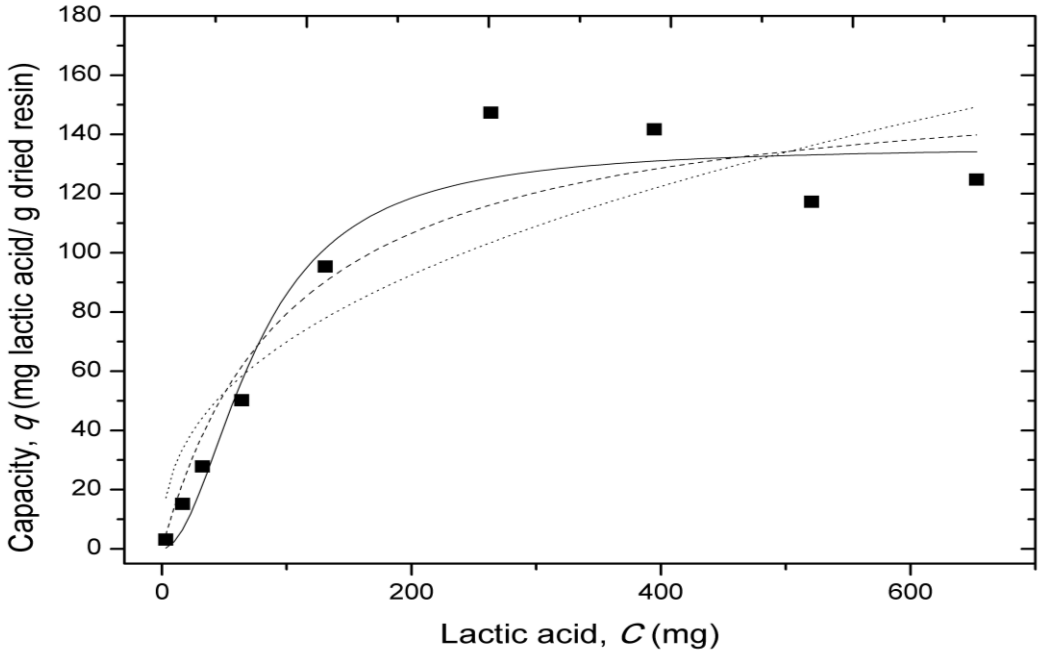


Figure 5-3: Effect of temperature on lactic acid binding by four anionic resins; initial pH for Amberlite® IRA67: 3; Diaion® WA30: 2; Amberlite® IRA400: 6 and Dowex® Marathon ™ MSA: 4

5.3.1.1 Adsorption isotherms

The two resins that demonstrated the highest binding capacity, Amberlite® IRA67 and Diaion® WA30, were further tested; adsorption isotherms were generated at 25 °C, which are shown in Figure 5-4a and 4b, respectively. Different kinetic models (Langmuir, Freundlich and Langmuir – Freundlich) were then used to fit the data, and the model parameters are presented in Table 5-2.

(a)



(b)

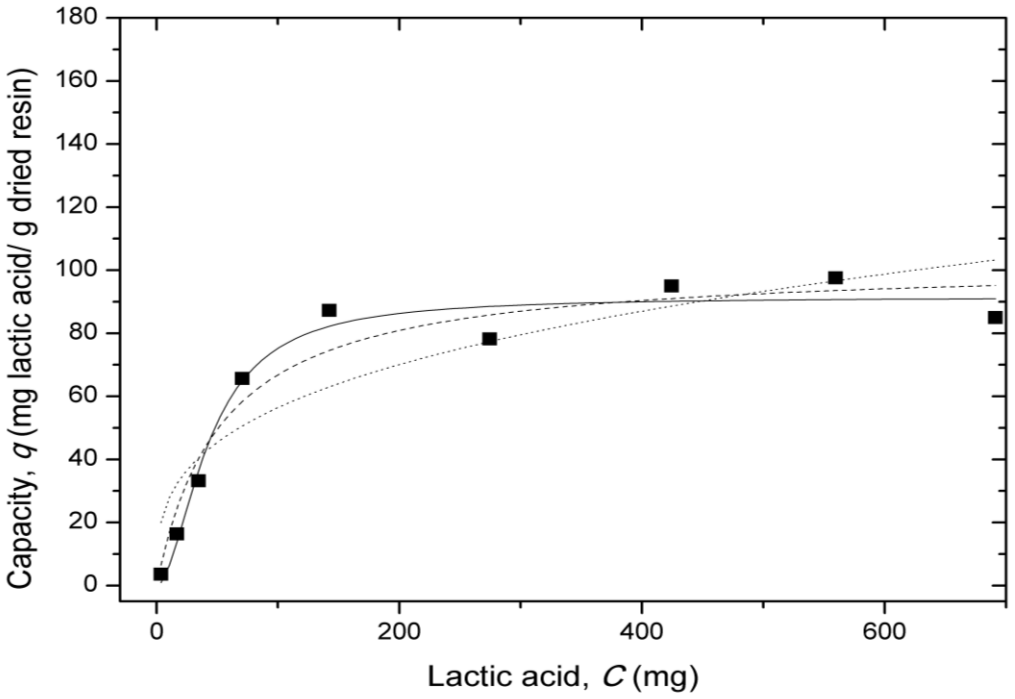


Figure 5-4: Adsorption isotherms of lactic acid to (a) Amberlite® IRA67 and (b) Diaion® WA30 at 25ºC; - - - Langmuir model, …… Freundlich model, ___ Langmuir-Freundlich model

Table 5-2: Langmuir, Freundlich and Langmuir – Freundlich isotherm parameters describing the adsorption of lactic acid to Amberlite® IRA67 and Diaion® WA30

Model	Parameter	Amberlite® IRA67	Diaion® WA30
Langmuir	q_{max} (mg/g)	162.09	102.47
	K	0.0096	0.019
	R^2	0.910	0.940
Freundlich	K_f	10.88	13.379
	n	2.475	3.200
	R^2	0.801	0.800
Langmuir-Freundlich	q_{max} (mg/g)	136.11	91.51
	K_{LF}	0.0002	0.001
	nLF	1.96	1.83
	R^2	0.940	0.965

The Langmuir and Langmuir – Freundlich models fitted better the data in the case of both resins ($R^2 > 0.9$), compared to the Freundlich model ($R^2 = 0.80$). The Langmuir – Freundlich model showed better fit than the Langmuir model for both the Amberlite® IRA67 ($R^2 = 0.94$) and the Diaion® WA30 resin ($R^2 = 0.965$). In the Langmuir model, it is assumed that the adsorption of a given adsorbate occurs as a monolayer sorption onto the surface of a resin containing a finite number of identical binding sites. The adsorbent has uniform binding sites and the adsorbate will only bind to the binding site (Foo & Hameed, 2010; Sala et al., 2014). In the Freundlich model, it is assumed that the binding of the adsorbate molecules onto the adsorbent is at infinite capacity. The adsorption is

not uniform and can occur in a multilayer, with the binding site that has stronger bond energy being occupied first (Dethe et al., 2006; Foo & Hameed, 2010). In the present study, the adsorption capacity of the resin was specific due to the strong interactions between lactic acid and the functional groups present in the Cl^- form of the resin. As a result, the Freundlich model did not fit as well as the other models the kinetic data. According to Sala et al. (2014), the Langmuir-Freundlich model is the simple generalisation of both isotherms, modelling the adsorption cooperativity of the two different binding mechanisms. This level of cooperativity can be determined from the *nLF* value, where, when $nLF > 1$, a positive cooperativity is indicated. When $0 < nLF < 1$, a negative cooperativity in binding process is indicated, whereas when *nFL* value = 1, it is assumed that the adsorption is purely independent, and no interaction takes place between absorbents. In this study, the *nFL* values for both resins were > 1, with 1.96 for Amberlite® IRA67and 1.83 for Diaion® WA30, indicating a positive cooperativity of the two binding mechanisms.

