Co-Fermentation of Wheat and Whey Permeate for Ethanol Production and Chemical Characteristics of the Resulting Dried Distillers' Grains with Solubles

by

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Abstract

The vast majority of global bio-ethanol production uses food crops as the primary feedstocks, which leads to the high dependence of ethanol production cost on the price of the crop-based carbon resources. The demand, and interests, to use cheaper non-crop-based carbon sources for the ethanol industry are increasing globally. Whey permeate, a residue of the cheese industry, can be a promising alternative feedstock for ethanol production. A major component in whey permeate is lactose which is a readily available fermentable carbon source. To investigate this possibility, the main goal of this study was to utilize whey permeate as a co-substrate to wheat for ethanol production. It is hoped that this approach will not only help alleviate the issues related to crop-based feedstocks for the ethanol industry but also create a profitable market to consume whey permeate for the cheese industry.

In this study, whey permeate was used as a co-substrate to replace part of the wheat for ethanol production by *Saccharomyces cerevisiae*. The simultaneous saccharification and fermentation occurred with β-galactosidase added at the onset of the fermentation to conduct whey lactose hydrolysis. *Aspergillus oryzae* and *Kluyveromyces lactis* β-galactosidases were two enzymes selected and used in the co-fermentation respectively for the comparison of their effectiveness on lactose hydrolysis. Ethanol yield of the co-fermentation of wheat and whey permeate was evaluated. It was found that *A. oryzae* β-galactosidase was efficient for lactose hydrolysis during the co-fermentation and the ethanol yield efficiency was enhanced by the whey permeate supplementation.

Due to the additional cost associated with the usage of soluble *A. oryzae* β-galactosidase, the possibility and reusability of immobilized β-galactosidase in the co-

fermentation was investigated. The immobilized β-galactosidase was shown to be as effective as the soluble enzyme for the co-fermentation and can be reused for at least three cycles without compromising ethanol yield.

Dried distillers' grains with solubles is an important co-product from the ethanol production with dry-grind process, which substantially contributes to the economical and sustainable ethanol manufacturing. A comprehensive evaluation of chemical characteristics of the dried distillers' grains with solubles produced from the fermentation of lactose containing batches was conducted. Differences in the chemical characteristics were observed between the dried distillers' grains with solubles derived from the fermentation of wheat only and the co-fermentation of wheat and whey permeate.

In summary, the supplementation of whey permeate contributed to ethanol production as a co-substrate to wheat with *A. oryzae* β-galactosidase added for lactose hydrolysis during the fermentation. The immobilized β-galactosidase can be effectively used in the co-fermentation with good reusability. Though the supplementation of whey permeate changed the chemical characteristic profile of the resulting dried distillers' grains with solubles, the nutritional values were still within the range of the variations published.

Preface

This thesis is an original work by Yiqiong Jin. No part of this thesis has been previously published. Dr. Archana Parashar contributed to manuscript edits. The fermentations conducted in 5-L bioreactors (presented in Chapter 3) were carried out with bioreactor handling and technical support provided by Alexandra MacDonell-Whiddon and Stephen Andersen. The mineral contents of the dried distillers' grains with solubles in Chapter 5 were analyzed by the Natural Resources Analytical Laboratory, the Department of Renewable Resources, University of Alberta. The contents of amino acids in Chapter 5 were determined and provided by the Agricultural Experiment Station Chemical Laboratories, University of Missouri.

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List of Abbreviations

ANOVA = analysis of variance

2,3-BD = 2,3-butanediol

BOD = high biological oxygen demand

CDIC = Canadian Dairy Information Centre

COD = chemical oxygen demand

CRFA = Canadian Renewable Fuels Association

CWP = cheese whey powder

DDGS = dried distillers' grains with solubles

DEPC = diethyl pyrocarbonate

DLP = delactosed permeate

DM = dry matter

DNFB = dinitrofluorobenzene

FAO = Food and Agriculture Organization

FID = frame ion detector

GC = gas chromatography

GOPOD = glucose oxidase-peroxidase

GOS = galacto-oligasaccharides

HMF = hydroxymethylfurfural

HPLC = high performance liquid chromatography

ICP-OES = inductively coupled plasma-optical emission spectrometry

LAU = lactase activity unit

NRC = Natural Resources Canada

OECD = Organization for Economic Co-operation and Development

PHAs = polyhydroxyalkanoates

PHB = poly-3-hydroxybutyrate

PHBV = poly-3-(hydroxybutyrate-co-hydroxyvalerate)

PLBA = pseudolactobiouronic acid

PVA = polyvinyl alcohol

RFA = Renewable Fuels Association

SHF = separate hydrolysis and fermentation

SSF = simultaneous saccharification and fermentation

USDE = U.S. Department of Energy

WPC = whey protein concentrate

WPI = whey protein isolate

1 Introduction

1.1 Project background

Whey is a by-product from the cheese industry. The major components in whey are lactose and whey protein which account for about 75% and 10%, respectively of the total solids (Mawson, 1994). Whey protein can be separated from whey by ultrafiltration, resulting in a lactose-rich stream referred to as whey permeate. Lactose is the most abundant component in whey and whey permeate besides water. Current whey and whey permeate utilization consumes only a small portion of lactose generated. It is quite plausible that the large amount of whey lactose surplus can be used as a low-cost and promising substrate for industrial chemical and fuel production.

The bio-ethanol industry has significantly expanded worldwide over the last few decades. In North America, ethanol is mainly produced from grains, such as corn and wheat. The production cost is highly related and controlled by the price of these grains resulting in less competitiveness of ethanol than fossil fuels. Therefore, numerous attempts of using lignocelluloses as an alternative cheaper substrate have been made to reduce the production cost of ethanol. However, the current technologies for lignocellulosic ethanol have several major challenges such as the presence of inhibitory compounds in hydrolysates caused by thermo-chemical pre-treatment (Parawira and Tekere, 2011), relatively high cost of enzymatic hydrolysis, and inefficient pentose fermentation (Viikari, *et al*., 2012). Lactose, a simple disaccharide consisting of glucose and galactose, is significantly easier to hydrolyze to sugar for fermentation than lignocellulosic derived sugars. Utilization of whey lactose as a low-cost substrate for

ethanol production will be a more immediate and feasible strategy to alleviate current economic strains on ethanol production using grains.

The commercial production of ethanol from whey lactose can date back to 1970s (Lyons and Cunningham, 1980; Guimaraes *et al*., 2010). Lactose-assimilating *Kluyveromyces* strains were used for the commercial production, particularly *K. marxianus*. The low ethanol titer of 2.5-4.2% (v/v) was the major drawback associated with the commercial protocol, which requires high energy input in the distillation process (Guimaraes *et al*., 2010). That is mainly due to the low starting lactose concentration (approximate 5%) in the whole whey used. However, considerably prolonged fermentation time was observed for the fermentation with increased substrate concentrations by *Kluyveromyces* yeasts (Hahn-Hagerdal, 1985; Zafar and Owais, 2005).

The majority of the studies on ethanol production have used whey lactose as the sole carbon source (Guimaraes *et al*., 2010). So far, only a few attempts of co-fermenting whey lactose with other substrates have been made (Gibbons and Westby, 1988; Oda and Nakamura, 2009; Kadar *et al*., 2011). This was probably due to the insufficient amount of whey/whey permeate produced in one cheese plant or cheese plants in the same region to support the ethanol production using whey lactose as the sole carbon source. For example, the annual cheese production of western Canada in 2013 (January-November) was 74,490 kg (CDIC, 2013), resulting in whey production of 670,410 kg which was calculated according to the cheese to whey production ratio of 1:9 (Kosikowski, 1979). It was reported that the average lactose content in whey is around 5% (w/w, wet basis) (Matthew 1978; Clark, 1988). So approximate 33,520 kg lactose was present in the 670,410 kg of whey. If the theoretical lactose-to-ethanol yield efficiency can be obtained,

1 g of lactose will produce 0.537 g of ethanol. Lactose of 33,520 kg would give 18,000 kg of ethanol (equivalent to 22,814 L at 20 °C) which is significantly lower than the ethanol production capacity of 673,000,000 L per year in western Canada (CRFA, 2013). Therefore, co-fermenting whey lactose with other substrates is a more practical and feasible solution for whey lactose utilization in the regions with insufficient whey/whey permeate for full replacement of grains for ethanol production as it also allows, if collocated, the reuse of process water as well. In addition, supplementation of other nutrients, such as lipids and nitrogen source, was required for the fermentation of whey/whey permeate alone due to the limitation of nutrients in whey/whey permeate (Koushki *et al*., 2012). While co-fermentation of whey lactose with grains can enable yeasts acquire the nutrients from the grains supplied, resulting in less nutrient supplementation.

Based on the above, it was hypothesized that whey lactose can be used as a co-substrate along with grain starch for ethanol production by *S. cerevisiae* without any additional nutrient supplementation. In this study, whey permeate was used as the source of whey lactose for partial replacement of wheat and process water used in the conventional wheat-to-ethanol fermentation. The overall objective was to evaluate the effect of whey permeate supplementation on ethanol production as well as the nutritional value of dried distillers' grains with solubles (DDGS), an essential by-product from ethanol production with dry-grind process. The short-term and long-term objectives of this study were stated as follows.

1.2 Objectives

1.2.1 Short-term objectives

1) To establish a simultaneous hydrolysis and fermentation process for utilization of lactose from whey permeate;
2) To incorporate whey permeate into wheat-to-ethanol fermentation to replace part of wheat and process water;
3) To reuse β-galactosidase for lactose hydrolysis during fermentation by the immobilization in LentiKats Biocatalyst;
4) To evaluate the nutritional values of DDGS from the co-fermentations of wheat and whey permeate.

1.2.2 Long-term objectives

1) To reduce production cost of ethanol fermented from wheat by substituting whey permeate for part of wheat and process water;
2) To create a promising and profitable off-take market for whey permeate.

1.3 Significance

1.3.1 Bio-ethanol industry

Incorporation of whey permeate into wheat-to-ethanol fermentation not only reduces the amount of wheat and process water used for ethanol production, but also lessens enzyme consumption for viscosity reduction, liquefaction, and saccharification of wheat grain. In addition, the viscosity of mash will decrease significantly as part of the viscosity-contributing wheat grain is replaced by the low-viscosity whey permeate,

leading to less energy input for mechanical mixing during mash preparation and fermentation.

1.3.2 Cheese industry

Since direct whey/whey permeate disposal into fields and rivers is a severe environmental threat due to the associated high biological oxygen demand (BOD) and chemical oxygen demand (COD) of whey/whey permeate. Sewage treatment of whey/whey permeate is required prior to its disposal. The strategy developed in this project can help the cheese industry to make profits out of whey permeate, instead of investing capital for waste abatement, which substantially alleviate the economic burden for the cheese industry.

2 Literature Review

2.1 Bio-ethanol production

2.1.1 Global bio-ethanol production and marketplace

The interest of using ethanol as an automotive fuel emerged as early as the 19th century through the first ethanol-based engines invented by Samuel Morey and Nicholas Otto (Demirbas *et al*., 2009). The Ford Motor Company continued the effort of developing a flexible vehicle which can be driven using ethanol. In the beginning of the 20th century, the first flexible cars capable of using any combination of ethanol and gasoline were built (Solomon *et al*., 2007). Since then, the use of ethanol as a fuel additive has been initiated in Europe and the United States, however, with a significantly small market.

Rapid growth of the ethanol industry has been observed starting from the 1970s. Due to the global oil crisis and overproduction of sugar, Brazil initiated the National Alcohol Program (ProAlcohol) which aimed to utilize sugarcane as the feedstock for large-scale ethanol production in 1975. The United States showed interest in promoting ethanol production and building its ethanol industry in the early of 1980s (Mussatto *et al*., 2010). As shown in Fig. 2-1, the annual ethanol production of the United States gradually increased during the period of 1980-2006, followed by a dramatic enhancement from 2007 to 2011. The ethanol production of 2012 and 2013 decreased slightly, which was largely attributed to the widespread severe drought (RFA, 2014a). Currently, the United States is the number one ethanol producer in the world, followed by Brazil, the European Union, China, India, and Canada (RFA, 2014b).

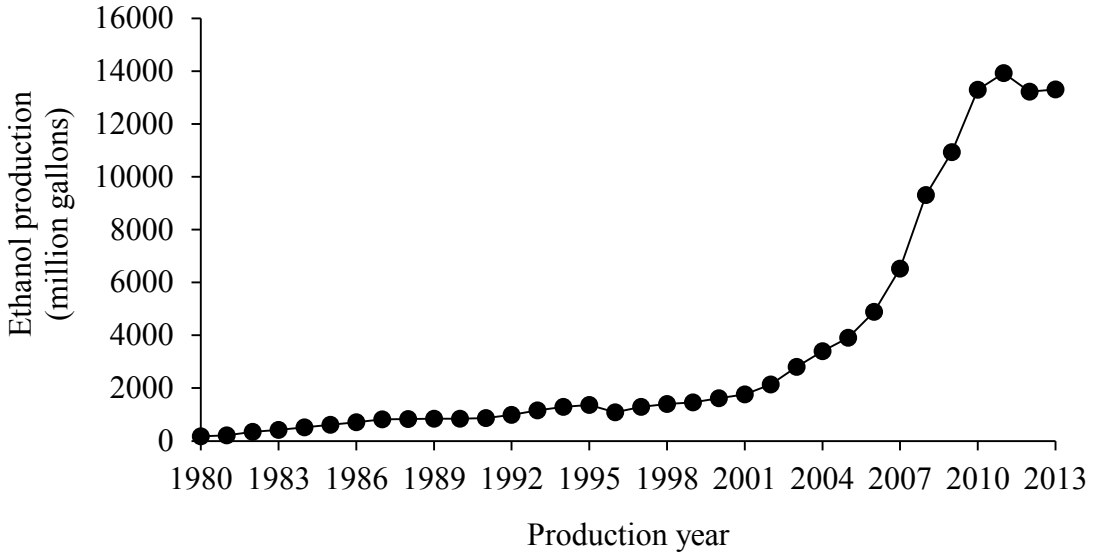


Figure 2-1 Historic ethanol production of the United States during the period of 1980-2013. (Data source: Renewable Fuels Association)

2.1.2 Feedstocks

The vast majority of current industrial ethanol output was derived from the input of sugar- and starch-based feedstocks such as sugar cane, sugar beet, sorghum, corn, wheat, cassava, rice, and others (Sanchez and Cardona, 2008; Mussatto *et al*., 2010). The selection of these crop-based raw materials for ethanol production substantially depends on the cultivation conditions required by the crops. For instance, ethanol in Brazil is produced exclusively from sugar cane, a tropical crop widely planted in Brazil (Mussatto *et al*., 2010). Other than the sugar- and starch-based feedstocks, lignocellulosic biomass as an alternative raw material has attracted the considerable attention of both the ethanol industry and academic research owing to its large availability, low cost, and lack of competition with food production (Viikari *et al*., 2012).

The major three processes involved in ethanol production are the conversion of raw materials to fermentable sugars, the fermentation of fermentable sugars to ethanol, and

the recovery of ethanol. In sugar-based crops, fermentable sugar-containing juice can be simply obtained by milling and pressing, such as sucrose from sugar cane. However, the ethanol production using starch-based crops requires hydrolysis of starch to glucose, as the yeast strain *S. cerevisiae* used cannot utilize starch directly due to the absence of the genes for α-amylase and amyloglucosidase expression (Inlow *et al*., 1988). Similar to starch, cellulose and hemicellulose, two major components of lignocellulosic materials that hold great interest and promise for ethanol production, are polysaccharides for which the hydrolysis is indispensable for the subsequent yeast fermentation. Though numerous studies have been carried out to utilize lignocellulosic biomass for ethanol production, there are still a few major technological and economical bottlenecks associated with the conversion process (Sanchez and Cardona, 2008; Chiaramonti *et al*., 2012; Viikari *et al*., 2012).