The maximum binding capacity of a resin (q_{max}) can also be predicted from the Langmuir and Langmuir–Freundlich models, however it cannot be obtained from the Freundlich model as this model assumes that lactic acid binding to the resin is unlimited. The highest q_{max} values for Amberlite® IRA67 were 162.09 and 136.11 mg/g resin based on the Langmuir and Langmuir–Freundlich models, respectively. This value, however, was lower than the q_{max} value reported by Garrett et al. (2015), i.e. 203 mg/g of resin, as predicted by the Langmuir model.

5.3.1.2 Effect of HCl strength on the recovery of lactic acid

The strength of the eluent on the recovery of lactic acid plays a key role for ensuring that all of the lactic acid that is bound to the resin is detached. In this study, HCl was used as an eluent to recover lactic acid from Amberlite® IRA67, which was deemed from the previous work to be the most suitable resin. Figure 5-5 depicts the elution profiles of lactic acid at different HCl concentrations. At 0.05 M and 0.1 M HCl, only 5.1 and 22.1% lactic acid was recovered from Amberlite® IRA67, respectively. However, at 0.5 and 1.0 M, ~ 96% and 100% recovery of lactic acid was achieved, respectively, indicating that the strength of the eluent plays critical role for the detachment of lactic acid from the resin. Based on these results, 0.5 M HCl was selected to recover lactic acid in subsequent experiments.

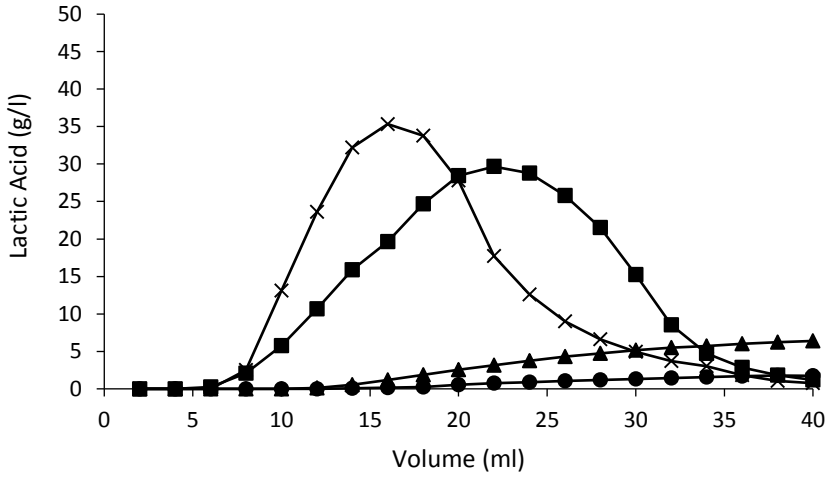


Figure 5-5: Elution profiles of lactic acid from the Amberlite® IRA67 resin (saturated with 20 ml of 30 g/l lactic acid) at different concentrations of HCl at 25°C; (—●—) 0.05M, (—▲—) 0.1M HCl, (—■—) 0.5M HCl and (—×—) 1.0M HCl.

5.3.2 Separation and purification of D-lactic acid from fermentation broth

5.3.2.1 Colour removal by activated carbon

After determining the conditions leading to maximum lactic acid adsorption and recovery using the Amberlite® IRA67 resin and model lactic acid solutions, the aim was to purify lactic acid from fermentation broths of *L. coryniformis* subsp. *torquens*, where DDGS hydrolysate was used as the fermentation medium. Fermentation broths usually contain besides the component of interest, residual sugars and proteins, as well as by-products of the fermentation process. In this particular case, fermented DDGS hydrolysate contained residual sugars (xylobiose, xylobiose, arabinose), organic nitrogen in the form of proteins, peptides or amino acids, polyphenols and acetic acid, all of which contributed into the dark brown colour of the fermentation broth (Qin et al., 2017). It is likely that a significant proportion of the dark brown colour of the medium is due to the dark colour of DDGS generated during the drum drying step in the DDGS production process. Therefore, prior to ion exchange purification, the fermented DDGS hydrolysate was initially subjected to activated carbon treatment, aiming to remove the components that were responsible for the dark colour. Figure 5-6 shows the effect of various activated carbon concentrations on the colour of the fermentation broth. A positive correlation can be observed as the activated carbon concentrations increased up to 5% (w/v) and the colour of the fermentation broths, which became notably lighter. For higher activated carbon concentrations (7 and 10%, w/v) no significant changes in colour were observed compared to 5% (w/v) of activated carbon.

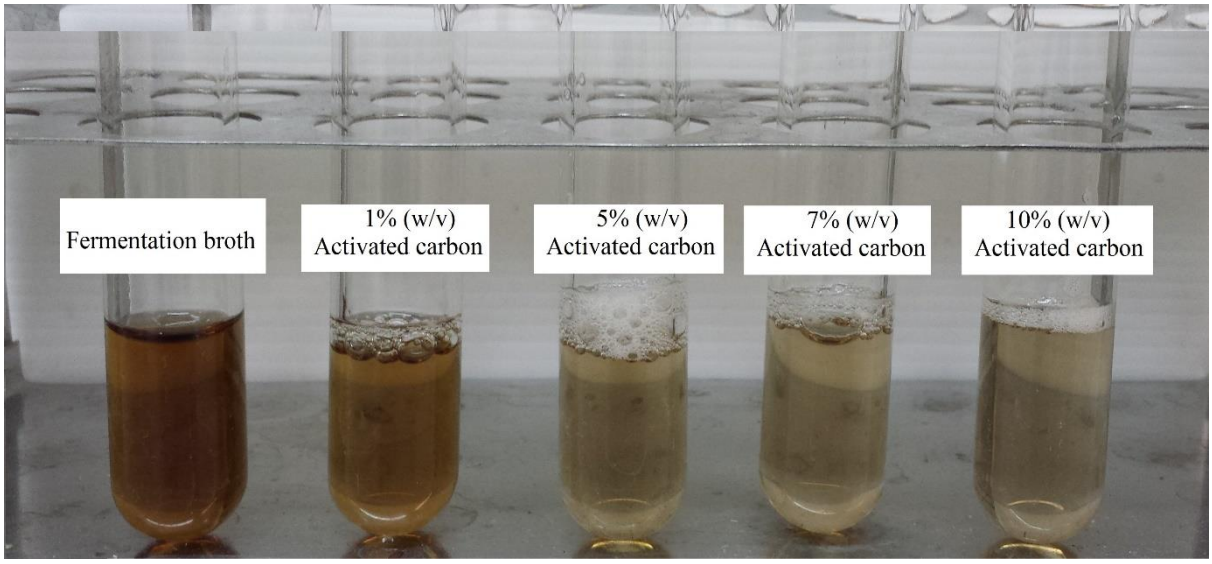


Figure 5-6: Fermentation broth treated with different concentrations of activated carbon at 25 ˚C for 1.5 h