Ethanol derived from sugar-based crops accounted for approximately 29% of the global ethanol production in 2013; while at least 68% was manufactured using starch-based crops, contributed mainly by the United States, China, Canada, and the European Union (RFA, 2014b). Up to now, only two ethanol plants in the United States, KL Process Design Group and BP Biofuels North America, are operating on a lignocellulosic ethanol platform. Together they provide an operational capacity of 3.0 million gallons per year, which is only 0.02% of the total ethanol output of the United States in 2013 (RFA, 2013). As the majority of ethanol has been produced from starch-based crops, and ethanol is produced exclusively from wheat and corn in North America (RFA, 2014b; CRFA, 2013), the starch-to-ethanol process was selected for further discussion in the following section.

2.1.3 Current technologies for starch-to-ethanol production

The procedure of ethanol production using starch-based crops includes milling, cooking, liquefaction, saccharification, fermentation, and distillation. Since distillation procedures are fairly standard throughout the industry, numerous efforts and attempts have been made to improve the processing steps prior to the distillation. Compared to the ethanol production using sugar-based crops, more technologies were developed for starch hydrolysis and fermentation process involved in the starch-to-ethanol production.

2.1.3.1 Wet mill and dry grind

Wet milling has been employed by some ethanol plants using corn as the feedstock (Jacques *et al*., 2003). First, the corn kernel is soaked in water and dilute sulfurous acid for 24-48 h, which is referred to as "steeping". Steeping enables the subsequent separation of major components in the corn, such as germ, fiber, gluten, and starch. The segregated starch is subject to liquefaction, saccharification, and fermentation for ethanol production. The germ is recovered for the preparation of corn oil while the fiber and gluten are sold as feed ingredients to the livestock industry (RFA, 2014c). In the dry grind process, the grain kernel is directly ground by a hammer mill without the separation of different components (Jacques *et al*., 2003). All of the nutrients in the grain undergoes liquefaction, saccharification, and fermentation. The components which cannot be used by the yeast (e.g. fiber and gluten) remain in DDGS, the valuable co-product of the ethanol production using dry grind process.

Both the wet mill and dry grind processes are commercially adopted for the ethanol production with the key difference between these two processes being the separation of different components in grain kernels. Due to the additional steps involved in the wet mill

process, higher capital investment is required (Bothast and Schlicher, 2005). The rapid growth of the ethanol industry has been in large part due to the dry-grind ethanol plants because of their lower capital costs. Currently, more than 80% ethanol plants in the United States are running based on the dry grind process (USDE, 2013).

2.1.3.2 Enzymes for starch hydrolysis

Acids were traditionally used for starch hydrolysis (Honsch, 1957). However, acid hydrolysis has been predominantly substituted by enzymatic hydrolysis since the 1970s because of acid hydrolysis's extreme conditions and undesirable secondary reactions (Sanchez and Cardona, 2008; Robertson *et al*., 2006). Thermostable α-amylase and glucoamylase are the two enzymes responsible for enzymatic starch hydrolysis. The process with α-amylase added for breaking down starch polymers into dextrins is called liquefaction which is first carried out during mash cooking at 90-120 °C in a jet cooker. α-Amylase is added to the slurry prior to cooking and when the temperature of the slurry rises above the starch gelatinization temperature, the starch granules start to absorb water and swell, resulting in a loose starch structure for α-amylase to access. After cooking, additional α-amylase is added for further liquefaction, followed by the saccharification using glucoamylase which break down dextrins to free glucose (Jacques *et al*., 2003; Bothast and Schlicher, 2005).

In the mid-2000s, the concept of native or raw starch hydrolysis (also known as granular starch hydrolysis or cold hydrolysis) was developed and has been discussed by Robertson *et al*. (2006). So far, two commercial products of granular starch hydrolyzing enzymes (GSHE) have been used for raw starch hydrolysis in the dry grind ethanol production (Wang *et al*., 2005; Sharma *et al*., 2007; Vidal Jr. *et al*., 2009; Gibreel *et al*.,

2009). The commercial offerings include STARGEN™ marketed by Genencor International and GSHE NS50040 from Novozyme. Both enzyme cocktails include mixtures of α-amylases and glucoamylases. It is estimated that the energy used in the cooking process is about 10-20% of the energy of ethanol produced (Robertson *et al*., 2006). Therefore, the energy cost is substantial and provides justification for the adoption of an efficient raw starch hydrolysis process at considerably lower temperature than the conventional enzymes.

2.1.3.3 Simultaneous saccharification and fermentation

Process integration is one of the most important approaches used to develop and improve the processes for cost-effective ethanol production (Cardona and Sanchez, 2007). The process integration targets the combination of independent steps in a single unit such that the number of operating units and energy input can be significantly reduced. Simultaneous saccharification and fermentation (SSF) is a strategy developed essentially based on reaction-reaction integration, of which the saccharification of biomass and the conversion of fermentable sugars to ethanol are carried out synchronously.

The SSF process was first proposed by Takagi *et al*. (1977) for ethanol production from cellulose. However, the significant advantages of the SSF process has been well demonstrated by the starch-to-ethanol industry. Conventionally, separate hydrolysis and fermentation (SHF) was adopted for the starch-to-ethanol production, in which liquefaction, saccharification, and fermentation were conducted separately. Since at least 2003, an individual tank for saccharification of starch has been rarely used in starch-to-ethanol processes (Jacques *et al*., 2003). It is reported that the SSF process not only

alleviates the product inhibition on the starch-hydrolyzing enzymes by avoiding the accumulation of glucose, but also reduces the overall operation time (Kobayashi *et al*., 1998).

In addition to the advantages stated above, there is another essential benefit associated with the SSF process. It is lower osmotic stress to the yeast compared to that of the SHF process, as glucose release and consumption occur simultaneously during the fermentation leading to a lower sugar concentration (Cardona and Sanchez, 2007). This kinetic property of the SSF process makes high gravity and very high gravity technologies possible for the ethanol industry. These two technologies are developed to reduce process water usage, increase final ethanol concentration, and thus reduce the cost of downstream processes. For example, less energy cost is required for ethanol distillation and treatment of effluent.

The high gravity fermentation was first suggested in the 1980s, in which the initial dissolved solids concentration was over 200 g/L (Bai *et al*., 2008). The high gravity technology has been successfully applied in the industrial ethanol production, resulting in a dramatic increase of final ethanol concentration from 7-8% (v/v) to 10-12% (v/v) (Puligundla *et al*., 2011). The very high gravity fermentation was proposed by the research group of Ingledew at the end of the last century, which is defined as the fermentation of mashes containing 27 g or more dissolved solids per 100 g mash (Thomas *et al*., 1993; Wang *et al*., 1999). The current very high gravity technology adopted by the ethanol industry can obtain a final ethanol titer of more than 15% (v/v) (Jacques *et al*., 2003; Puligundla *et al*., 2011).

2.2 Whey

Whey is the residue produced by the precipitation of casein from milk, which is the principal operation of cheese and casein production. About 55% of milk nutrients are retained in whey (Siso, 1996). The major components are lactose, whey protein, milk salts, and fat. Due to the variety of methods adopted to manufacture individual cheese or casein products, the compositions of whey slightly differ from each other (Mawson, 1994). The approximate compositions of different whey are shown in Table 2-1.

Table 2-1 Approximate composition of whey from different sources[a]

Constituent (%, w/w, wet basis)	Cheese whey	Cheddar cheese whey	Casein whey	Lactic acid casein whey	Sulphuric acid casein whey	Rennet casein whey
Total solids	6.4	5.6	6.5	5.6-6.4	6.3	5.8-6.5
True protein[b]	0.55	0.55	0.55	0.56	0.56	0.62-0.73
Lactose	4.8	4.0	4.9	3.8-4.4	4.7	4.5-5.2
Ash	0.5	0.5	0.8	0.66-0.76	0.8	0.42-0.49
Lactate	0.05	0.08	0.4	0.63-0.73	-	0.02

[a]Data taken from Matthews (1978), Clark (1988), and Teknotext (1995).

[b]True protein = (Total nitrogen – Non-protein nitrogen) × 6.38

Whey is classified into two main types - acid and sweet whey. The whey obtained from casein production using acid-assisted precipitation is classified as acid whey with a pH of 4.3-4.6. The low pH is achieved biologically by lactic acid produced during the fermentation of the starter or chemically by the addition of mineral acids, such as hydrochloric acid and sulphuric acid (Bylund, 1995). Whey with a pH at 5.9-6.6 is known as sweet whey which is produced from cheese and rennet casein manufacture. As coagulum formation to recover cheese and rennet casein is caused by the enzyme activity of rennet instead of acids, the pH of the solution remains close to the original pH of milk.

More than 80% of whey is generated from cheese output (International Dairy Federation, 2010). The production of 1 kg cheese is reported to generate approximately 9 kg whey (Kosikowski, 1979). The latest data of dairy processing given by the Organization for Economic Co-operation and Development (OECD) and the Food and Agriculture Organization (FAO) of the United Nations in 2013 indicates that the average annual cheese output of 2010-2012 was around 20.4 million metric tonnes and it was projected to have a moderate increase to reach 23.4 million metric tonnes in 2022 (OECD-FAO, 2013). Therefore, an enormous amount of cheese whey (about 183.6 million metric tonnes per year) would have been produced in 2010-2012 from cheese manufacturing alone.

The high content of organic compounds and output of whey reveals the remarkable potential to utilize it as a feedstock for the production of industrial chemicals and fuels; however, it also can be considered as a heavy burden on the environment if disposed without any treatment. The BOD and COD of whole whey are 35000 - 45000 mg/L and 60000 - 70000 mg/L, respectively (Mawson, 1994). Recovery of protein from whey only reduces around 15% of the BOD and COD, as the high values of these two parameters are significantly contributed by the lactose retained from milk (Siso, 1996).

2.3 Options for Whey Utilization

The earliest uses of whey were emitting into aquatic systems, spreading over fields, and feeding animals as drinking water. With the increasing awareness of environmental protection, legislations regarding safe disposal of industrial waste have been enacted globally, exerting additional cost over cheese and casein manufacturers. Currently, whey disposal into aquatic systems and spreading onto fields is rarely adopted by the cheese

industry (Jelen, 2003). The applications of whey as soil fertilizer and animal feeds are not favorable approaches due to the high transportation cost and these applications' small capacities to consume the large supply of whey. Therefore, the exploration of efficient and profitable applications of whey has a strong appeal to both industrial manufacturers and the academic community at large.

In addition to the traditional treatments stated above, various value-added enhancements of whey were described in many reviews (Siso, 1996; Audic *et al.*, 2003; Yang and Silva, 1995; Panesar and Kennedy, 2012). Pathways for the commercial and lab-scale utilization of whey are shown in Fig. 2-1. According to the composition in the final products, the utilization of whey can be classified into two groups including direct and indirect use. The direct use is defined as the direct application or consumption of the nutrients from whey. The indirect use of whey is classified as the use of compounds which have been modified chemically or biologically and derived from the original components in whey.

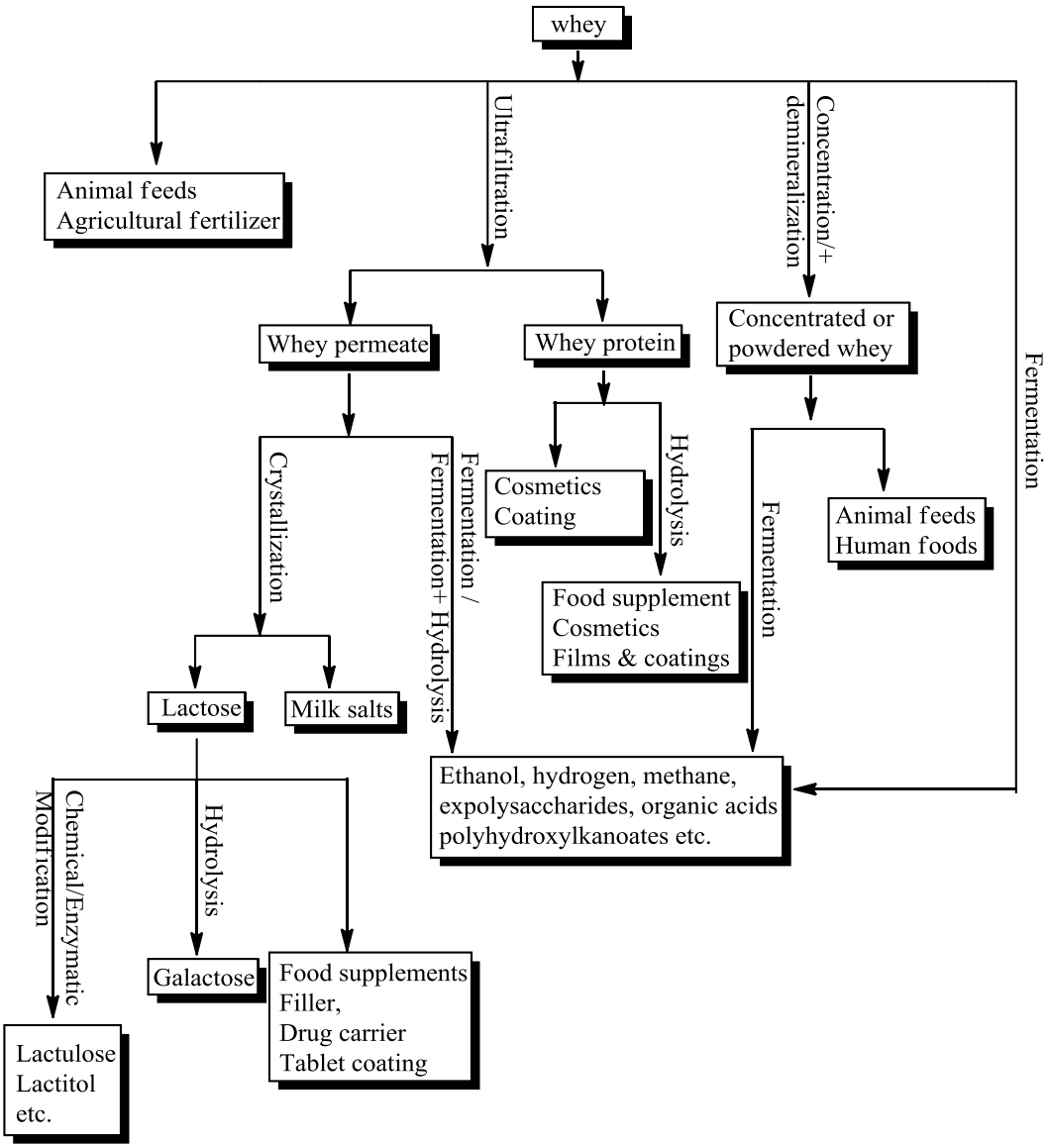


Figure 2-2 The commercial and lab-scale whey utilization

2.3.1 Direct use

The direct use whey products are obtained with no further processing or from physical treatments after liquid whey is harvested. The conventional use of whole whey as agricultural fertilizer and animal feed without treatment is included in this group. In order to add value, various technologies are employed to further process whey. Reverse osmosis and vacuum evaporation are often conducted for whey concentration followed

by spray-drying to produce condensed or powdered whey (Bylund, 1995). The prime markets for these products are animal feeds and ingredient supplements to human foods (Siso, 1996; Marwaha and Kennedy, 1988). Demineralization can be carried out to eliminate the excessive saline taste, thereby expanding its application into dietetic or baby foods (Siso, 1996). Moreover, concentrated whey is utilized as the raw material to produce whey cheese, for example, Ricotta and Mysost (Jelen, 2003).

Compared to the whole whey products, the fractionation of nutrients for high value-added products is economically attractive. Fractions include whey protein, lactose, and milk salts (Pesta *et al.*, 2007). Ultrafiltration is commonly adopted for protein recovery to produce whey protein concentrate (WPC) or isolate (WPI) (Siso, 1996; Marwaha and Kennedy, 1988). The protein content of WPC is 30-90%, while WPI possesses higher protein content of > 90% (Audic *et al.*, 2003). They are suitable for various human foods as a protein supplement, including even dietetic or baby foods (Siso, 1996). In 1996, Smithers *et al.* discussed the potential application of whey protein as functional foods. Today, whey protein is a popular dietary supplement available on the market. In terms of non-food uses, whey protein can be applied in cosmetology and pharmacology as hydrating and anti-wrinkle agents, edible films, and coating (Audic *et al*., 2003).

Lactose, the most abundant nutrient in whey and whey permeate, can be crystallized and refined. Lactose from whey permeate is typically used in the US, while the whole whey is preferred by manufacturers in Western Europe (Yang and Silva, 1995). Ingredient supplementation in infant formula and filler or coating agent for tablets are two principal applications of lactose (Audic *et al*., 2003). Pesta *et al.* (2007) reported an interesting possibility for utilization of delactosed permeate (DLP), a byproduct of lactose

crystallization. Further processing can be carried out to derive the milk salts from the DLP which are consumed not only as a source of salty taste but also as a nutritional supplement (Pesta *et al.*, 2007). For instance, Lactoval® HiCal, a milk minerals complex rich in calcium, phosphorus, and magnesium, is marketed as a natural calcium source for the fortification of a wide variety of food products by DMV International (DMV, 2014).

2.3.2 Indirect use

Indirect use whey products are value-added compounds derived from the original components in whey through either chemical or biological conversions. Whey lactose is the component at which most of the studies on whey utilization have been focused. The availability of lactose in whey and whey permeate is more than the demand required for the food applications, making its non-food applications of prime interest (Audic *et al*., 2003). Lactose derivatives, such as lactulose, lactitol, lactobionic acid, and galacto-oligosaccharides, have been produced commercially using chemical and enzymatic reactions (Gaenzle *et al.,* 2008). However, microbial fermentation is more attractive to the manufacturers and researchers, as it significantly expands the range of profitable products obtained from whey (see Fig. 2-1). Hydrolysis of lactose in whey can be carried out prior to fermentation in order to increase the variety of products, because many microorganisms lack the lactose permease-expressing genes as well as the genes for β-galactosidase (Siso, 1996). The conversion of lactose to value-added products will be further discussed in the following session, with a focus on the biological conversions.

2.4 Utilization of Lactose from Whey

2.4.1 Chemical and enzymatic modification of lactose

Lactose, known as "milk sugar", is a disaccharide consisting of glucose and galactose. The glycosidic linkage, reducing group of glucose, free hydroxyl groups and carbon-carbon bonds are the reactive sites for chemical and enzymatic modifications of lactose (Audic *et al*., 2003). Production of some lactose derivatives has been already commercialized, for example, lactulose, lactitol, lactobionic acid, lactosucrose and galacto-oligasaccharides (Audic *et al*., 2003; Gaenzle *et al.,* 2008). They are considered as the conventional products of chemical and enzymatic modification of lactose (Fig. 2-2).

Lactulose is a disaccharide produced by the isomerization of lactose, where the glucose residue of lactose is replaced by fructose, as shown in Fig. 2-2. It is a non-digestible sugar to mammalian digestion system and is used as a prebiotic. The physiological effects of lactulose are mainly attributed to the selective metabolism by some species of the colonic microflora (Schuster-Wolff-Buhring, 2010). Chemical isomerization of lactose is exclusively adopted for commercial production of lactulose (Aider and Halleux, 2007). The original synthesis was conducted in alkaline solutions and yielded approximately 30% lactose conversion, while the optimized synthesis employs borate or aluminate as chelating agents for lactulose under alkaline conditions giving lactose conversion of around 80% (Schuster-Wolff-Buhring, 2010). A novel and alternative approach to chemical isomerization is enzymatic catalysis which significantly alleviates the requirement on lactose purity in the raw materials (Schuster-Wolff-Buhring, 2010). It is carried out based on transgalactosylation by β-glycosidase using

lactose and fructose as the substrates. Moreover, the study of Hua *et al.* (2010) demonstrated the dual-enzymatic synthesis of lactulose using lactase and glucose isomerase. In this case, lactose was used as the sole substrate and no fructose addition was required.

Lactitol is yielded from catalytic hydrogenation of lactose, where the aldose residue (glucose) is reduced to polyol (sorbitol). It has similar physiological effects as lactulose (Schaafsma, 2008). Due to its noncariogenicity, low calorie, soft mouthfeel, and insulin-independent metabolism, lactitol is also used as a bulk sweetener in food products, especially diabetic foods (Yang and Silva, 1995). The industrial hydrogenation of lactose is catalyzed by Raney nickel with hydrogen pressure of approximately 40 bar at 100 °C. Because of the insufficient stirring problems caused by solution viscosity and the low solubility of lactose, the initial concentration of substrate is limited to around 30% dry matter content (Wilson, 2000). In this case, Wilson (2000) used a mixture of lactose and inulin/starch hydrolysates as the initial solution for lactitol production. It was explained that the anti-crystallizing action of inulin/starch hydrolysates increased the concentration of substrate and decreased the viscosity of the reaction solution to generate condensed lactitol syrup. In addition, Kuusisto *et al*. (2007) investigated the effects of reaction conditions and catalyst choice towards lactitol yield. It was concluded that catalysis of ruthenium on carbon or sponge nickel under 110-120 °C with over 50 bar of hydrogen pressure was optimal for lactitol production.

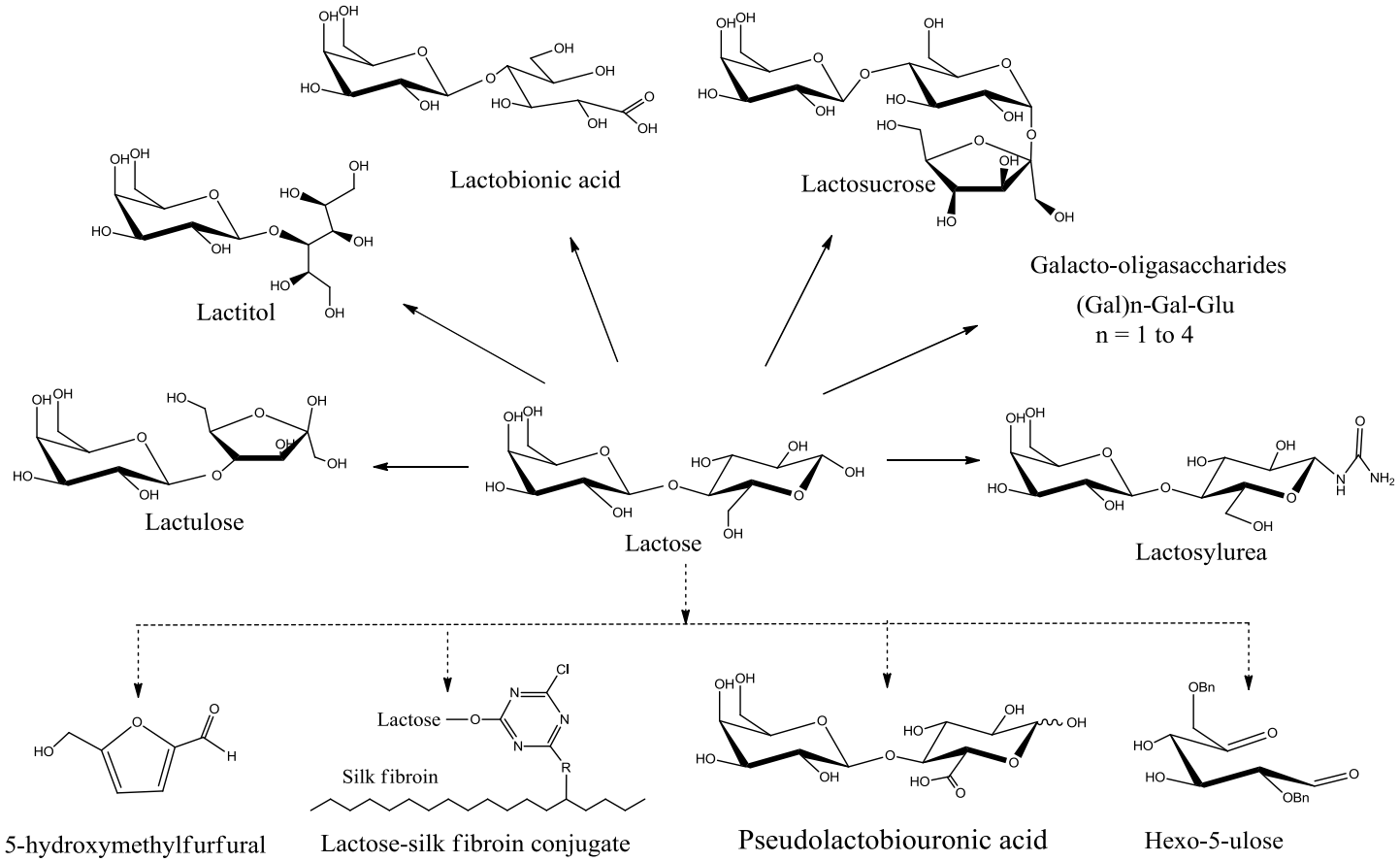


Figure 2-3 Conventional and novel products from chemical and enzymatic modification of lactose
(Arrows with solid line: conventional products; arrows with broken line: novel products)

Lactosucrose is a trisaccharide of galactose, glucose, and fructose. It is widely used as a sweetener in the food industry because of the high quality taste similar to sucrose and low calories contained (Arakawa *et al*., 2002). Lactosucrose is also considered as a prebiotic because of its bifidogenic effect, non-digestibility, and non-absorbability properties. Lactosucrose is derived from transfructosylation of lactose. It is commercially produced in Japan. The industrial synthesis of lactosucrose was established based on the catalysis of β-fructofuranosidase from *Arthrobacter* sp. K-1 over sucrose and lactose (Arakawa *et al*., 2002). The fructosyl residue of sucrose (donor) is transferred by the enzyme to lactose (acceptor) to form lactosucrose. Invertase-deficient yeast is added together with the enzyme to consume glucose produced from sucrose hydrolysis, as glucose is able to inhibit lactosucrose production (Arakawa *et al*., 2002).

Galacto-oligasaccharides (GOS) are the products resulting from the hydrolysis of lactose followed by transgalactosylation where the galactose moiety of lactose is transferred to an acceptor molecule (galactose, lactose, and previous formed disaccharides or oligosaccharides). The primary physiological property of GOS is acting as a prebiotic. GOS have a beneficial regulatory effect on intestinal microbiota, which has been confirmed by the *in vitro* and *in vivo* experiments for its prebiotic activities (Gibson *et al*., 2004). The prime commercial applications of GOS are ingredient supplements in the food industry, especially infant foods (Torres *et al*., 2010).

The industrial production of GOS is performed by using lactose as the raw material and fungal β-galactosidases as the catalyst (Gaenzle *et al.,* 2008). The β-galactosidases are responsible for both hydrolysis of lactose and transgalactosylation. Lactose concentration, temperature, and enzymes can be manipulated to obtain a high yield of

GOS synthesis. High lactose concentration favours transgalactosylation over hydrolysis. High temperature, resulting in increased lactose solubility, demonstrates the same reaction trend towards transgalactosylation as high lactose concentration. The enzyme used is not only essential for efficient synthesis, but also accounts for the linkage formed with GOS. β-Galactosidases from different microorganisms catalyze different predominant linkages formation (Placier *et al*., 2009).

Lactobionic acid is the product derived from the oxidation of glucose moiety of lactose. Lactobionic acid possesses strong mineral-complexing properties, which convey its use in calcium supplements (Schaafsma, 2008; Gerling, 1997) and organ preservation solutions (Southard and Belzer, 1995). The commercial production of lactobionic acid employs chemical oxidation of lactose over noble metal catalysts in alkaline solutions (Gerling, 1997). Catalyst screening conducted by Kuusisto *et al*. (2007) demonstrated that the highest yields of lactobionic acid were achieved using supported gold catalysts.

Lactosyl urea is recommended for ruminant feed supplements as non-protein nitrogen due to its palatability and low toxicity (Merry *et al*., 1982). It was prepared chemically from lactose and urea under acidic conditions (Illanes, 2011; Yang and Silva, 1995). Whey was used as a lactose source for the preparation of lactosyl urea by Merry *et al*. (1982) and Torkash Vand *et al*. (2007). Mole ratio of urea and lactose, lactose purity, temperature, time, and lactose concentration were the main variables studied to have effect on the yield of lactosyl urea.

In addition to the conventional products, the synthesis of other lactose-based bioactive compounds were investigated including pseudolactobiouronic acid (PLBA) and lactose conjugates. However, the application of the bioactive products only utilizes a

small portion of recovered lactose (Schaafsma, 2008). As a consequence, a few studies have been carried out to utilize lactose as the starting material for the preparation of synthetic intermediates, such as 5-hydroxymethylfurfural (5-HMF) and hexos-5-uloses, with a purpose of significantly increasing the market demand of lactose.

PLBA is an acidic galactooligosaccharides composed of galactose and glucuronic acid. PLBA is presumed to have physiological benefits on health maintenance. The first approach for PLBA synthesis proposed by Chiba *et al*. (1974) was to predominantly modify the C-6 position of lactose. In 2002, a novel preparation of PLBA was introduced, which was much more efficient with 67% overall yield (Attolino *et al*., 2002). The selective protection of eight hydroxyl groups (Catelani *et al*., 2000) and selective oxidation of the primary alcoholic function of the reducing unit in lactose (Attolino *et al*., 2002) were employed for PLBA production.

Lactose conjugates were prepared with an aim of immobilizing β-galactose residue onto the matrixes, as it is reported that β-galactose residue can be effectively recognized by receptors on the surfaces of cells (Varki, 1993; Yang *et al*., 2002). Lactose conjugates have been demonstrated to have potential applications in the pharmaceutical field. For example, lactose-conjugated polyion complex micelles was shown to possess gene transfecting effect against cultured HepG2 cells (Wakebayashi *et al*., 2004); lactose-modified cellulose films were used as a scaffold for cell culture (Esaki *et al*., 2009). Lactose-silk fibroin conjugate was examined to be an effective scaffold for hepatocyte attachment (Gotoh *et al*., 2004).

Hexos-5-uloses, a group of dicarbonyl hexoses, are essential intermediates for synthesis of some valuable compounds such as inositol (Pistara *et al*., 2000) and

iminosugar (Baxter and Reitz, 1994). Most of the information about hexos-5-uloses production from lactose was reported by the research group of Catelani (Guazzelli *et al*., 2010; Catelani *et al*., 2003). The hexos-5-ulose was previously converted from methyl-β-D-galatopyranoside which is available on market but expensive (Barili *et al*., 1992). A new and simple synthesis method for transformation of lactose into 2,6-di-O-benzyl-L-*arabino*-hexos-5-ulose was invented by Corsaro *et al*. (2003); however, the reaction was associated with two by-products.

5-HMF is an important intermediate that has an enormous range for potential applications. The conversion to useful derivatives, incorporation into polymers, and production of renewable fuels significantly contribute to the continuous studies on 5-HMP production (Lewkowski, 2001; Roman-Leshkov *et al*., 2007). It was demonstrated that lactose was hydrolyzed into glucose and galactose followed by the conversion of these two monosaccharides to 5-HMF (Binder *et al*., 2010). However, lactose-to-HMF conversion was much less active than glucose-to-HMF, probably resulting from low conversion rate of galactose-to-HMF. The mechanistic insight acquired by Binder *et al*. (2010) suggested that new catalysts development is required for improving the conversion rate.