The effect of activated carbon concentration on the recovery of D-lactic acid, protein, oligosaccharides and monosaccharides is shown in Table 5-3. The recovery of D-lactic acid gradually decreased as the concentration of activated carbon increased. More specifically at 1, 5, 7 and 10% (w/v) of activated carbon, 95, 90, 88 and 85% lactic acid was recovered from the fermentation broth, respectively. A relatively small reduction in monosaccharides and proteins (< 30% removal) concentrations was overall noted when the fermentation broth was treated with activated carbon, even as high as 10% (w/v) concentration. Stone and Kozlov (2014) reported that activated carbon can only adsorb low molecular weight proteins, in which is in line with protein removal data obtained in this study. On the other hand, oligosaccharides (xylotriose and xylobiose) were completely removed at 7% (w/v) activated carbon. According to Boon et al. (2000), activated carbon has higher affinity for trisaccharides and disaccharides compared to monosaccharides with a capacity of 133 mg/g and 117 mg/g of activated carbon, respectively. Based on the above, 7% (w/v) activated carbon was selected as the best

concentration to treat the fermentation broth to ensure a good recovery of lactic acid and the removal of the oligosaccharides and some of the proteins and monosaccharides.

Table 5-3: Recovery of D-lactic acid, protein, oligosaccharides and monosaccharides after treatment with activated carbon at different concentrations

Activated carbon (%, w/v)	***Recovery (%)**					
	D-lactic acid	Protein	Oligosaccharides		Monosaccharides	
			Xylotriose	Xylobiose	Xylose	Arabinose
0	100 ± 0.0	100 ± 2.5	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
1	95 ± 0.4	94.0 ± 0.2	77.4 ± 0.5	87.6 ± 0.0	90.4 ± 0.9	83.4 ± 1.6
5	90 ± 0.3	83.6 ± 1.3	0	42.8 ± 1.0	85.7 ± 0.6	79.9 ± 0.9
7	88 ± 3.4	83.0 ± 1.2	0	0	82.2 ± 3.0	76.7 ± 2.7
10	85 ± 1.9	80.2 ± 2.8	0	0	72.7 ± 1.7	70.0 ± 1.4

*Initial concentration in the fermentation broth: D- lactic acid, 25.9 g/l; xylotriose, 1.0 g/l; xylobiose, 3.8 g/l; xylose, 4.8 g/l; arabinose, 0.5 g/l and protein, 152.8 mg/l (FAN)

5.3.2.2 Acidification of fermentation broth by cation exchange chromatography

In lactic acid fermentations, the pH of the culture is normally controlled to prevent microbial growth inhibition and ensure the good growth for lactic acid bacteria in the fermentation media (Bustos et al., 2004; Nguyen et al., 2013). NaOH is often selected as the neutralising agent because of its low cost and the fact that no gypsum is generated as a by-product which is the case when $Ca(OH)_2$ is used (Wang et al., 2013); as a result of using NaOH sodium lactate is formed in the fermentation broth. In order to recover lactic acid from the fermentation broth by anion exchange chromatography, the pH of the broth needs to be reduced to below pH 3 in order for lactic acid to be in its undissociated form rather than in the form of salt. To achieve this and taking into account that the pH of the fermentation broth used in this study was ~5.5, the fermentation broth was passed through the Amberlite® IRA120 (H^+) cation exchange resin, in order to exchange the sodium ions

and release lactate ions into the solution. Figure 5-7 depicts the compositional profile of the solution during passing through the resin.

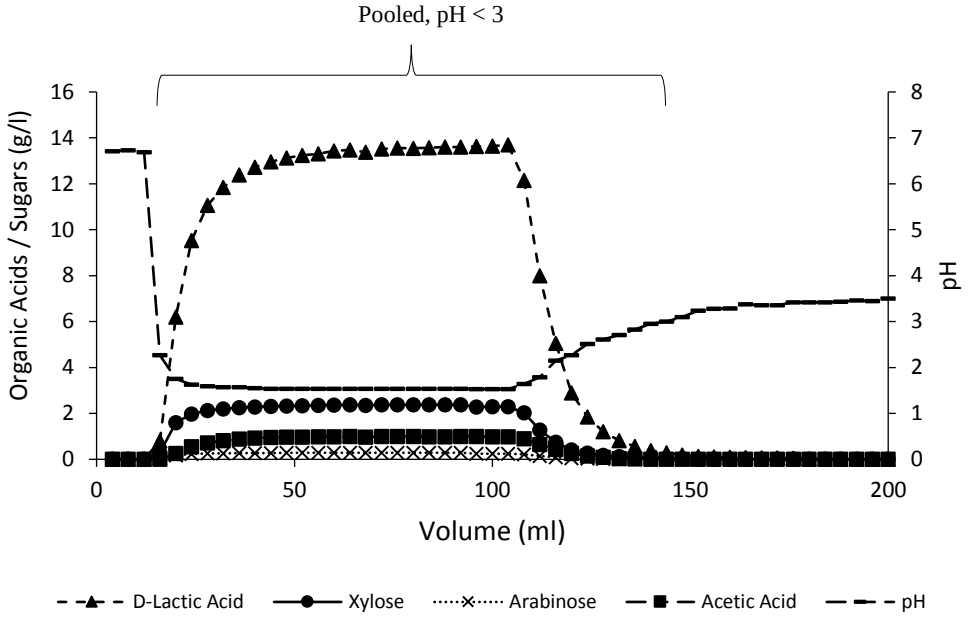


Figure 5-7: Elution profile of fermentation broth during cation exchange chromatography with Amberlite IRA120 (H^+) resin

In cation exchange chromatography, no eluent is needed as lactic acid does not bind to Amberlite IRA® 120 (H^+). Amberlite IRA® 120 is a strong acidic cation exchange resin that contains sulfonic acid functional groups. It is assumed that the hydrogen ions (H^+) that are attached to the resin's functional group will bind to sodium lactate and convert it to lactic acid in the solution. At the same time, the cation of the lactate salt (Na^+) will be transferred to the cation exchange resin and transformed through the reaction: $P\text{-}H^+ + Na^+La^- \rightarrow P\text{-}Na^+ + H^+La^-$, where P is the polymer matrix (Eyal & Elankovan, 2007). In the first 12 ml of the fractions collected, no organic acids and sugars were detected and the pH of the collected fraction was around 6.5, indicating that the fraction contained only water (already present in the column). The pH of the solution then dropped sharply and when it reached ~2.3, the presence of D-lactic acid and other

compounds such as acetic acid, xylose and arabinose were detected. The pH of eluate in the collected fraction dropped further with time, to ~1.5, indicating that sodium lactate was successfully converted to undissociated lactic acid. Fractions that had pH below 3 were then pooled together yielding a fraction with a pH of 1.67.

5.3.2.3 Recovery of D-lactic acid by anion exchange chromatography resin

The undissociated form of D-lactic acid collected from Amberlite® IRA120 (H^+) was then subjected to Amberlite® IRA67 (Cl^-). According to Bishai et al. (2015), Amberlite® IRA67 has significant commercial potential as it is a robust resin where the amines present in the functional group do not easily detach from the polymer matrix, it is easy to regenerate, and provides higher recovery of lactic acid compared to other resins. The results from this study (Figure 5-8) show that during the binding stage, no lactic acid was present in the collected fraction, while other compounds such as xylose and arabinose were detected. The pH of the effluent collected during the binding stage was around 5.5 to 6.0, indicating that the organic acids present in the fermentation broth (lactic acid and acetic acid) were adsorbed to the resin. After washing the column with water, lactic acid was desorbed with 0.5 M HCl. The first acid to be eluted was acetic acid, followed by lactic acid. Fractions that contained only lactic acid were pooled together, as shown in Figure 5-8.