The preparation of these novel products from lactose helps expand the scope of lactose utilization, especially, 5-HMF-an excellent building block platform. The efficient conversion of lactose to 5-HMF can provide great opportunities for the production of renewable fuels and chemicals. However, the current preparation of these novel products using lactose was carried out in the laboratory scale and inefficient conversion was observed for some of them. Thus, further investigation and optimization are necessary to

improve the productivity and efficiency of lactose conversion, as well as the feasibility of the applications in the commercial scale.

2.4.2 Fermentative conversion of lactose

Unlike chemical and enzymatic modification, microbial conversion of lactose yields a much wider and broader range of products, such as alcohols, biogas, polymers, organic acids, amino acids, enzymes, vitamins and so forth. So far, various microorganisms have been employed in the value-added utilization of whey lactose, targeting at different products (Panesar and Kennedy, 2012). The major products are identified below.

2.4.2.1 Ethanol

Ethanol is one of the most widely produced compounds from whey lactose. It can be used for not only alcoholic beverage production, but also motor fuels in which ethanol serves as a fuel additive. The dependence of economic development on fossil fuels and global warming significantly promote the growth of the bio-ethanol market. Currently, the vast majority of global bio-ethanol production is based on the fermentation of starch- and sugar-based feedstocks, leading to the production cost fluctuation with the price of these food sources. Lactose is considered as a cheaper alternative carbon source, which can substantially contribute to the reduction of ethanol production cost. Therefore, ethanol production has attracted the attention of both industrial manufacturers and academic researchers among all the products from whey lactose.

The commercial production of ethanol from whey dates back to at least 1970s (Lyons and Cunningham, 1980; Guimaraes *et al*., 2010). The simplest process at that time was based on the batch fermentation by strains of *Kluyveromyces marxianus* using untreated whey as the substrate (Mawson, 1994). A number of industrial-scale plants in Ireland,

New Zealand, United States, and Denmark are reported to manufacture ethanol using whey or whey permeate (Guimaraes *et al*., 2010). Carbery Group, a whey-to-ethanol plant in Ireland, started its production of potable ethanol in 1972 and initiated the manufacturing of fuel ethanol for E85 and E5 blends in 2005 (Doyle, 2005). Anchor Ethanol, a subsidiary of the dairy Fonterra Cooperative Group in New Zealand, also expanded their production to include fuel ethanol. It has been providing fuel ethanol for E10 blend since 2008 (Ling, 2008).

Apart from the industrial development, numerous studies on whey-to-ethanol conversion have been performed in recent years. The four yeasts used are *K. marxianus*, *K. fragilis*, *Candida pseudotropicalis*, and *Saccharomyces cerevisiae* (Table 2-2). In fact, *K. fragilis* and *C. pseudotropicalis* were reported as the synonyms of *K. marxianus* (Lachance, 1998; Fonseca *et al*., 2008). Therefore, lactose-to-ethanol production is discussed based on the following two fermentation platforms - *Kluyveromyces* yeasts and *S. cerevisiae*.

(1) *Kluyveromyces* yeasts

Kluyveromyces yeasts (*K. marxianus* and *K. fragilis*) and *C. pseudotropicalis* are three of a few microorganisms which can assimilate lactose directly. Fermentation of whole whey containing about 5% lactose is economically infeasible, as a high cost is required for the downstream processing, especially distillation (Guimaraes *et al*., 2010). A low lactose content in the media leads to a low final concentration of ethanol. Thus, concentrated whey or whey permeate with high initial lactose concentrations have been used for the fermentation (Ferrari *et al*., 1994; Hahn-Hagerdal, 1985). Recently, cheese whey powder was suggested for ethanol production (Table 2-2) as it has several

advantages such as excellent stability, easy transportation, and high concentration of ethanol in the output (Kargi and Ozmihci, 2006; Dragone *et al*., 2011). However, the high initial lactose concentration had two negative impacts including substrate and product inhibitions which negatively affected fermentation. A series of investigations have been conducted by Kargi and Ozmihci to optimize the fermentation by *Kluyveromyces* yeasts (Ozmihci and Kargi, 2007a; Ozmihci and Kargi, 2007b; Ozmihci and Kargi, 2007c; Ozmihci and Kargi, 2007d). Fed-batch operation was demonstrated to be an effective approach for alleviation of substrate inhibition (Ozmihci and Kargi, 2007d), as shown in Table 2-2. Moreover, oxygen restriction during fermentation is one of the factors responsible for the yeast growth and ethanol yield in *K. marxianus* fermentation (Silveria *et al*., 2005). A high ethanol yield was obtained with an initial lactose concentration of ≥ 50 g/L under hypoxic and anoxic conditions, indicating the importance of low oxygen levels to high lactose-to-ethanol conversion rate (Silveria *et al*., 2005). Efforts have also been made to improve the fermentation through investigations of different bioreactors/operation types, immobilization of *Kluyveromyces* yeasts, and co-immobilization of *Kluyveromyces* yeasts and *S. cerevisiae* (Guimaraes *et al*., 2010 for details). Above all, the reasons for researchers and manufacturers to select *Kluyveromyces* yeasts for ethanol production are mainly based on their two advantageous metabolism features including autologous enzymes for lactose assimilation and potential resistance to catabolite repression (also known as glucose repression which causes diauxic growth).

(2) *S. cerevisiae*

S. cerevisiae is the microorganism of choice for industrial ethanol production due to its well-known fermentative capabilities (Cot *et al*., 2007). Compared to *Kluyveromyces* yeasts, it is more resistant to osmotic pressure caused by high substrate and product concentrations, resulting in elevated growth rate and ethanol yield. Moreover, the comprehensive scientific knowledge about *S. cerevisiae* allows for a great potential of metabolic engineering modifications with the aim of boosting its fermentation efficiency.

The application of *S. cerevisiae* in whey utilization could be undertaken with pre-hydrolysis of lactose (Cote *et al*., 2004), as there are no genes for lactose assimilation present in *S. cerevisiae*. Enzymatic hydrolysis by β-galactosidase gives a much milder environment for further microorganism growth than acidic hydrolysis. However, enzymatic hydrolysis requires additional enzyme, time, space, and personnel, resulting in higher production costs. With the purpose of circumventing the obstacles associated with the absence of β-galactosidase-expressing genes in *S. cerevisiae*, the use of co-immobilized yeast and β-galactosidase for ethanol fermentation was studied. In addition, protoplast fusion of *Kluyveromyces* yeasts and *S. cerevisiae* was used to create cells which can secrete the enzyme for direct fermentation with better ethanol production performance than the parental *Kluyveromyces* yeasts (Farahnak *et al*., 1986; Ryu *et al*., 1991).

Table 2-2 Lactose-to-ethanol production by *Kluyveromyces* yeasts and *S. cerevisiae* (modified from Guimarães *et al*., 2010)

Organism	Medium	Operation type	Approach	Ethanol yield (g/L)	Reference
C. pseudotropicalis	Cheese whey + lactose (15% lactose)	Batch	Micro-aeration	40-45	Ghaly and El-Taweel (1995)
K. marxianus	CWP (15% lactose)	Shake-flasks	Condition optimization	80	Kargi and Ozmihci (2006)
K. fragilis	Deproteinized CWP solution	Shake-flasks	Conditions optimization		Dragone *et al*. (2011)
K. marxianus	CWP (10-12.5% lactose)	Continuous	Reactor development	29	Ozmihci and Kargi (2007c)
K. marxianus	Whey permeate (17% lactose)	Batch	Conditions optimization	76-80	Silveira *et al*. (2005)
K. fragilis	Concentrated whey permeate (15% lactose)	Continuous	Cell immobilization	13	Hahn-Hägerdal (1985)
S. cerevisiae with β-galactosidase	Concentrated whey permeate (15% lactose)	Continuous	Co-immobilization with enzymes	52	Hahn-Hägerdal (1985)
S. cerevisiae expressing *lacA*	Synthetic medium (10% lactose)	Batch	Gene modification	30	Ramakrishnan and Hartley (1993)
S. cerevisiae expressing *lacA*	Semi-synthetic medium (5% lactose)	Batch	Gene modification	25	Domingues *et al*. (2002)
S. cerevisiae expressing *lacA*	Semi-synthetic medium (5% lactose)	Continuous	Gene modification and reactor development	20	Domingues *et al*. (2005)
S. cerevisiae expressing *lacZ and* overexpressing *GAL4*	Yeast extract + peptone +2% lactose	Shake-flasks	Gene modification	18	Porro *et al*. (1992)
S. cerevisiae expressing *LAC4* and *LAC12*	Synthetic medium (5% lactose)	Continuous	Gene modification	16	Domingues *et al*. (1999)
S. cerevisiae expressing *LAC4* and *LAC12*	CWP solution (15% lactose)	Batch	Gene modification and adaptive evolution	55	Guimarães *et al*. (2008a)

CWP: cheese whey powder

The prime focus of the studies on whey-to-ethanol fermentation by *S. cerevisiae* is the gene modification by recombinant DNA technology. The genetic strategies used can be divided into two groups: extracellular and intracellular β-galactosidase expression in *S. cerevisiae*. Gene *lacA* from *Aspergillus niger* has particularly attracted the interest of researchers for extracellular β-galactosidase expression, which was first expressed in *S. cerevisiae* in the early 1990s (Kumar *et al*., 1992; Ramakrishnan and Hartley, 1993). Further investigations of *S. cerevisiae* transformants with *lacA* were mostly conducted by the research group of Guimaraes to obtain strains with enhanced β-galactosidase activity and higher ethanol production (Domingues *et al*., 2000; Domingues *et al*., 2002; Domingues *et al*., 2004; Domingues *et al*., 2005), as shown in Table 2-2.

As for intracellular β-galactosidase expression, it can be further classified into two approaches, depending on where lactose is hydrolyzed. The first approach is to express β-galactosidase within cells using the genes from *K. lactis* or *E. coli*, then to release it into the medium by cell lysis. So lactose is hydrolyzed in the medium. In order to lyse the cells, *GAL4* in *S. cerevisiae* was over-expressed together with *lacZ* (gene encoding intracellular β-galactosidase) from *E. coli*, as over-production of Gal4 could stimulate lysis of some yeast cells (Porro *et al*., 1992). The other approach is to construct a recombinant *S. cerevisiae* strain with the genes for intracellular β-galactosidase and lactose permease. In this case, lactose is transported from the medium into the yeast cells by lactose permease and then hydrolyzed into glucose and galactose within the cells. *LAC4* and *LAC12* (genes encoding for β-galactosidase and lactose permease) of *K. lactis* were commonly used to construct the lactose-fermenting *S. ceresiviae* strain, as shown in Table 2-2. The attempts of recombinant lactose-fermenting *S. cerevisiae* for whey-to-

ethanol fermentation were turned out to be ineffective (Sreekrishna and Dickson, 1985; Rubio-Texeira *et al*., 1998; Domingues *et al*., 1999). However, evolutionary engineering adaptation was found to be useful to improve fermentative capacities of lactose-fermenting *S. cerevisiae* (Domingues *et al*., 2001; Guimaraes *et al*., 2008a; Silva *et al*. 2010).

As opposed to the efforts made on enhancing the desirable property of lactose hydrolysis, no attempt has been made to eliminate the potential catabolite repression in the fermentation of whey lactose by *S. cerevisiae* so far. The hydrolysis of lactose can lead to a mixture of glucose and galactose. The presence of glucose is responsible for transcriptional repression of the GAL genes which account for galactose metabolism (Johnston, 1994). Even for *K. marxianus*, catabolite repression is associated with its conversion of biomass containing various sugars (Rodrussamee *et al*., 2011; Dong and Dickson, 1997).

2.4.2.2 2,3-Butanediol

2,3-Butanediol (2,3-BD) is a remarkable and promising fuel and bulk chemical, which has a variety of practical applications. During World War II, it was used as a starting material for synthetic rubber production (Celinska and Grajek, 2009). 2,3-BD is an excellent intermediate for numerous products, such as acetoin and diacetyl (flavouring agents), methyl ethyl ketone, butadiene, moistening agents, and so forth (Perego *et al*., 2000). Moreover, 2,3-BD is used in the fuel industry as a liquid fuel additive; it is more suitable and effective than ethanol (Martinez and Speckman, 1988).

Only a few bacterial species were reported for whey/whey permeate fermentation: *Baciillus polymyxa*, *Klebsiella pneumonia*, *Enterobacter aerogenes*, and *K. oxytoca*

(Speckman and Collins, 1982; Lee and Maddox, 1984; Lee and Maddox, 1986; Champluvier *et al*., 1989; Barrett *et al*., 1983; Martinez and Speckman, 1988; Perego *et al*., 2000). The first study on 2,3-BD fermentation using whey/whey permeate was conducted by Speckman and Collins (1982). Low product concentration and yield were obtained by the direct utilization of untreated sweet whey using *B. polymyxa*. Then, lactose pre-hydrolysis was adopted to increase the 2,3-BD yield by *K. pneumonia* (Lee and Maddox, 1984). The effect of lactose pre-hydrolysis was further proved by Champluvier *et al*. (1989). Lactose and a mixture of galactose and glucose were used individually as the substrate for 2,3-BD production to assess the fermentative capacity of *K. oxytoca*. Compared to the undetected 2,3-BD production from lactose fermentation, the final concentration of 18.8 g/L obtained from galactose-glucose mixture supported the importance of pre-hydrolysis.

2.4.2.3 Hydrogen and methane

Hydrogen is a well-known clean energy source. In addition to its application for energy supply, hydrogen is widely used for production or modification of chemicals (Kapdan and Kargi, 2006; Guo *et al*., 2010). The present industrial approach to generate hydrogen is expensive and energy-intensive. To find an effective and cost-efficient alternative method, hydrogen production from agricultural and food industry wastes has gained much attention in recent years (Kapdan and Kargi, 2006; Guo *et al*., 2010). In this case, whey/whey permeate is also one of the interesting candidate feedstock for hydrogen generation.

There are a few studies conducted to explore whey valorization for hydrogen production recently. Dark fermentation, the key technology for biological hydrogen

production, was widely adopted in most of the studies (Davila-Vazquez *et al*., 2008; Davila-Vazquez *et al*., 2009; Azbar *et al*., 2009a; Azbar *et al*., 2009b; Calli *et al*., 2008; Antonopoulou *et al*., 2008; Venetsaneas *et al*., 2009; Stamatelatou *et al*., 2011). Pure cultures of *Clostridium saccharoperbutylacetonicum*, *C. thermlacticum*, and recombinant *E. coli* were also carried out to produce hydrogen from whey (Collet *et al*., 2004; Ferchichi *et al*., 2005; Manuel *et al*., 2010). In dark fermentation, mixed culture originating from the waste treatment field is added as the inoculum. Compared to traditional pure culture, mixed culture possesses several advantages, such as no sterilization requirements and adaptive capacities due to diverse microorganisms, which significantly contribute to its preference by researchers (Kleerebezem and van Loosdrecht, 2007).

The major problem associated with hydrogen production is that most organic compounds in whey are retained in the form of organic acids, as shown in the two chemical equations below. In other words, the high COD of whey is still remaining. From the waste abatement point of view, hydrogen fermentation is not an effective strategy. Consequently, two-stage anaerobic process (hydrogen fermentation followed by methane production) was suggested to obtain a satisfactory result. The COD of whey can be reduced up to 95% (Venetsaneas *et al*., 2009; Antonopoulou *et al*., 2008; Cota-Navarro *et al*., 2011).

$$C_{12}H_{22}O_{11} + 5H_2O \rightarrow 4CH_3COOH + 4CO_2 + 8H_2 \qquad (2\text{-}1)$$

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4CH_3CH_2CH_2COOH + 4CO_2 + 4H_2 \qquad (2\text{-}2)$$

The two-stage process is a frequently used strategy of industrial wastewater treatment. Hydrolysis, acetogenesis, and methanogenesis are the three steps involved in

lactose biomethanation for the production of hydrogen and methane (Fig. 2-3). Acetogenesis and methanogenesis are carried out in two separate reactors by virtue of the different sets of activities, which is the reason why this strategy is named two-stage process. The separation of the two stages helps to reduce the cost, enhance biogas generation, increase waste abatement efficiency, and improve stability of anaerobic reactors (Ke *et al*., 2005). Hydrogen is the product given by lactate- and ethanol-consuming acetogenic bacteria, while methane is produced by methanogenic bacteria through hydrogen, formate, and acetate comsumption (Chartran and Zeikus, 1986).