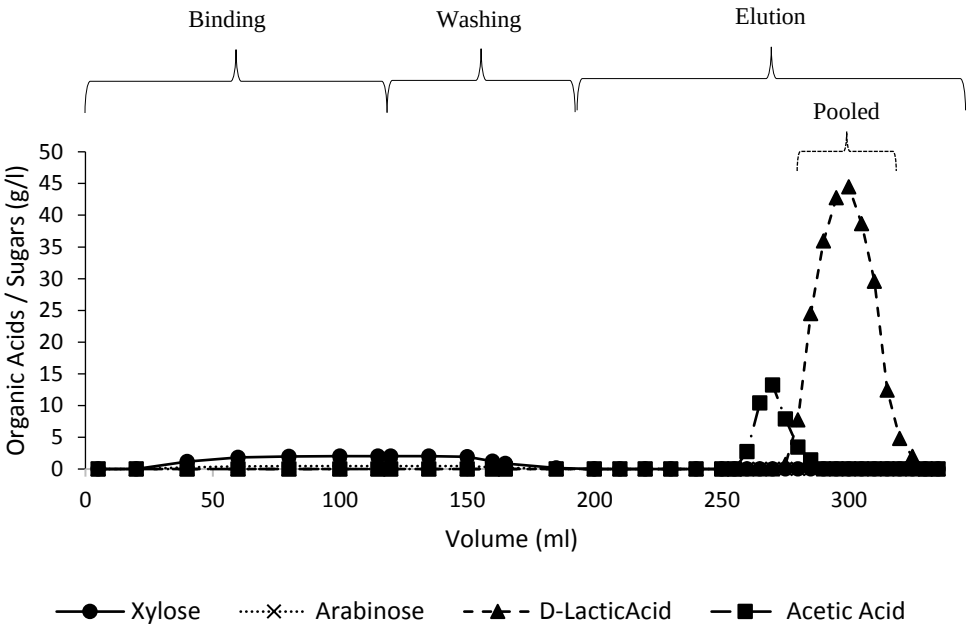


Figure 5-8: Adsorption and elution profile of fermentation broth during anion exchange chromatography with Amberlite® IRA67 (Cl^-) resin

Table 5-4 presents the recovery and purity of D-lactic acid, as well as the percentage of total sugars and protein removal after each stage of the purification process. After the fermentation broth was treated with 7% (w/v) activated carbon, a ~96% recovery of D-lactic acid recovery was observed and a 9.6% increase in D-lactic acid purity, the latter due to the complete removal of oligosaccharides and of 55% of the proteins that were initially present in the fermentation broth. During the cation exchange stage, a ~93% recovery of D-lactic acid compared to the D-lactic acid concentration in the fermentation broth was observed, and although only 1% increase in D-lactic acid purity was detected, ~21% of total sugar and ~44% of proteins were successfully removed at this stage. Cation exchange did not increase the D-lactic acid purity as its main objective was to acidify the fermentation broth. During the anion exchange step a ~80.4% recovery of D-lactic acid was observed, while 74% of the sugars were removed, leading to a cumulative sugar removal of 100%. As a consequence, the purity of D-lactic acid in

the eluent was significantly increased during this step, reaching 91.8%, with the remaining components consisting most likely of small amounts of organic molecules, e.g. proteins, acetic acid and other microbial metabolites. Optical purity values for D-lactic above 90% acid are considered appropriate for the synthesis of PLA (Nguyen et al., 2013; Niaounakis, 2015) and demonstrate that the proposed multi-step downstream process has significant potential for scaling up and commercialisation.

Table 5-4: Recovery and purity of D-lactic acid from the fermentation broth during the different downstream processing stages

Purification stages	**Volume (ml)**	**D-lactic Acid**			**Total Oligosaccharides**		**Total Monosaccharides**		**Protein**	
		g/l	Recovery (%)	Purity* (%)	g/l	Cumulative removal (%)	g/l	Cumulative removal (%)	mg/l	Cumulative removal (%)
Fermentation broth	50	25.9	100	54.6	4.5	0	5.3	0	152.8	0
7% Activated carbon	92	13.5	95.9	64.2	0	100	2.7	4.5	37.8	55
Cation exchange chromatography (Amberlite® IRA120, H^+)	132	9.1	92.5	65.2	0	100	1.7	26.2	0.6	99
Anion exchange chromatography (Amberlite® IRA67, Cl^-)	29	36.0	80.4	91.8	0	100	0	100	0.4	99

*The purity (%) of lactic acid in the eluate was determined according to Equation 7.

5.4 Conclusions

This study demonstrated the effectiveness of employing a multi-stage downstream process for the purification of microbially produced D-lactic acid, involving activated carbon, cation and ion exchange chromatography. Activated carbon successfully reduced the dark colour of the fermentation broth and at the same time removed other impurities such as oligosaccharides. The cation exchange chromatography using the cationic resin Amberlite® IRA120 was able to acidify the fermentation broth to a pH of approximately 1.67 and convert sodium lactate to undissociated lactic acid. The adsorption of lactic acid to the anionic resin Amberlite® IRA67 resin occurred below its pKa value (3.86), i.e. at a pH of 3, and the adsorption data were modelled effectively by the Langmuir-Freundlich model. At the end of the overall purification process, approximately 80.4% D-lactic acid was recovered with 91.8% purity, indicating the effectiveness of the downstream process developed in this study.

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Chapter 6 - Synthesis of Low Molecular Weight Poly-D-Lactic Acid (PDLA) by Azeotropic Polycondensation Process

Abstract

This study reports the polymer synthesis of poly-D-lactic acid (PDLA) from microbially derived D-lactic acid, via an azeotropic polycondensation process. A clear yellowish solid polymer with a low molecular weight of 3010 Da was obtained after 80 h of the polymerisation process using tin (ll)-2-ethylhexanoate as catalyst. Such low molecular weight polylactic acid (PLA) can find applications in various sectors, including the biomedical and agricultural sectors.

6.1 Introduction

Polylactic acid (PLA) is a biodegradable plastic that has received considerable attention recently due to its versatile applications in the food packaging, biomedical, pharmaceutical, automobile and household sectors. PLA offers advantages over conventional petrochemical-derived plastics being biodegradable and compostable (Garlotta, 2001; Sin et al., 2013a), and reduces the reliance on fossils fuel for the production of plastics (Arfat, 2017; Jamshidian et al., 2010). Moreover, PLA production has a lower environmental impact compared to conventional petroleum derived polymers, as the carbon emissions and the energy consumption are reduced by 15 to 60% and 25 to 55%, respectively (Dorgan et al., 2006; Tawakkal et al., 2014). The low toxicity of PLA,

along with its positive environmental characteristics, has rendered PLA as an ideal material for application in various fields, including in the food, biomedical and agricultural fields (Conn et al., 1995; Jamshidian et al., 2010).

Ring opening polymerisation and direct polycondensation are the most common methods used to synthesise PLA from lactic acid. In ring opening polymerisation, PLA is polymerised through a cyclic lactide intermediate. Companies such as NatureWorks® LLC (United States) and Corbion N.V. (Netherlands), produce PLA through this route (Corbion N.V., 2017; NatureWorks LLC, 2017; Vink et al., 2003). This protocol is of interest as it produces high molecular weight PLA. However, the procedure is complicated and time consuming because it involves several polymerisation steps and requires strict purity of the lactide monomer prior to PLA synthesis (Kim & Kim, 1999; Xiao et al., 2012). On the other hand, direct polycondensation offers significant advantages as the polymerisation process is simpler and easier in this case. In direct polycondensation, only one step for polymer synthesis is involved, during which the lactic acid solution is heated at 130 – 140 ^{o}C. Through this process, normally low molecular weight PLA (< 5000 Da) is produced with relatively weak mechanical properties; this is due to difficulties in removing the water from the reaction mixture as the polymerisation process progresses (Lasprilla et al., 2012; Sin et al., 2013b; Södergård & Inkinen, 2011). However, Ajioka et al. (1995) successfully produced high molecular weight PLA (> 300000 Da) using a single step synthesis using organic solvent with a catalyst (tin, Sn, powder) in azeotropic condition. Azeotropic polycondensation involved refluxing of the solvent under reduced pressure to remove the condensation water that was generated during polymer synthesis. This method had been patented and used by Mitsui Toatsu Chemicals (Japan) to synthesise PLA under the commercial name LACEA (Jamshidian et al., 2010; Kawashima et al., 2002; Vink et al., 2003).

Recently, there is also growing interest for PLA production from renewable sources. As lactic acid, the monomer for PLA, can be produced via fermentation, the use of renewable biomass resources that are rich in cellulosic or hemicellulosic components as fermentation substrates is a promising approach. Renewable biomass resources such as corn stover (Jiang et al., 2016; Öhgren et al., 2007), wheat straw (Garde et al., 2002; Saito et al., 2012), rice bran (Gao et al., 2008) and sugarcane bagasse (Adsul et al., 2007; Sasaki et al., 2012) have been investigated for lactic acid production. Depending on the microorganisms used for fermentation, the isomeric forms D-, L-, or mixtures of DL-lactic acid can be produced. Consequently, different polymers can be synthesised from these precursor molecules, namely poly-L-lactic acid (PLLA), poly-D-lactic acid (PDLA) and poly-DL-lactic acid (PDLLA) (Griffith, 2000). Among these, the PLLA polymer has received most attention and is commercially available in the market (Vink et al., 2003). However, PLLA has been associated with low thermostability, which limits its application in certain areas such as heat resistant food packaging (i.e. coffee and instant noodle cups and cutleries) (Gruber, 2001; Xu et al., 2006). However, Ikada et al. (1987) reported that a 1:1 polymer blend of PLLA and PDLA increased the melting temperature of the PLA polymer by 50 ^{o}C to 230 ^{o}C due to a the formation of a stereocomplex via van der Waals forces. The increase in thermostability of PLA due to the addition of PDLA has increased the research interest on D-lactic acid and its polymerisation to PDLA (Pivsa-Art et al., 2013; Quynh et al., 2009).

The main objective of the present study was to synthesise PDLA using a single step polycondensation process employing an azeotropic distillation approach. As starting material for PDLA synthesis purified D-lactic acid was used, which was produced by microbial fermentation using Dried Distillers Grains with Solubles (DDGS) as the substrate.

6.2 Materials and Methods

6.2.1 Materials

The D-lactic acid used in this study was obtained from the fermentation of *L. coryniformis* subsp. *torquens* using DDGS hydrolysate as substrate, as described in **Chapter 4**. D-lactic acid was purified following a series of purification using activated carbon, Amberlite® IRA120, H^+ (cation exchanger) and Amberlite® IRA67, Cl^- (anion exchanger), as described in **Chapter 5**. D-lactic acid was then concentrated in rotary evaporator at 45 ^oC. Finally, D-lactic acid with 91.8% purity, determined as described in **Chapter 5**, was obtained and was used for polymer synthesis.

6.2.2 Direct polymerisation of PDLA

Polycondensation of PDLA was conducted as described by Ajioka et al. (1995) with minor modifications. Figure 6-1 shows the experimental set up used for the lactic acid polymerisation process. 2 g of D-lactic acid were mixed with 40 ml toluene in 100 ml reaction flask, equipped with a Dean-stark apparatus and a mechanical stirrer. In the first step of the polycondensation process, the mixture was azeotropically dehydrated at 140 ^oC for 3 hours to remove the free water. After removing the condensed water that was trapped in the Dean-stark apparatus, the tube was packed with molecular sieve (4 Å) and calcium chloride in layers to remove small amounts of water dissolved in the organic solvent that was produced during PLA synthesis. 0.2 g tin (ll)-2-ethylhexanoate (stannous octoate) was added to the reaction mixture and then returned to reflux at 140 ^oC for another 80 hours. The polymer produced was then recovered by filtration and vacuum

dried using Bünchner funnel apparatus, followed by freeze drying (VisTis Sentry 2.0, Warminster, PA).

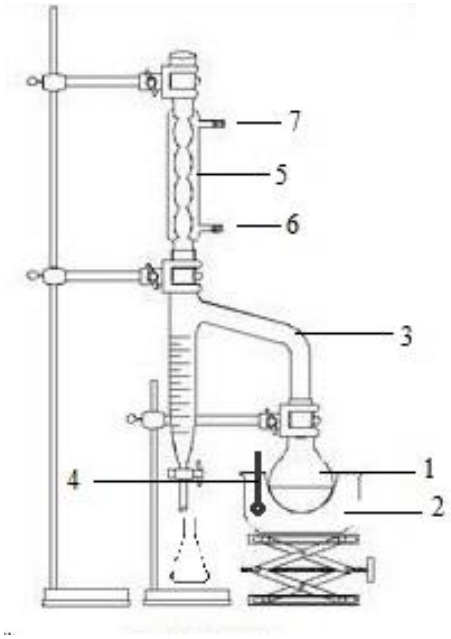

Figure 6-1: Experimental set up for the azeotropic polycondensation process. (1) reaction flask, (2) electrical heater with temperature control, (3) Dean-stark apparatus, (4) thermocouple, (5) condenser, (6) water in, and (7) water out

6.2.3 Gel Permeation Chromatography

The molecular weight and poly dispersity index (PDI) of PLA was determined by gel permeation chromatography (GPC), using an Agilent 1100 Series chromatography system that was equipped with a refractive index RID 1200 detector (35 ^{o}C). The flow rate was set at 1.0 ml/min and the molecular size was determined using a PL gel 5µM mixed-D column (300 X 7.5 mm) and a PL gel 5µM guard column (50 X 7.5 mm). Chloroform was used as the eluent. The PLA obtained was first dissolved in chloroform prior to analysis. The sample was analysed at room temperature using 20 µl injection volume. A calibration curve was generated using polystyrene standards with molecular weights ranging from 580 to 483,400 Da.