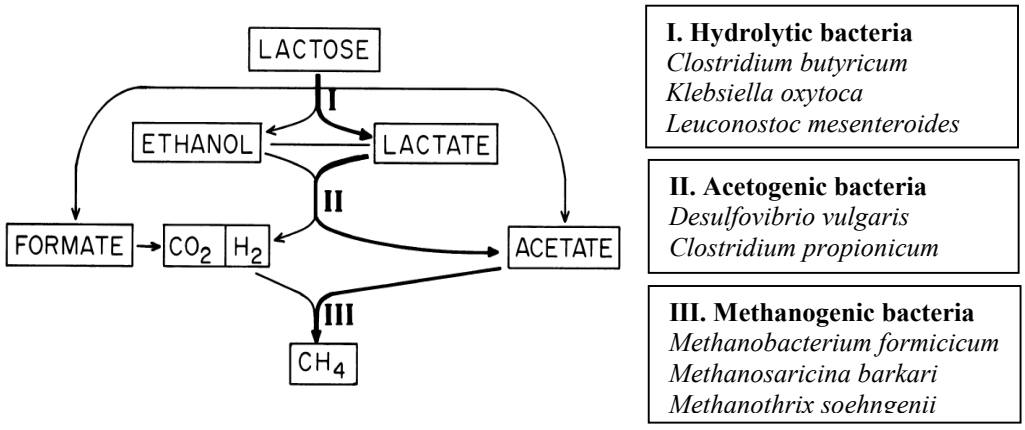


Figure 2-4 The microbial food chain responsible for biomethanation of lactose in three distinct but simultaneous trophic phases
The heavy arrows indicate the major flux of carbon and electrons during biomethanation. (Taken from Chartran and Zeikus, 1986)

Compared to other whey valorization approaches, methane production is a much more economical and suitable choice for medium size cheese factories due to its low investment costs (Saddoud *et al*., 2007). Successful whey-to-methane fermentation has been established by a number of plants (Fitzmaurice *et al*., 1987; Kemp and Quickenden,

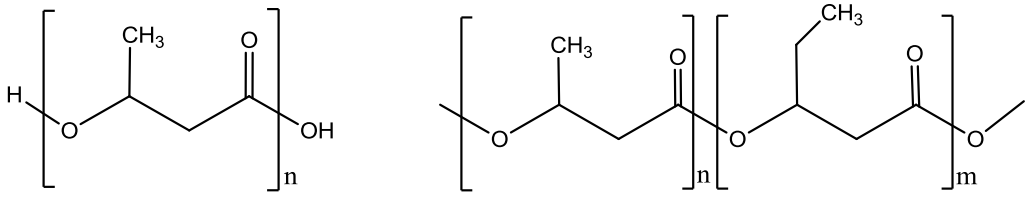
1989; Mawson, 1994). Methane was reported to be consumed as an energy source *in situ*; thus it substantially reduced the energy input of the factories where whey was generated (Siso, 1996; Mawson, 1994). For example, the biogas at the Tirau plant was able to satisfy 25% of the factory's heat demand (Fitzmaurice *et al*., 1987). Moreover, methane production can contribute to over 95% COD reduction of the influent (Siso, 1996). Thereby, methane production is considered as an effective energy-saving and pollution-eliminating approach for whey utilization. However, there remain several problems associated with the anaerobic digestion of whey to methane including long hydraulic retention time, instability of the reactor attributed to high influent concentration (Yan *et al*., 1989), and the need for post-treatment required for disposal (Audic *et al*., 2003).

2.4.2.4 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are a family of the linear polyesters produced by bacteria as carbon and energy storage materials. They are novel environmentally friendly bioplastics. The complete biodegradability and thermoplastic properties give PHAs great potential to be promising substitutes of petrol-derived plastics in the future (Lee, 1996). PHAs production has been recently commercialized by a few companies, such as Tianan, Bio-on, Biomer, and Tepha (Tianan, 2014; Bio-on, 2014; Biomer, 2014; Tepha 2014). High production cost remains the major disadvantage of current industrial manufacture, making PHAs less economically competitive than the traditional plastics (Chen, 2009).

Utilization of cheaper carbon sources instead of glucose for PHAs production is one of the strategies suggested for lowering the production cost (Chen, 2009; Salmanca-Cardona *et al*., 2014). Whey, a prime by-product from the dairy industry, has a huge potential to be utilized as an alternative substrate for microbial production of PHAs. Kim

(2000) estimated that the use of whey as a substrate for PHAs production could reduce the overall cost by up to 50%. Furthermore, there have been a number of studies carried out to generate PHAs based on whey. Poly-3-hydroxybutyrate (PHB) and poly-3-(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV) are the two bacterial PHAs obtained in these studies (structure shown in Fig. 2-4). PHB, composed of pure 3-hydroxybutyric acid, is the most well-known and popular microbial PHA in the research field. PHBV is the copolymers of 3-hydroxybutyrate (3-HB) and 3-hydroxyvalerate (3-HV) constituents with various ratios. In order to synthesize PHBV, addition of 3-HV precursors such as valerate or valeric acid is required for some bacteria strains (Koller *et al*., 2007; Koller *et al*., 2008). The principal mechanism of PHAs biosynthesis is the condensation of acetyl-CoA units from hexose catabolism. The basic metabolic pathway of lactose-to-PHB is briefly elucidated in Fig. 2-5.



(A) PHB (B) PHBV

Figure 2-5 The chemical structures of (A) PHB and (B) PHBV

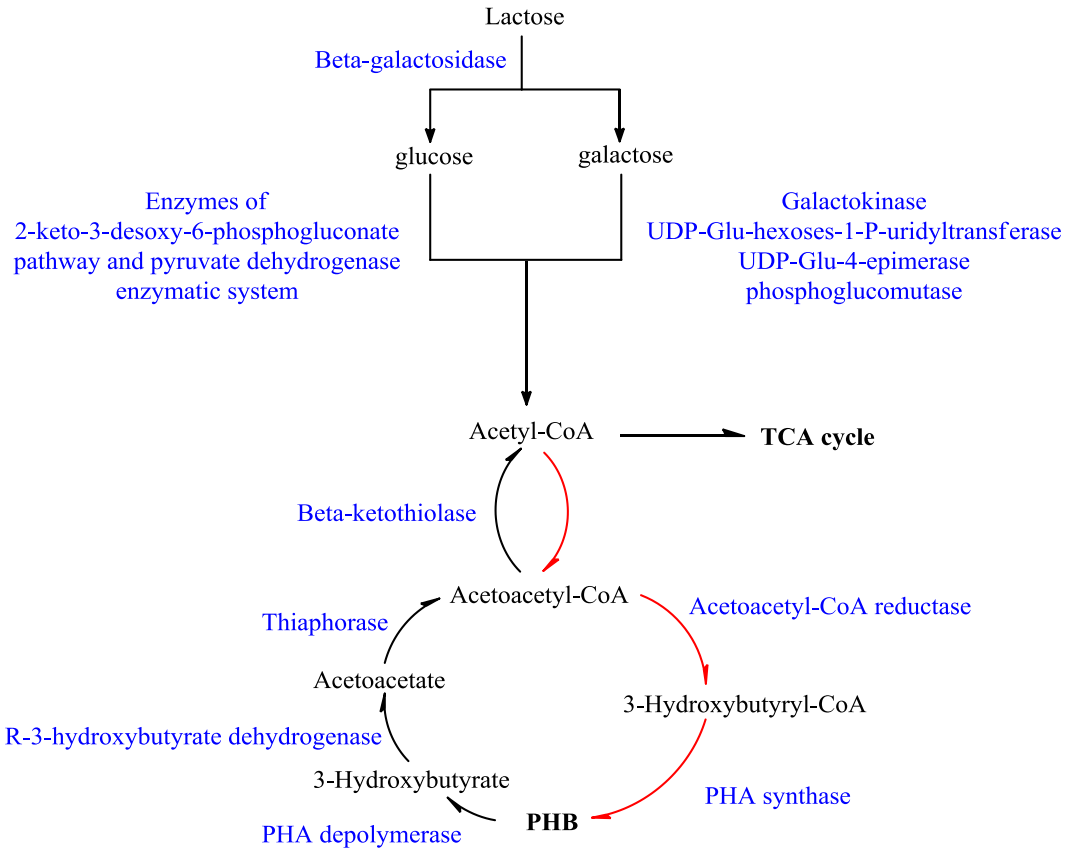


Figure 2-6 The basic metabolic pathways from lactose to PHB (Taken from Koller *et al*., 2007)

Red arrows represent the metabolic flow of PHB synthesis.

The approaches for PHAs production from whey can be classified into two routes: direct conversion of lactose and conversion of hydrolyzed lactose. Wild-type or recombinant bacteria strains, capable of expressing sufficient β-galactosidase, are applied in the direct conversion of lactose. The wild-type strains of *Hydrogenophaga pseudoflava*, *Sinorhizobium meliloti*, *Bacillus megaterium*, *Azotobacter chroococcum*, and *Thermus thermophilus* were investigated (Povolo and Casella, 2003; Nath *et al*., 2008; Koller *et al*. 2007; Kim, 2000; Pantazaki *et al*., 2009). Povolo and Casella (2003)

assessed the PHA-producing capacities of collection strains and three isolates from soil. *H. pseudoflava* was demonstrated to be a good candidate for PHAs production as it is able to accumulate not only PHB but also copolymers with 4-HB and/or 3-HV. *T. thermophilus*, a new strain in the field of PHAs production, was demonstrated to be able to produce significant amounts of PHAs, however, with low molecular weight (Pantazaki *et al*., 2009). As for recombinant strains for PHAs production, two metabolic engineering strategies were adopted. One is to express genes of PHA synthesis in non-PHA-producing strains which have adequate β-galactosidase activity, while the other involves expression of exogenous β-galactosidase in PHA-producing strains that cannot hydrolyze lactose originally. Recombinant *E.coli* harbouring *Ralstonia eutropha* or *Alcaligenes latus* PHA synthesis genes is the best example of the former strategy. A series of studies on PHB production of recombinant *E. coli* were conducted by the research group of Lee (Lee *et al*., 1997; Wong and Lee, 1998; Ahn *et al*., 2000; Ahn *et al*., 2001). It is published that a final PHB concentration of 168 g/L was achieved by recombinant *E. coli* in a cell recycle membrane system using a concentrated whey solution containing 280 g/L of lactose (Ahn *et al*., 2001). The second genetic engineering strategy was illustrated by the recombinant *Cupriavidus necator* DSM 545, a well-known PHAs producer carrying *LacZ*, *lacI*, and *lacO* genes of *E. coli* (Povolo *et al*., 2010). As for the conversion of hydrolyzed lactose to PHAs, *Pseudomonas hydrogenovora* was demonstrated to have a disadvantage of low final PHA concentrations at 1.3-1.44 g/L (Koller *et al*., 2007; Koller *et al*., 2008); while high robustness and stability of *Haloferax mediterranei* in PHAs production was reported by Koller *et al*. (2007) with a comparably higher final PHA concentration of 5.5 g/L than that of *P. hydrogenovora*.

2.4.2.5 Other products

The substances listed above are only a part of the whole range of the microbial products obtained from whey/whey permeate. Organic acids can be produced using whey lactose, for example, citric acid, gluconic acid, acetic acid, propionic acid, lactic acid, and succinic acid (El-Samragy *et al*., 1996; Mukhopadhyay *et al*., 2005; Huang and Yang, 1998; Czaczyk *et al*., 1996; Kosmider *et al*., 2010; Panesar *et al*., 2007; Lee *et al*., 2000). Currently, organic acids have a small market due to prices limitations and infeasibility of large-scale production (Sauer *et al*., 2008). Efficient microbial production of organic acids with low-cost raw materials will considerably contribute to the market expansion. Whey-based productions of penicillin, vitamins (vitamin B12, folate), and amino acids (lysine, threonine) were also carried out by some researchers (Paul *et al*., 1998; Marwaha and Sethi, 1984; Hugenschmidt *et al*., 2010; Barrett *et al*., 2004; Ko and Chipley, 1983).

Except for these small molecular compounds, production of enzymes (β-galactosidase and penicillin acylase) has been studied (Oberoi *et al*., 2008; De Leon-Rodriguez *et al*., 2006). Microbial lipid (so-called "single-cell oil"), was possible to be derived from whey lactose fermentation (Vamvakaki *et al*., 2010). It has been also demonstrated that exopolysaccharides can be produced from whey by fermentative conversion (Dlamini and Peiris, 1997; Fialho *et al*., 1999; Shene *et al*., 2008).

2.5 Conclusions

Whey, a by-product of the dairy industry, is no longer considered as a waste for disposal, but a renewable resource due to its enormous potential for further processing. A multitude of products from lactose represent various choices for whey valorization through physical, chemical, or biological treatments. The selection of appropriate

industrial chemicals or fuels for whey utilization is substantially dependent on the scale of cheese and casein production. The amount of whey generated determines what products could be chosen to obtain an optimal revenue. Regardless of revenue maximization for whey utilization, industrial chemicals and fuels from biological production are more promising than those from chemical processing, as the interests of the chemical and other industries have been switched from petroleum and chemistry to biofuels and biomaterials. The transformation from fossil fuels and chemicals to 'biofactories' is the tendency. This review has demonstrated that the insights gained during this thesis have the potential to impact and unlock commercial feasibility of many different value-added pathways creating value from whey byproduct streams.

3 Simultaneous Hydrolysis of Whey Lactose and Co-Fermentation with Wheat for Ethanol Production

3.1 Introduction

Lactose is the most abundant component besides water in whey and whey permeate. Current whey and whey permeate utilization consumes only a small portion of lactose generated from cheese production. The large amount of whey lactose surplus can be used as a potential low-cost substrate for industrial chemical and fuel production. So far, numerous studies have focused on utilizing whey lactose for ethanol production. One of the first studies to ferment whey lactose for ethanol was carried out in 1940s (Browne, 1941; Rogosa *et al*., 1947), while the commercial ethanol production from whey lactose dated back to at least 1970s (Lyons and Cunningham, 1980; Guimaraes *et al*., 2010). Utilization of whey lactose for ethanol production has been considered as a promising strategy with both academic and industrial attempts. *Kluyveromyces* strains, particularly *K. marxianus,* have been used, which are able to ferment lactose directly. However, the low ethanol titre of 2.5-4.2% (v/v) with a low starting lactose content of 5.0% and the prolonged fermentation time with high lactose concentrations were the major drawbacks of lactose-to-ethanol production by *Kluyveromyces* yeasts.

Currently, the global ethanol supply mainly depends on fermentation utilizing natural sugars and starch as feedstocks (Bai *et al.*, 2008; Renewable fuels association, 2013). In Canada, the bio-ethanol industry has been dominated by grain-to-ethanol production which accounts for more than 95% of the annual production capacity (CRFA, 2013). Wheat is the most commonly used grain in western Canada and as such high costs and competition with the food industry remain. Integration of byproduct streams including

whey lactose into conventional wheat-to-ethanol production as a co-substrate is an interesting and attractive strategy for the whey lactose utilization in western Canada. Whey lactose can be supplemented to replace part of the wheat, leading to reduction of feedstock cost, less dependence of production cost on grain price fluctuation, and less cost associated with enzymes used for starch hydrolysis. In addition, incorporation of whey lactose into wheat-to-ethanol fermentation is highly compatible with the current process of ethanol production. Therefore, no setup of new major facilities is required, resulting in low construction cost for ethanol plant to adopt this approach.