6.3 Results and Discussion

This study demonstrated the polymerisation of D-lactic acid obtained from microbial fermentation of DDGS. The azeotropic dehydration polycondensation method was employed using toluene as solvent and tin (ll)-2-ethylhexanoate (stannous octoate) as catalyst. Tin compounds and protonic acid have been found to be the best catalysts for the direct polycondensation of high molecular weight PLA (Ajioka et al., 1995). However, stannous octoate is preferable as it is approved by U. S. Food and Drug Administration (FDA) in the list of Indirect Additives used in Food Contact Substances under the Code of Federal Regulation, Title 21 (Food and Drugs). In addition to that, the removal of water is crucial in the direct polycondensation process, as its presence could initiate transesterification reactions or chain terminating reactions of the PLA produced (Proikakis et al., 2002; Södergård & Inkinen, 2011). During azeotropic polycondensation, the water that was present in the feedstock (D-lactic acid) or generated during the polymerisation process was continuously distilled off from the reaction mixture through the Dean-stark trapped.

After 80 h of polymerisation, a solid yellowish crystal PDLA was produced as shown in Figure 6-2. The average molecular weight of the PDLA produced was 3010 Da with a PDI of 4.1, which categorised it as a low molecular weight PLA. In contrast, Kim and Woo (2002) produced ten times higher molecular weight of PLLA (33000 Da) after 72 h reaction when the same polycondensation method was used. A similar finding was reported by Marques et al. (2010), where 80,000 Da molecular weight of PLLA was produced after 70 h of polymer synthesis. The differences in the molecular weight produced in both cases might be due to the differences in the type of catalyst (tin (ll)

chloride dihydrate) and solvent (*m*-xylene) used during the polymerisation process in those cases. Moreover, other factors such as the purity of D-lactic acid, the presence of small amounts water during the polymerisation process and the reaction temperature might also have contributed to the generation of low molecular weight PDLA (Fomin et al., 2011; Inkinen et al., 2011). Table 6-1 compares the molecular weight of PLA produced from this and other studies, where various synthesis methods, monomers (D- or L- lactic acid) and catalysts were employed.

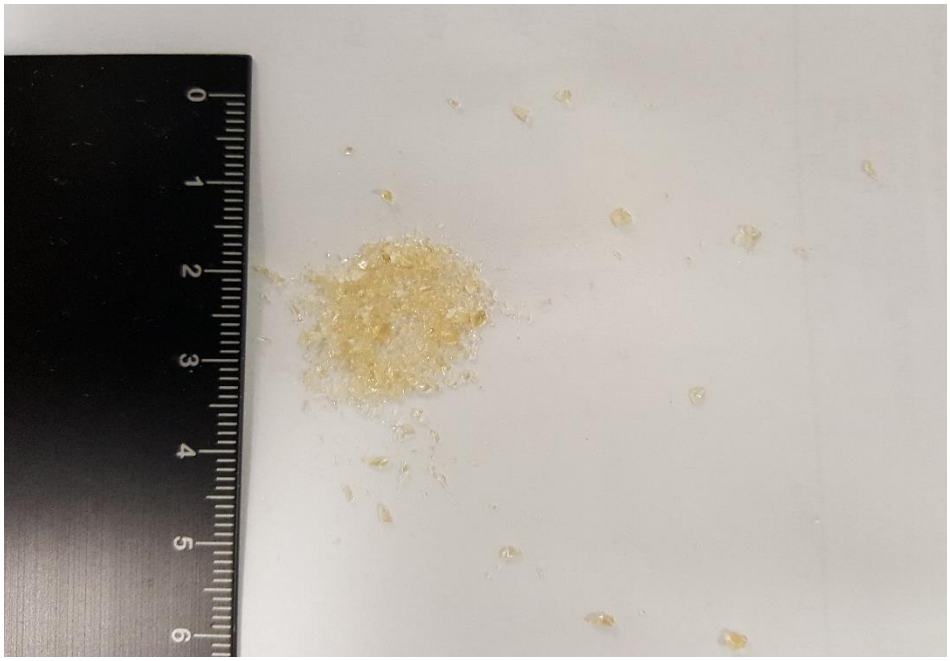

Figure 6-2: PDLA produced after 80 h polymerisation process at 140^{o}C in toluene

Table 6-1: Comparisons of molecular weight of PLA produced form this and other studies, using different synthesis methods, catalysts and monomers

Method	Monomer (lactic acid)	Catalyst	Polymer produced	Molecular weight, Da	References
Azeotropic polycondensation	D	Tin (ll)-2-ethylhexanoate, $SnC_{16}H_{30}O_4$	PDLA	3010	This study
	L	Tin (ll) chloride dihydrate, $SnCl_2.2H_2O$	PLLA	33000	Kim and Woo (2002)
	L	Tin (ll) chloride dihydrate, $SnCl_2.2H_2O$	PLLA	80000	Marques et al. (2010)
Direct polycondensation	L	-	PLLA	90000	Achmad et al. (2009)
	L	Antimony trioxide, Sb_2O_3	PLLA	67000	Kim and Kim (1999)
Melt polymerisation	DL	Tin (ll) chloride, $SnCl_2$	PDLLA	4100	Zhao et al. (2004)
1.Solid state polymerisation (SSP) 2.Melt polymerisation	L	Creatinine, CR	PLLA	120000	Huang et al. (2014)
1. Melt polymerisation 2. Solid state polymerisation (SSP)	D	2-Naphthalenesulfonic acid (2-NSA)	PDLA	33300	Pivsa-Art et al. (2013)
1.Ring opening polymerisation (ROP) 2.Chain extension	L	1.Tin (ll)-2-ethylhexanoate, $C_{16}H_{30}O_4Sn$ 2.Hexamethylene diisocyanate, HDI	PLLA	203000	Liu et al. (2013)
Enzymatic polymerisation	DL	-	PDLLA	2400	Lassalle and Ferreira (2008)
	L	-	PLLA	4500	Chuensangjun et al. (2012)

This is the first work demonstrating the synthesis of D-lactic acid polymer (PDLA) employing either a direct method or a one-step polymerisation (azeotropic polycondensation) process. Pivsa-Art et al. (2013) synthesised PDLA via a two-step polycondensation process, which included a melt polymerisation step followed by solid state polymerisation, and produced PLA with a molecular weight of 33300 Da. A number of interesting applications for low molecular weight PLA have been proposed in various fields, especially in the biomedical (Proikakis et al., 2002) and agricultural fields (Zhao & Wilkins, 2005). In the biomedical field, low molecular weight PLA is used as particles for parental controlled drug release in the human body in the form of microspheres, microcapsules, pellets or tablets. Using this approach, drugs are fabricated in a polymeric device (PLA) and the release of the drug is regulated by either diffusion through the polymer barrier or erosion of the polymer matrix (Jalil & Nixon, 1990). PLA is preferable compared to other polymers such as polyethylene and silicon rubber, as it does not require surgical retrieval, due to its natural degradation in the body. PLA is degraded by simple non-enzymatic hydrolysis (after exposure to moisture) to its monomer (lactic acid) (Lopes et al., 2012), which can be metabolised by the human body (Lopes et al., 2012; Proikakis et al., 2002). Drug delivery using low molecular weight PLA offers advantages over high molecular weight PLA as it has a weak retarding effect. Thus, the risk of material accumulation in tissues is reduced as PLA is relatively fast degraded to lactic acid (Lopes et al., 2012; Mainil-Varlet et al., 1997).