Our previous work has been focused on supplementing pre-hydrolyzed whey lactose into wheat-to-ethanol fermentation (Parashar *et al*., manuscript in preparation), which conducted lactose hydrolysis by β-galactosidase into glucose and galactose prior to fermentation. If applied in the ethanol plant, extra cost for facilities, personnel, and time associated with lactose pre-hydrolysis would be required. Furthermore, sugar loss and organic acid production during lactose hydrolysis at 30 °C could be a potential issue due to the presence of natural microorganisms in whey permeate, especially lactic acid bacteria. Lactose is consumed by the natural microorganisms, resulting in less fermentable carbon available for ethanol production. Meanwhile, lactic acid produced would induce the stress on yeast growth, rates of glucose consumption, and ethanol production, if the lactic acid concentration reached to 0.2-0.8% (w/v) (Graves *et al*., 2006; Narendranath *et al*., 2001).

In this study, whey lactose was directly blended with wheat for mash preparation without pre-hydrolysis. *S. cerevisiae* was used for ethanol production because of its high tolerance to substrate and product inhibition (Cot *et al*., 2007). *A. oryzae* or *K. lactis* β-

galactosidase was added at the onset of the fermentations for simultaneous lactose hydrolysis. The goal of this chapter was to supplement whey permeate for partial replacement of the wheat and process water used for ethanol production, and to evaluate the effectiveness of simultaneous lactose hydrolysis during the fermentations as well as the contribution of whey permeate to the ethanol production. To our best knowledge, this is the first study to apply simultaneous lactose hydrolysis in the co-fermentations of whey lactose and grains.

3.2 Materials and Methods

3.2.1 Grain, enzymes, and others

Spring wheat (AC Andrew) was provided by Seed Solutions (Viking, AB, Canada). Wheat was ground by a laboratory hammer mill (Model 3100, Perten, Sweden) equipped with a mill feeder (Model 3170, Perten, Sweden). Sieve sizes of 0.5 mm and 1.98 mm were used to mill wheat for STARGEN- and jet cooking-based fermentations, respectively. The moisture and starch contents of wheat flour were determined by AOAC official method 934.01 and Total Starch Assay Kit (Megazyme, Country Wicklow, Ireland), respectively. Whey permeate was provided by one of the top three dairy companies in Canada, which was shipped on ice and stored at 4 °C after receipt.

STARGEN™ 002 (enzyme blend of α-amylase and glucoamylase, 570 glucoamylase unit/g), Optimash™ TBG (thermostabe β-glucanase, 5625 U/g), GC 626 (acid α-amylase, 10,000 soluble starch unit/g), and Fermgen™ (protease, 1000 spectrophotometric acid protease unit/g) were provided by Genencor International (Palo Alto, CA, USA). Viscozyme Wheat FG (cellulase and xylanase), Liquozyme SC DS (α-amylase, 240 Kilo Novozymes Unit/g), and Spirizyme Ultra (glucoamylase, 900 amyloglucosidase unit/g)

were supplied by Novozymes (Franklinton, NC, USA). *Aspergillus oryzae* (8 U/mg) and *Kluyveromyces lactis* (2600 LAU/g) β-galactosidases were purchased from Sigma-Aldrich (St. Louis, MO, USA). *K. lactis* β-galactosidase (2600 LAU/g) is marketed as Lactozyme® 2600 L. D-Glucose Assay Kit and Lactose/D-Galactose Assay Kit were obtained from Megazyme (Country Wicklow, Ireland). SuperStart distiller's yeast was supplied from Lallemand Ethanol Technology (Milwaukee, WI, USA).

3.2.2 Standard and whey permeate-blended STARGEN-based fermentations

3.2.2.1 Fermentation in 250 g shake-flask scale

For the standard STARGEN-based fermentation of 30% (wt/wt, wet basis) wheat, wheat flour (0.5 mm) was weighed into a 500 mL Erlenmeyer flask and mixed thoroughly with distilled water. The pH of wheat mash was adjusted to 4.0 using 4 N HCl. The flask was covered with foil and heated in an incubator shaker (Innoca 44/44R, New Brunswick Scientific, Edison, NJ, USA) at 55 °C, 200 rpm. When the temperature of mash reached to 53-55 °C, Fermgen (940 µL/kg of grain), Optimash TBG (80 µL/kg of grain), and GC 626 (440 µL/kg of grain) were added. The flask was kept for 1 h at 200 rpm. After that, diethyl pyrocarbonate (DEPC, Sigma-Aldrich, St. Louis, MO, USA) was added as a chemical disinfectant with a dosage of 105 µL/kg mash. The flask containing mash was stored at 4 °C for 72 h before fermentation. As for the whey permeate-blended STARGEN-based fermentation, 25% (wt/wt, wet basis) wheat was prepared. A certain amount of whey permeate was added to contribute fermentable carbon from lactose in order to have the same amount of total fermentable carbon (carbon from wheat starch and whey lactose) as that of 30% wheat. Theoretically, 1 g of starch can be converted to 1.111 g of glucose containing 0.444g of fermentable carbon; while 1 g of lactose can

generate 0.526 g of galactose and 0.526 g of glucose having 0.421 g of fermentable carbon. Therefore, 1 g starch can be replaced by 1.05 g of lactose in the perspective of the same amount of fermentable carbon. The following mash treatments were identical to those of the standard fermentation stated above.

After 72-h storage at 4 °C, the mash was heated up to 53-55 °C in the incubator shaker at 200 rpm. STARGEN^{TM} 002 (2.8 mL/kg of grain) was added to the mash, followed by 1-h incubation at 53-55 °C, 200 rpm. Urea (1 M) was supplemented to the mash to obtain a final concentration of 16 mM in 250 g mash. When the temperature of the mash decreased to 30 °C, hydrated yeast was added to have an approximate initial cell concentration of 2×10^7 cfu/mL in the mash. β-Galactosidase was added together with the hydrated yeast to conduct simultaneous lactose hydrolysis for the whey permeate-blended group. The flask was then sealed with a gas trap which has a S-lock filled with water to allow CO_2 escape during fermentation. All the fermentations were carried out at 30 °C, 200 rpm for 72 h.

3.2.2.2 Fermentation in 5-L bioreactor

Mash was prepared and treated in the same way as that used in 250 g shake-flask scale, except for the amount. In addition, water bath (SWBR27, Sheldon Manufacturing Inc., Cornelius, OR, USA) and a spatula were used for mash heating and mixing, instead of the incubator shaker. Fermentations were carried out in 5-L bioreactors (Rose Scientific, Ltd., Mississauga, Ontario, Canada) at 30 °C with a stirring speed of 400 rpm for 72 h.

3.2.3 Analytical assays

3.2.3.1 Glucose, galactose, lactose, lactic acid, and acetic acid

Fermentation samples were centrifuged at 12,000 rpm (Model 5424, Eppendorf, Hamburg, Germany) for 10 min. Clear supernatant was transferred to a sealed tube and boiled for 5 min, followed by the filtration through 0.22 μm membrane filter (Mandel Scientific, Guelph, ON, Canada). The filtrate obtained was used for the analysis of sugars and organic acids. Glucose, lactic acid, and acetic acid concentrations were analyzed by high performance liquid chromatography (HPLC, 1200 series, Agilent Technologies, Mississauga, ON, Canada) equipped with refractive index detector (1100 series, Agilent Technologies, Mississauga, ON, Canada) and Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). The analyses were carried out using a mobile phase of 5 mM H_2SO_4 at 60 °C with a flow rate of 0.5 mL/min for 30 min. Different concentrations of glucose, lactic acid, and acetic acid (HPLC grade, Sigma-Aldrich, St. Louis, MO, USA) standard solutions were prepared and analyzed for standard curves preparation. Samples with a glucose concentration lower than 1 g/L were analyzed by D-glucose Assay Kit. Lactose and galactose concentrations were measured by Lactose/D-Galactose Assay Kit. The lactose concentrations in 0-h (initial) and 72-h (residual) fermentation samples were used for the calculation of lactose hydrolysis % as the following Equation (3-1):

$$\text{Lactose hydrolysis \%} = \frac{(\text{Initial lactose concentration} - \text{residual lactose concentration})}{\text{Initial lactose concentration}} \times 100$$

3.2.3.2 Ethanol

Ethanol was analyzed by gas chromatography (GC, 7890A, Agilent Technologies, Mississauga, ON, Canada) equipped with an autosampler (7693, Agilent Technologies, Mississauga, ON, Canada), Resteck Stabilwax-DA column [0.5 μm × 30 m × 0.53 mm] and frame ionization detector. Helium was used as the carrier gas with a constant pressure of 7.5 lb/in^2. 1-Butanol was used as the internal standard which helped to account for the ethanol loss during sample preparation. As for the external standard and blank groups, 10% (v/v) ethanol and double-distilled water were used, respectively, instead of the supernatant of fermentation samples. Split injection was selected with an injection volume of 1 μL and a split ratio of 20:1. The ratio of ethanol area to 1-butanol area was used as the response factor for ethanol content calculation.

Ethanol yield efficiency, also called fermentation efficiency, was calculated as the ratio of the actual ethanol yield to the theoretical ethanol yield (Wu *et al.*, 2006; Yao *et al.*, 2012). Theoretically, 1 g of glucose or galactose which contains 0.4 g of fermentable carbon can produce 0.511 g of ethanol. Therefore, the theoretical ethanol yield of 1 g fermentable carbon is 1.278 g. In this study, fermentable carbon from both starch and lactose in the fermentation was accounted for the calculation. The actual ethanol yield from 1 g fermentable carbon was derived as the ratio of the ethanol amount measured to the known amount of fermentable carbon input.

3.2.4 Fermentation of whey permeate-blended mash using *K. lactic* and *A. oryzae* β-galactosidases

Mash containing 25% wheat and whey permeate was prepared in 250 g shake-flask scale as section 3.2.2.1. The dosages of 65 LAU and 20 U per g of lactose were selected

for *K. lactic* and *A. oryzae* β-galactosidases, respectively, as the comparable lactose hydrolysis percentage of around 95% was obtained by these two dosages in pure lactose solution for 72 h. LAU, short for lactase activity unit, was defined by Novozymes. One LAU is the amount of commercial enzyme that can produce 1 μmol of glucose per minute under standard conditions: 4.7% lactose concentration and 30 °C in the standard milky buffer (pH 6.5). The enzyme activity unit (U) of *A. oryzae* β-galactosidase was given by Sigma. One U will hydrolyze 1 μmol of o-nitrophenyl-β-D-galactoside per minute at pH 4.5 at 30 °C. Lactose concentrations of 0-h and 72-h fermentation samples were measured to evaluate the effectiveness of *K. lactic* and *A. oryzae* β-galactosidases on lactose hydrolysis during fermentation.

3.2.5 Selection of *A. oryzae* β-galactosidase dosage on lactose hydrolysis during fermentation

The same medium as section 3.2.4 was used for selection of an appropriate dosage of *A. oryzae* β-galactosidase which was chosen based on the results obtained in section 3.2.4. Four dosages of 10, 20, 30, and 40 U per g of lactose were employed in the fermentations of whey permeate-blended wheat mash, individually. Lactose concentrations of 0-h and 72-h fermentation samples were determined to evaluate the effectiveness of each *A. oryzae* β-galactosidase dosage on lactose hydrolysis during fermentation.

3.2.6 Various wheat replacement by lactose from whey permeate

Mashes containing 30% wheat and 25% wheat with whey permeate were prepared as described in section 3.2.2.1. Mashes of 20% and 15% wheat blended with whey permeate were prepared similarly to that of 25% wheat with whey permeate. Less wheat was used,

while a corresponding increased amount of whey permeate was supplemented. The total fermentable carbon amount of each whey permeate-blended group was maintained at the same level as that of 30% wheat. *A. oryzae* β-galactosidase was added to the whey permeate-blended groups with a dosage of 20 U per g of lactose at the onset of the fermentation. Initial and residual glucose, galactose, lactose, lactic acid, and acetic acid concentrations in fermentation samples were analyzed. Ethanol content was measured to calculate ethanol yield efficiency.

3.2.7 Whey permeate-blended STARGEN-based fermentations in 5-L bioreactors

Fermentation with 25% wheat blended with whey permeate was selected for the scale-up in 5-L bioreactors. Both mashes of 30% wheat and 25% wheat with whey permeate were prepared according to section 3.2.2.2. The *A. oryzae* β-galactosidase dosage of 20 U per g of lactose was used. Fermentation samples were taken at 0.5, 3, 6, 12, 18, 24, 48, and 72 h to monitor sugars and organic acids concentration change during the fermentations. Ethanol content was analyzed by GC for the calculation of ethanol yield efficiency.

3.2.8 Whey permeate-blended jet cooking-based fermentations in 5-L bioreactors

In addition to granular starch hydrolyzing enzymes (STARGEN), the conventional α-amylase (Liquozyme) and glucoamylase (Spirizyme) have also been commercially available. The key difference between them is the operation temperature. Liquefaction by the conventional α-amylase is carried out at the temperature of 90-110 °C (jet cooking) which is almost double of the temperature used for granular starch hydrolyzing enzymes (Wang *et al.*, 2005; Sharma *et al.*, 2007). Both the conventional enzymes and granular starch hydrolyzing enzymes are widely used by the ethanol industry. Therefore, the whey

permeate supplementation in the jet cooking-based fermentation was investigated in this study with a purpose of expanding the scope of utilizing whey lactose in the ethanol industry.

Wheat flour (1.98 mm) was used for mash preparation which was carried out according to Gibreel *et al.* (2009) with a few modifications. Viscozyme wheat FG (300 μL/kg of grain), Fermgen (940 μL/kg of grain), and Liquozyme SC DS (21 μL/kg of grain, 1/4 of the total dosage) were used for the pre-treatment of mash before jet cooking. The jet cooking system was set up in Agri-Food Discovery Place (Edmonton, Alberta, Canada) with the jet cooker unit donated by Pick Heaters, Inc. (West Bend, WI, USA). After jet cooking, DPEC was added to mash, followed by 72-h storage at 4 °C in a sterile Nalgene® clearboy container (Nalge Nunc International Corp., Rochester, NY, USA). Whey permeate was blended into jet cooked wheat mash prior to yeast inoculation, which was used for the co-fermentation of wheat and whey permeate. Jet cooked wheat mash without whey permeate was used as the control. The total fermentable carbon amount of these two groups was maintained at the same level. Then, second liquefaction was carried out at 85 °C for 90 min by adding Liquozyme SC DS (63 μL/kg of grains, 3/4 of the total dosage), followed by a pre-saccharification step with Spirizyme Ultra (600 μL/kg of grains) at 30 °C for 15 min. Hydrated yeast was inoculated in the bioreactors to have an approximate initial cell concentration of 2×10^7 cfu/mL as soon as the 15-min pre-saccharification was complete. The *A. oryzae* β-galactosidase dosage of 20 U per g of lactose was used. Fermentation samples at 0.5, 6, 12, 24, 48, and 72 h were taken and analyzed to monitor sugar consumption and organic acid accumulation during the

fermentations. Ethanol content was analyzed by GC for the calculation of ethanol yield efficiency.

3.2.7 Statistical analysis

All the fermentations were carried out in triplicate. The difference of lactose hydrolysis percentage and ethanol yield efficiency for each fermentation of the same batch were assessed by ANOVA (Analysis of Variance) followed by Tukey's test with 95% confidence ($\alpha = 0.05$).