The same mode of application (controlled release) is also being used in the agricultural field, specifically the agrochemical sector, where PLA can serve as carrier for herbicides and pesticides that are released into the soil. The advantages of using low molecular weight PLA have been demonstrated by Zhao and Wilkins (2005), where delayed release of pesticides was observed in the early stages of application, which makes

it desirable for sensitive targets such as seed treatment. In their study, bromacil (pesticide) was incorporated into PLA in the form of granules and films, and the delayed release of bromacil was achieved via degradation and erosion of PLA. Since the degraded monomer (lactic acid) is safe and widely distributed in nature, the environmental problems of polymer disposal can be avoided through this approach (Jamshidian et al., 2010; Zhao & Wilkins, 2005).

In addition, Quynh et al. (2009) successfully produced PLA with high thermostability when commercial PLLA polymer (10400 Da) was crosslinked with low molecular weight PDLA (9830 Da). The stereocomplex formation of PLLA and PDLA was achieved via the melt polycondensation process, and increased the melting temperature of PLA from175.6 ^{o}C to 218.1 ^{o}C. This finding has widespread the application of PLA in other areas such as in the production of computer casings, automotive components and heat resistant food packaging (Sin et al., 2013a).

6.4 Conclusions

Low molecular weight PDLA polymer (3010 Da) was obtained via a single step azeotropic polycondensation process. Even though the molecular size of the PDLA produced was low, such low molecular weight is favourable for specific applications. Moreover, the advantage of the PDLA polymer is that it is produced from a low value agricultural biomass, i.e. DDGS, through a microbial fermentation process which reduces the dependency on petrochemical-derived plastics. However, research and development

on the PDLA properties, investigation of its potential applications and marketability as a biodegradable polymer should be continued.

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Chapter 7 - General Discussion, Future Perspectives and Conclusion

7.1 General Discussion

Polylactic acid (PLA) is a polymer with an expanding market demand due to its attractive physicochemical properties such as biodegradability, mechanical strength, biocompatibility and environmental-friendly nature. Moreover, PLA can be produced from bio-derived monomers originating from plant-based renewable materials, and such an approach provides alternative ways to replace petroleum-based polymers. To this end, renewable sources such as dried distiller's grains with solubles (DDGS) hold a potential as substrates for microbial lactic acid production due to their high polysaccharide content, which can be hydrolysed to fermentable simple sugars. DDGS production is increasing as a result of the rapid development of bioethanol plants and as such, DDGS is expected to reach a surplus, exceeding the demand for its utilisation as animal feed. As a consequence the need to identify alternative routes for DDGS valorisation is considered imperative (Villegas-Torres et al., 2015). The present work demonstrated an efficient strategy of exploiting wheat DDGS as raw material for polymer (poly-D-lactic acid, PDLA) production. The process involved a DDGS pretreatment and hydrolysis step, followed by microbial fermentation and lactic acid production, purification of D-lactic acid and finally, polymerisation process towards PDLA synthesis. As research on D-lactic acid for polymer synthesis is rarely been conducted, this research provided a fundamental knowledge of possible biotechnological approach that could be taken to produced high quality PDLA.

Alkaline pretreatment was proven successful in helping enzyme reaction in order to improve the hydrolysis of cellulose to glucose. Specifically, the DDGS cellulose to glucose conversion yield increased significantly after alkaline pretreatment, which shows the efficient of the pretreatment strategy; this can be further explored for other types of biomass samples. The aims of alkaline pretreatment were to assist the enzymatic hydrolysis by: i) removing the polymer barriers (hemicellulose and lignin) around cellulose, ii) increasing the internal surface area of biomass by swelling of the fibre, and iii) decreasing the cellulose crystallinity index by cleaving the hydrolysable linkages in cellulose and hemicellulose. The advantages of alkaline pretreatment are that it is relatively simple, scalable and low cost process. Moreover, the generated carbohydrate-rich solids have the potential to be used as substrates for other types of fermentation with commercial potential besides D-lactic acid, such as for the production of ethanol, platform chemicals (e.g. succinic acid) and carotenoids.

Fermentation is a commonly used method for the industrial production of lactic acid. This work studied three different lactic acid producers, namely, *Lactobacillus coryniformis* subsp. *torquens*, *L. pentosus* and *L. brevis*. However, only *L. coryniformis* produced exclusively D-lactic acid, while the other two produced mixture of D and L – lactic acid. As the optical purity is one of the most important parameters in determining the properties of PLA, subsequent experiments focused on *L. coryniformis*. Two fermentation methods were compared in terms of D-lactic acid production, namely, the conventional separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). The SSF process was more efficient than SHF, resulting in higher yields, concentrations and productivity of D-lactic acid. Apart from a simple, one-step bio-processing procedure, SSF is also industrially desirable as it may reduce the total production time and the processing steps for lactic acid production.

Moreover, SSF also reduced to accumulation of hydrolysis products (cellobiose, glucose), which have been reported to reduce the efficiency of enzymatic hydrolysis.

In terms of the recovery and purification of D-lactic acid obtained from the SSF process, the unavoidable dark colour of the fermentation broth was successfully reduced through treatment with activated carbon, followed by conversion of sodium lactate to lactic acid by strong acid cation exchange resin (Amberlite® IRA120, H^+). Subsequently, the acidified fermentation broth was further purified by adsorption using a weak base anion exchange resin (Amberlite ® IRA67, Cl^-). Around 80.4% of D-lactic acid of 91.8% purity was recovered from the initial fermentation both at the end of purification process, demonstrating the effectiveness of the designed purification stage. The use of activated carbon and adsorption resins for lactic acid purification offers significant advantages as they are safe (stable and resistant to organic fouling), reusable, and do not require sophisticated equipment for their operation.

Finally, the purified D-lactic acid was used as monomer for PDLA synthesis employing a single step azeotropic polycondensation process. The direct polycondensation process is possible since lactic acid has both hydroxyl (OH) and carbonyl (C=O) groups (Nampoothiri et al., 2010). A transparent, yellowish PDLA with molecular weight of 3,010 Da was obtained at the end of the polymerisation process. Even though the molecular weight of the synthesised PDLA was low, its application in various fields including biomedical (for controlled drug release in human body), and agricultural industries (for controlled herbicides/pesticide release in the soil) is desired as it provides shorter degradation times compared to high molecular weight PLA (Lopes et al., 2012).

The developed alternative routes for PDLA production from DDGS could have considerable potential for industrial applications. The use of low cost renewable sources as starting materials, combined with the simultaneous hydrolysis and fermentation (SSF) approach, by the application of reusable ion exchange resins for purification, and the direct polymer synthesis method, could impact on reducing the total PLA production cost. Even though in this study the work on polymer synthesis is preliminary and low molecular weight PDLA was generated at the end of polymerisation process, its potential application in biomedical and agriculture fields were identified. Moreover, this work also demonstrated that the production of environment-friendly biodegradable polymer could possibly reduce the negative impact of waste disposal on the environment by efficiently re-utilising the biomass.

7.2 Future Perspectives

The work presented in this thesis has demonstrated the successful utilisation of an industrial by-product, DDGS, as raw material for production of a biodegradable polymer (PDLA). However, some limitations were identified in this study that could potentially lead to future research as follow-up investigations. For example, alkaline pretreatment was proven a successful strategy to increase the yield of glucose hydrolysis during enzymatic breakdown of DDGS (**Chapter 3**). However, glucose losses were noted during the pretreatment process especially at high alkaline concentrations and temperatures (up to ~ 30%, w/w) which is not favourable as it resulted in less available carbon source for microbial fermentation. Therefore, future work could be directed towards investigating alternative pretreatment strategies, focusing on lowering the alkaline concentration and

reaction temperatures by combining other potential methods such as ultrasonication or microwaves, with the aim of reducing glucose losses. Moreover, other potential pretreatment approaches such as steam explosion and liquid hot water treatment should also be explored as these protocols do not require the use of reagents (alkaline / acid) and have previously shown significant effect on hemicellulose solubilisation (Kim et al., 2009; Yang et al., 2011). The pretreated mixture can then be directly used for enzymatic hydrolysis and subsequently for microbial fermentation.

Hemicellulosic materials, as in the case of DDGS, are mainly composed of cellulose and hemicellulose polymers. As a consequence, the enzymatically produced hydrolysates contained a mixture of hexoses (glucose) and pentose sugars (xylose, arabinose) that can be used as carbon sources in microbial fermentations. However, most homofermentative lactic acid bacteria, including the one used in this study (*L. coryniformis* subsp. *torquens*), catabolise only glucose as carbon source, thus leaving xylose and arabinose unconsumed in the fermentation media. For the efficient utilisation of lignocellulosic biomass as a raw material for microbial fermentations, the complete utilisation of all available carbon sources is required. To this end, co-fermentation with pentose-consuming lactic acid bacteria was conducted in this study (**Chapter 4**), which led to higher total lactic acid production, but was accompanied by the production of acetic acid as by-product and a reduced optical purity of D-lactic (mixtures of D- and L- lactic acid were obtained at the end of the fermentation). Future work should be directed towards complete sugar utilisation in complex media (as in the case of DDGS hydrolysates) while retaining the optical purity of lactic acid. One way to achieve this is through genetic modification of homo-D-lactic acid bacteria, through the introduction of xylose assimilation genes, for pentose sugar utilisation and redirection of the phospohoketolase pathway to the pentose-phosphate pathways in order to avoid by-

product formation as demonstrated by Okano et al. (2009). In addition, nitrogen is an important nutrient for cell multiplication during microbial fermentations. In this study, yeast extract (commercial nitrogen source) was used to support the growth of *L. coryniformis* subsp. *torquens* in DDGS hydrolysate as the protein from DDGS was extracted out during alkaline pretretment. However, the high cost of yeast extract ($0.15 to $0.35 per gram) (Kightlinger et al., 2014) is not favourable as it may negatively impact on the production costs of lactic acid. Since DDGS is a rich source of protein (~ 28%, w/w), future work can be directed towards i) preserving the protein in DDGS during pretreatment by using non-chemical pretreatment approaches such as ultrasonication, steam explosion or hot water treatment, and directly use the mixture media for subsequent enzymatic hydrolysis and fermentation, or ii) recover the protein that was fractionated into the liquid stream after alkaline pretreatment and utilise it as a supplement during fermentation. The latter process requires an in-depth study on protein recovery, assimilability and solubility prior its utilisation as nitrogen source in microbial fermentations.

The purification of lactic acid from the fermentation media is a multi-step process, as it involves transformation of lactate salts to lactic acid and at the same time removal of impurities from the fermentation broth. This study demonstrated a successful purification process with 92% purity of D-lactic acid and 100% sugar and protein removal; the quality of the purified D-lactic acid was sufficient enough to allow subsequent polymerisation process to take place (**Chapter 5**). A future approach could focus on combining all steps into one single purification step. This can be attained via sequential column chromatography (filled with activated carbon or resins). Even though, the feasibility of the proposed method is unknown as it has never been conducted before, lower costs are expected as the investigated resins are reusable and relatively low priced.

Singh et al. (2016) reported that downstream processing in industry contributes to 30 – 40% of total lactic acid production costs.

In this study, low molecular PDLA was generated via the direct azeotropic polycondensation process (**Chapter 6**). Although, the applications of low molecular weight PLA are identified in the biomedical and agricultural field, PLA with high molecular weight is always on demand as it is associated with strong mechanical properties, high melting temperature and slow degradation rate. These features can broaden the PLA applications in other areas such as food packaging, automotive and telecommunications. Future research should be directed to optimise the azeotropic polycondensation process parameters (catalyst, reaction time and temperature and type of organic solvent) as these could lead to the generation of high molecular weight PLA.

Overall, future work should focus on improving the biotechnological process involved in PDLA production. Starting from pretreatment of raw material to the synthesis of polymer, all steps at each stage have to be carefully examined in order to improve the quality of the end products Moreover, the utilisation of PDLA or mixtures of PDLA and PLLA as food packaging materials should also be explored as part of future work. This can be started by moulding the PLA into desired shapes, such as containers, films or sheets for food packaging, followed by studying the effect of utilising PLA as food contact materials on food products (sensory attributes and the shelf life of PLA).

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Appendix

List of conferences/seminars, rewards/awards, professional memberships and publication during PhD program:

A. Conferences / Seminars:

- Poster presentation at Grain, Cereal and Bakery Waste Valorisation Meeting on 7th June 2017 in University of Reading, UK, under title "Simultaneous Saccharification and D-lactic Acid from DDGS"

- Oral Presentation at International Conference of Bioprocess and Fermentation Technology on 21st-22nd June 2017 in Vienna, Austria, under title "Simultaneous Saccharification and Fermentation (SSF) for D-Lactic Acid Production from Dried Distillers Grains with Solubles (DDGS)"

- Oral presentation at International Symposium of Green Chemistry on 13th-19th May 2017 in La Rochelle, France, under title "Alkaline Pretreatment: Effective Approach for Cellulose Digestibility in Dried Distillers Grains with Soluble"

- Oral Presentation at Department of Food and Nutritional Sciences Seminar on 15th February 2017 in University of Reading, UK, under title "Alkaline Pretreatment of Wheat DDGS for Improved Cellulose Digestibility".

- Participant in Research Image Competition, Doctoral Research Conference 2016 on 23rd June 2016 in University of Reading, under title "Morphological Structure of Dried Distillers Grains with Solubles".

- Poster Presentation at SCI Young Researchers in Agri-Food 2016: Food Quality and Sustainability from Plough to Plate on 12th May 2016 in University of Reading, UK, under title "Dried Distillers Grains with Solubles (DDGS) as a Potential Substrate for D-Lactic Acid Production".

- Oral Presentation at Food Bioscience Research Seminar, Department of Food and Nutritional Sciences on 5th May 2016 in University of Reading, UK, under title "Production of D-Lactic Acid from Dried Distillers Grains with Solubles (DDGS)".

B. Rewards/ Awards:

- Netherfield Travel Award for attending International Conference of Bioprocess and Fermentation Technology in Vienna on 21st - 22nd June 2017
- RSC Travel Grant for attending International Symposium of green Chemistry in La Rochelle, Paris on 16th - 19th May 2017
- Graduate School Travel Grant, University of Reading

C. Professional Membership:

- Associate Member of Royal Society of Chemistry (RSC)
- Early Career Scientist, Society for Applied Microbiology (sfam)
- Member of FoodWasteNet, UK

D. Publication:

Zaini, N. A. M., Chatzifragkou, A. and Charalampopoulos, D. 2018. Alkaline fractionation and enzymatic saccharification of wheat dried distiller's grains with solubles. Submitted to Waste and Biomass Valorisation Journal.

Zaini, N. A. M., Chatzifragkou, A. and Charalampopoulos, D. 2018. Microbial production of D-lactic acid from Dried Distiller's Grains with Solubles (DDGS). Submitted to Engineering in Life Sciences Journal.