3.3 Results and Discussion

3.3.1 Comparison of lactose hydrolysis by *K. lactic* and *A. oryzae* β-galactosidases

Lactose concentrations in 0-h and 72-h fermentation samples were shown in Table 3-1 with a purpose of selecting a more effective β-galactosidase for lactose hydrolysis under the fermentation conditions. The initial lactose concentrations obtained by the fermentations using *A. oryzae* and *K. lactis* β-galactosidases were not significantly different. The fermentation using *A. oryzae* β-galactosidase had a residual lactose concentration at 5.3 g/L, resulting in 85.5% lactose hydrolysis. While considerably higher residual lactose concentration was obtained by the fermentation using *K. lactis* β-galactosidase, leading to a lactose hydrolysis percentage of 13.7% which was significantly lower than that obtained by the fermentation with *A. oryzae* β-galactosidase. That is likely attributed to their different enzyme activities at the pH of the wheat-to-ethanol fermentation. The optimum pH of *A. oryzae* β-galactosidase at 4.5 (Grosova *et al.*, 2008a) is much closer to the fermentation pH of 4.0 than that of *K. lactis* β-galactosidase at 6.5-7.3 (Mahoney, 1997). *A. oryzae* β-galactosidase was more suitable

and efficient for lactose hydrolysis at an acidic fermentation condition of pH 4.0 than *K. lactis* β-galactosidase, which has not been addressed by the reported studies on whey-to-ethanol fermentation. However, it is advised to the food industries that fungal β-galactosidases are suitable for the processing of acid whey instead of yeast enzymes because of the optimum pH range of 2.5-5.4 (Panesar *et al*., 2010). Therefore, *A. oryzae* β-galactosidase was selected for the future work on co-fermentation of wheat and whey permeate.

Table 3-1 Lactose concentration in 0-h and 72-h samples from the co-fermentations using *K. lactis and A. oryzae* β-galactosidases[a]

	Enzyme source	Enzyme dosage (U* per g of lactose)	Lactose (g/L)	
			0 h	72 h
Enzyme selection	*A. oryzae*	20	36.8 ± 0.6	5.3 ± 0.6
	K. lactis	65 LAU**	36.3 ± 2.4	31.3 ± 1.5
Dosage selection	*A. oryzae*	10	35.6 ± 1.0	11.0 ± 1.1
	A. oryzae	20	34.2 ± 1.8	4.0 ± 0.2
	A. oryzae	30	34.0 ± 0.8	3.9 ± 0.4
	A. oryzae	40	35.4 ± 1.6	3.4 ± 0.6

[a]All the fermentations were carried out using 25% wheat blended with whey permeate in the scale of 250 g shake-flask.

*U: unit for *A. oryzae* β-galactosidase which is measured at pH 4.5, 30 °C.

**LAU: unit for *K. lactis* β-galactosidase which is measured at pH 6.5, 30 °C.

3.3.2 Selection of *A. oryzae* β-galactosidase dosage

Lactose concentrations of 0-h and 72-h samples from the fermentations using four different *A. oryzae* β-galactosidase dosages were presented in Table 3-1. The initial lactose concentrations were comparable among these four groups. Whereas there was a significant difference of residual lactose concentration among the four fermentation

groups. The fermentation with 10 U per g of lactose possessed a significant higher residual lactose concentration than the fermentations with 20, 30, and 40 U per g of lactose. The lactose hydrolysis percentages were calculated according to Equation (3-1), showing 69.0%, 88.4%, 88.6%, and 90.2% lactose hydrolysis for the fermentations using 10, 20, 30, and 40 U per g of lactose, respectively. Compare to 69.0% lactose hydrolysis obtained by the fermentation with the dosage of 10 U per g of lactose, a considerable increase of lactose hydrolysis to 88.4% was observed for that using 20 U per g of lactose. When β-galactosidase dosage increased from 20 to 40 U per g of lactose, Lactose hydrolysis was enhanced slightly. However, the hydrolysis percentages of 88.4%, 88.6%, and 90.2% were not significantly different. Therefore, the *A. oryzae* β-galactosidase dosage of 20 U per g of lactose was selected.

3.3.3 Various wheat replacement by whey permeate

As shown in Table 3-2, glucose concentrations at 72 h were considerably lower than 1 g/L, indicating complete glucose consumption at the end of the fermentations. However, there was galactose residue at 72 h for the co-fermentations of wheat and whey permeate, which is possibly due to glucose repression and low galactose consumption rate. It is reported that *S. cerevisiae* consumes glucose before galactose and the uptake of galactose is much slower than that of glucose (Ramakrishnana and Hartley, 1993; Staniszewski *et al*., 2009). As for lactose, the initial concentration increased proportionally with wheat replacement percentage by whey permeate. A similar trend was also observed for the residual lactose concentration. Based on these values, 86.5%, 85.8%, and 85.4% lactose hydrolysis were calculated for the fermentations using 25%, 20%, and 15% wheat blended with whey permeate, respectively. The percentage of

lactose hydrolysis was maintained at the comparable level as expected, since the same dosage of *A. oryzae* β-galactosidase was used for all the co-fermentations of wheat and whey permeate. The presence of lactic acid in 0-h samples of the fermentation using whey permeate-blended wheat was detected (Table 3-2), which can be explained by lactic acid originating from whey permeate. Lactic acid bacteria are the natural microorganisms present in whey permeate, which can consume lactose and produce lactic acid. It was observed by Wongso (1993) that the lactic acid content in whey/whey permeate became even higher when there was gross bacterial contamination or whey/whey permeate were concentrated. In addition, the increase of initial lactic acid concentration corresponded with the increase of wheat replacement by whey permeate, as expected. The more whey permeate added, the higher initial lactic acid concentration.

Table 3-2 Glucose, galactose, lactose, lactic acid, and acetic acid concentrations in 0-h and 72-h samples of the fermentations with various wheat replacements by whey permeate[a]

Wheat concentration (%, w/w)	Wheat replacement %*	Glucose (g/L)		Galactose (g/L)		Lactose (g/L)		Lactic acid (g/L)	
		0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
30	0	91.6 ± 5.1	0.11 ± 0.00	-	-	-	-	-	0.3 ± 0.0
25	16.7	72.5 ± 2.5	0.07 ± 0.00	1.6 ± 0.4	3.7 ± 0.7	32.3 ± 1.3	4.3 ± 0.3	0.8 ± 0.1	0.9 ± 0.2
20	33.3	49.5 ± 0.6	0.09 ± 0.00	2.0 ± 0.3	5.2 ± 0.2	64.3 ± 1.9	9.2 ± 0.1	1.3 ± 0.0	1.8 ± 0.0
15	50	37.4 ± 0.5	0.11 ± 0.02	3.7 ± 0.3	5.6 ± 0.2	97.9 ± 2.8	14.4 ± 0.4	1.8 ± 0.1	2.6 ± 0.0

*Equation (3-2): $\text{Wheat repacement \%} = \frac{(30\% - \text{Actual wheat concentration})}{30\%} \times 100$

[a]All the four fermentations contained the same amount of fermentable carbon. Wheat of 30% was carried out as the control.

The ethanol yield efficiency of the fermentation using 25% wheat blended with whey permeate was significantly lower than 89.5% of the control (Fig. 3-1). That is likely due to lower lactose-to-ethanol conversion than that of starch-to-ethanol, which is possibly attributed to incomplete lactose hydrolysis and residual galactose. Based on the comparison among the three whey permeate-blended fermentations, the ethanol yield efficiency decreased from 83.9% to 77.8% with the increased wheat replacement by whey permeate. As stated above, it is less efficient to convert lactose to ethanol than starch. Therefore, the more whey permeate was added to replace wheat, the lower the overall ethanol yield efficiency fell.

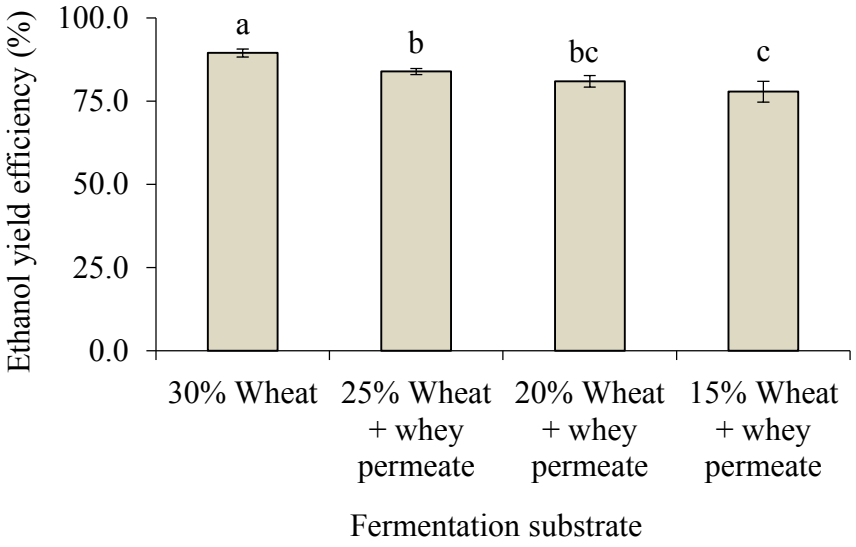


Figure 3-1 Ethanol yield efficiency of the fermentations with various wheat replacements by whey permeate[a]

[a]All the four fermentations contained the same amount of fermentable carbon. Wheat of 30% (wt/wt, wet basis) was carried out as the control. Means with the same letter are not significantly different ($p<0.05$).

Regardless of reduced ethanol yield efficiency of the whey permeate-blended fermentations, the contribution of whey lactose to ethanol production was obvious. If there is no ethanol produced from whey lactose, ethanol would only be generated from wheat starch. As shown in Table 3-3, the observed amount of ethanol produced from wheat and whey permeate was higher than the expected amount of ethanol from wheat alone in the co-fermentations, indicating that the additional ethanol production was attributed to the whey permeate supplementation. More ethanol was contributed by lactose-to-ethanol production when increased amount of whey permeate was supplemented to replace more wheat.

The published studies on co-fermenting whey lactose with other substrates have not clearly demonstrated and explained the contribution of whey lactose to the overall ethanol yield (Gibbons and Westby, 1988; Kadar *et al*., 2011; Nakamura *et al*., 2012). For example, cheese whey was used as process water for the preparation of rye and wheat mashes individually by Kadar *et al.* (2011). Both rye and wheat mashes were fermented by *K. marxianus* for ethanol production, of which the fermentation was completed by 190 h. Apart from the long fermentation time, significant lower overall ethanol yield efficiencies of 72% and 56-57% were obtained for the fermentations of rye and wheat respectively, compared to the reported around 90% ethanol yield efficiency from grains (Gohel and Duan, 2012; Gibreel *et al.*, 2009). It was explained that the ethanol yield efficiency was calculated based on starch and lactose. Therefore, it can be identified that the low ethanol yield efficiency was due to the incorporation of whey. The amount of whey lactose added to rye and wheat mashes was around 40% of the total substrates which was much less than 50% wheat replacement in the co-fermentation of 15% wheat

with whey permeate. However, the overall ethanol yield efficiencies achieved by Kadar *et al*. (2011) were even lower than 77.8% obtained by the co-fermentation of 15% wheat with whey permeate in this study.

In addition, compared to the previous results of the fermentations with pre-hydrolyzed whey permeate (Parashar *et al*, manuscript in preparation), the fermentations of 20% and 15% wheat with whey permeate in this study possessed a significant increase of ethanol yield efficiency, which indicated that the strategy of wheat replacement by lactose instead of glucose and galactose, resulting in a lower initial monosaccharide concentration and lower osmotic stress caused to the yeast, was effective to improve ethanol yield efficiency when more wheat was replaced. As for the case of replacing wheat starch by free glucose and galactose in pre-hydrolyzed whey permeate, the growth rate of yeast cells probably was compromised under a high osmotic pressure from the substrate sugar at the beginning of the fermentation, because the metabolism of the yeasts mainly focused on the synthesis of glycerol, a so-called compatible solute or osmolyte (Zhao and Bai, 2009; Parmar *et al*., 2012).

Table 3-3 Estimated amount of ethanol produced from whey permeate in the co-fermentations with various wheat replacement by whey permeate

Substrate	Expected ethanol from wheat[a] (g)	Observed ethanol from wheat + whey permeate[b] (g)	Estimated ethanol from whey permeate[c] (g)
30% Wheat	22.4	-	-
25% Wheat + whey permeate	18.7	21.0	2.3
20% Wheat + whey permeate	14.9	20.3	5.4
15% Wheat + whey permeate	11.2	19.5	8.3

[a]The ethanol amount produced from wheat in the co-fermentation was calculated based on the known amount of fermentable carbon from wheat starch and the ethanol yield efficiency of 89.5% from the control. With the actual ethanol yield of 89.5%, 1.144 g of ethanol can be produced from 1 g of fermentable carbon.

[b]The observed ethanol amount produced from wheat and whey permeate was measured as section 3.2.3.2.

[c]The estimated ethanol from whey permeate of each co-fermentation was calculated by Equation (3-3):

$$\text{Estimated ethanol from whey permeate} = \text{observed ethanol from wheat and whey permeate} - \text{expected ethanol from wheat}$$

3.3.4 STARGEN-based fermentations of wheat only and wheat blended with whey permeate in 5-L bioreactors

Fig. 3-2 illustrated the time courses of glucose, galactose, lactose, lactic acid, acetic acid, and ethanol concentrations for the STARGEN-based fermentation of wheat control and wheat blended with whey permeate in 5-L bioreactors. The pattern of glucose consumption was identical for both the control and whey permeate-blended fermentations. The initial glucose was generated from wheat starch hydrolysis by STARGEN^{TM} 002 in the pre-saccharification step. The glucose concentration went up during 0-3 h, resulting from faster glucose release from starch than glucose consumption by yeast. It decreased dramatically during 3-12 h due to the accelerated glucose consumption by yeast and the reduced glucose release from less remaining starch (Wu *et al*., 2005). Subsequently, the glucose concentration was maintained at a low level of less than 1 g/L until the end of the fermentation, demonstrating the complete glucose consumption.

Lactose was gradually hydrolyzed during the fermentation, resulting in 83.8% lactose hydrolysis which was comparable to those achieved in the shake-flask fermentations. Galactose was one of the products generated from lactose hydrolysis, of which the concentration went up during the first 12 hours (Fig. 3-2 C). In the period of 0-12 h, lactose concentration decreased from 37.0 g/L to 21.8 g/L, indicating that 15.2 g/L of lactose was hydrolyzed to give 8 g/L glucose and 8 g/L galactose. In practice, galactose of 7.8 g/L was detected at 12 h which was fairly close to the theoretical value of 8 g/L, demonstrating that galactose was hardly taken up by yeast during the first 12 hours, which was possibly caused by glucose repression (catabolite repression). The presence of glucose in high concentration will down-regulate and inhibit the enzyme

synthesis for assimilation of other sugars present in medium (Jacques, *et al*, 2003). Lactose concentration decreased from 21.8 g/L to 6.0 g/L during 12-72 h, which would bring up the galactose concentrations by 8.3 g/L, if there was no galactose uptake by yeast. However, galactose concentration was maintained at a comparable level of about 8 g/L without a significant increase after 12 h (Fig. 3-2 C), illustrating that galactose was slowly consumed by yeast after 12 h when the presence of glucose was at a considerably low concentration (<1 g/L), which was again confirmed by glucose repression (Ostergaard *et al*., 2000). Nonetheless, not all of the galactose was assimilated by yeast, leading to the residual galactose at 72 h. That is likely attributed to low galactose consumption rate controlled by galactose induction (Sanchez *et al.,* 2010). No noticeable galactose consumption before 12 h and residual galactose at the end of the fermentation were also observed in the previous study on the fermentations using pre-hydrolyzed whey permeate (Parashar *et al*, manuscript in preparation).

As shown in Fig. 3-2 B and D, lactic acid was produced and accumulated in both the fermentations of wheat only and wheat blended with whey permeate, which is possibly due to natural lactic acid bacteria in the mashes as the mashes prepared for STARGEN-based fermentation did not go through sterilization (Wu *et al*., 2005). The production of lactic acid during the fermentation was also detected in the studies of Wu *et al*. (2006), however, by monitoring the decrease of pH. The presence of acetic acid in 0-h samples was observed for both the fermentations of wheat only and wheat blended with whey permeate, which could be generated by natural acetic acid-producing microorganisms during the mash preparation or storage. The higher initial lactic acid and acetic acid concentrations in the whey permeate-blended fermentation were contributed by lactic

acid and acetic acid from whey permeate which were produced by the natural microorganisms during its shipment and storage (Wongso, 1993).

Ethanol was produced throughout the 72-h fermentations shown in Fig. 3-2 A and C. The final ethanol concentration obtained by the whey permeate-blended fermentation was lower than that of the control. However, the comparison between the final ethanol concentrations cannot be directly used to evaluate the effectiveness of whey lactose utilization for ethanol production, as the percentages of solids in the control and whey permeate-blended fermentations were different. Therefore, ethanol yield efficiency was determined for a comprehensive evaluation. The ethanol yield efficiency of the control and whey permeate-blended fermentations was presented in Fig. 3-3. Similar to the results achieved in section 3.3.3, 84.9% ethanol yield was obtained by the co-fermentation of wheat and whey permeate, which was significantly lower than 92.6% of the fermentation with wheat only. An ethanol yield of 198.9 g was expected from wheat in the co-fermentation according to the starch-to-ethanol yield efficiency of the control, while the observed ethanol production from wheat and whey permeate was determined to be 218.8 g. Therefore, the ethanol produced from whey permeate in the STARGEN-based co-fermentation was estimated to be 19.9 g (equation shown in Table 3-3), indicating the contribution of whey permeate to ethanol production.

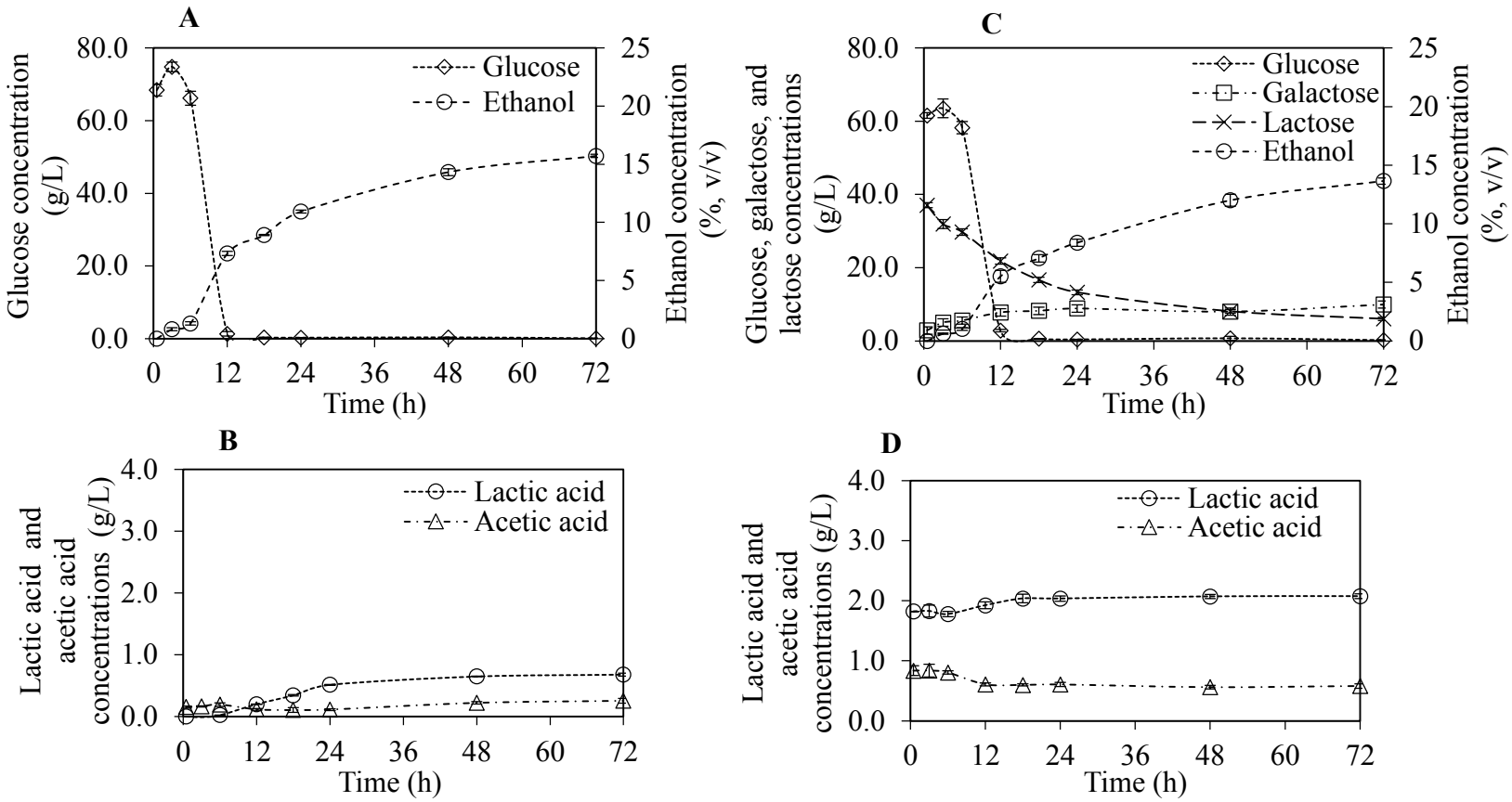


Figure 3-2 Ethanol production, sugar consumption, and organic acid accumulation during the STARGEN-based fermentations in 5-L bioreactors*. (A) Ethanol production and glucose consumption, (B) lactic acid and acetic acid accumulation during the fermentation of 30% (wt/wt, as is) wheat; (C) ethanol production and consumption of glucose, lactose, and galactose, (D) lactic acid and acetic acid accumulation during the fermentation of 25% wheat blended with whey permeate.

*The two fermentations contained the same amount of total fermentable carbon.

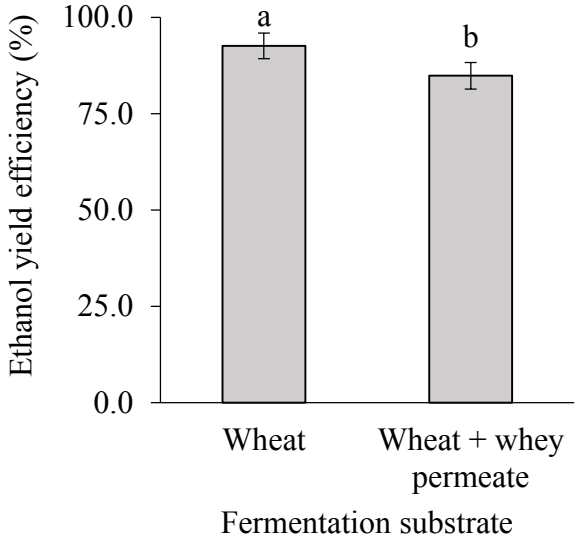


Figure 3-3 Ethanol yield efficiency of the STARGEN-based fermentations of wheat only and wheat blended with whey permeate.
Means with the same letter are not significantly different (p<0.05).

3.3.5 Jet cooking-based fermentations of wheat only and wheat blended with whey permeate in 5-L bioreactors

The time courses of glucose, galactose, lactose, lactic acid, and ethanol concentrations during the jet cooking-based fermentations of wheat only and whey permeate-blended wheat were shown in Fig. 3-4. Glucose consumption patterns of these two fermentations were similar, with a peak at 6 h and a considerable low glucose concentration starting at 24 h. Lactose was gradually hydrolyzed with a final hydrolysis efficiency of 88.2%, which was comparable to those obtained by the co-fermentations in shake flasks and 5-L bioreactors. The galactose concentration increased during 0-24 h, followed by a stable concentration at around 7 g/L (Fig. 3-4 C). The trend of galactose concentration was similar to that of the STARGEN-based fermentation. The major

difference between the STARGEN- and jet cooking-based fermentations was the time point when galactose concentration started to maintain at a stable level. The galactose concentration stabilized at 24 h for the jet cooking-based fermentation, instead of 12 h. It was likely due to prolonged glucose repression caused by the high glucose concentration of 14.0 g/L at 12 h in jet cooking-based fermentation, unlike the considerably low glucose concentration at 12 h in the STARGEN-based fermentation. Lactic acid was produced and accumulated in both the fermentations of wheat only and whey permeate-blended wheat, as shown in Fig. 3-4 B and D. The initial lactic acid of the whey permeate-blended fermentation was originated from whey permeate as stated in section 3.3.3 and 3.3.4. Interestingly, acetic acid was not detected in the jet cooking-based fermentations, which was probably because of the sterilization effect of jet cooking process which inactivated the natural acetic acid-producing microorganisms present in the mashes.

The production of ethanol was illustrated in Fig. 3-4 A and C. The final ethanol concentrations reached by the control and whey permeate-blended fermentations were used for calculating the ethanol yield efficiency. Fig. 3-5 showed that the ethanol yield efficiencies of 90.6% and 83.3% were achieved by the jet cooking-based fermentations with wheat only and whey permeate-blended wheat, respectively. The lactose-to-ethanol contribution from whey permeate was also observed for the jet cooking-based fermentation of wheat and whey permeate. An ethanol yield of 178.3 g expected from wheat was estimated according to the starch-to-ethanol yield efficiency of 90.6%. While the observed ethanol from wheat and whey permeate was 196.7 g, illustrating that 18.4 g of ethanol was predicted to be produced from whey permeate in the co-fermentation.

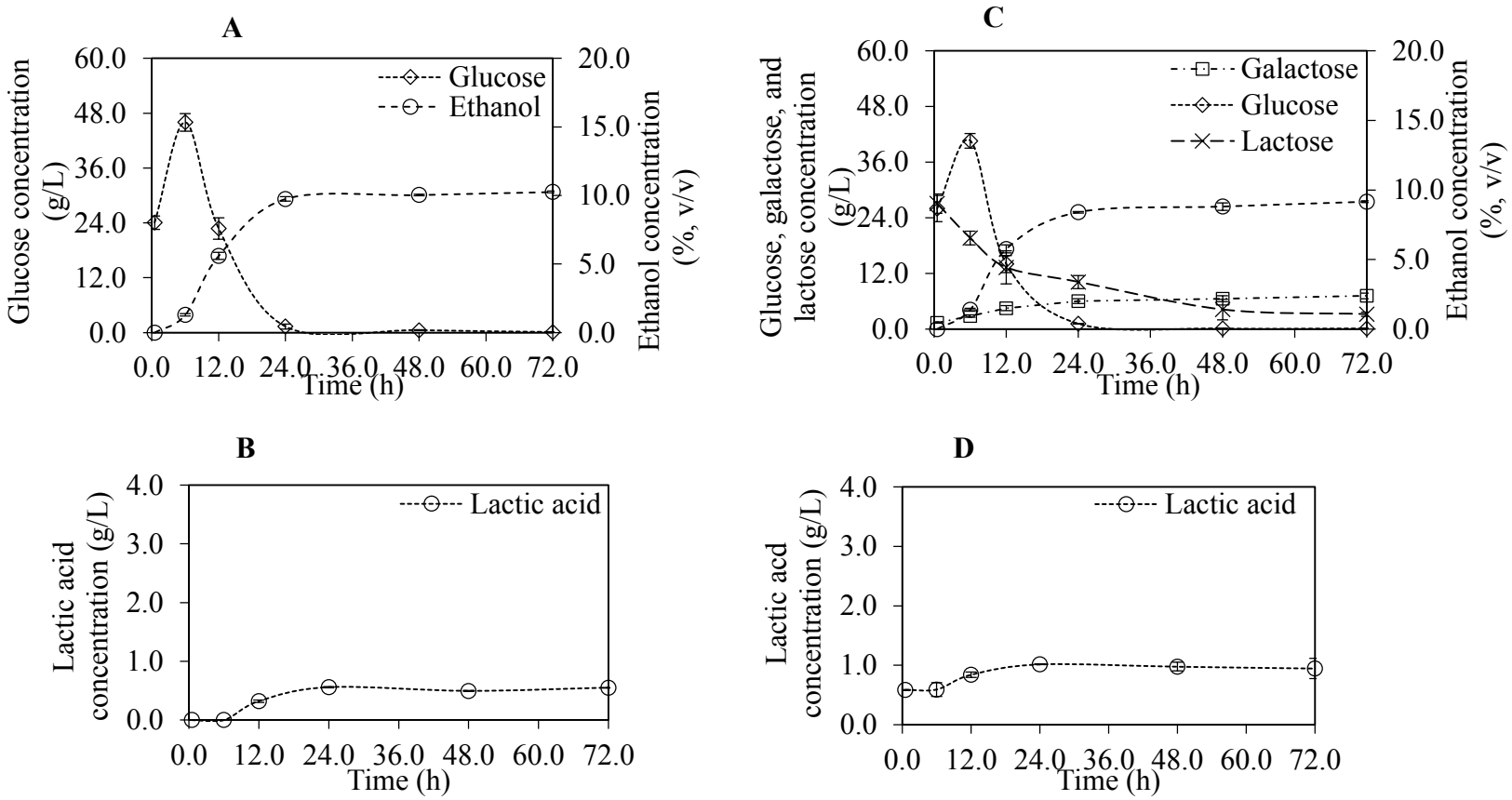


Figure 3-4 Ethanol production, sugar consumption, and organic acid accumulation during the jet cooking-based fermentation in 5-L bioreactors[a]. (A) ethanol production and glucose consumption; (B) lactic acid accumulation during the fermentation of jet-cooked wheat only; (C) ethanol production and consumption of glucose, lactose, and galactose; (D) lactic acid accumulation during the fermentation of jet cooked wheat blended with whey permeate.

[a]The two fermentations contained the same amount of total fermentable carbon.

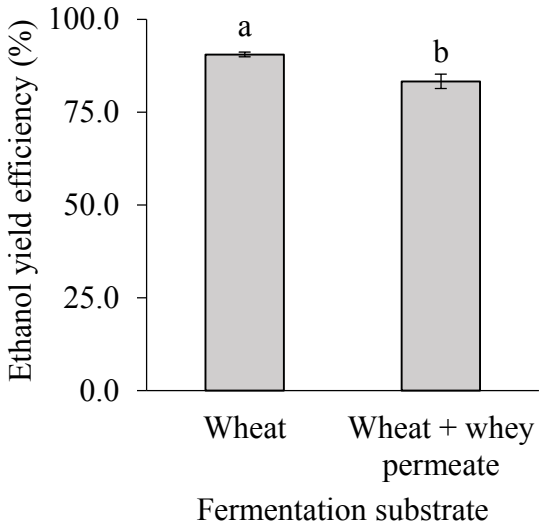


Figure 3-5 Ethanol yield efficiency of the jet cooking-based fermentations of wheat only and wheat blended with whey permeate.

Means with the same letter are not significantly different (p<0.05).

3.4 Conclusions

The supplementation of whey permeate as a co-substrate to wheat can contribute to the ethanol production in the conventional wheat-to-ethanol fermentation with *A. oryzae* β-galactosidase added for simultaneous lactose hydrolysis during the fermentation. *A. oryzae* β-galactosidase was selected due to its significantly higher efficiency for lactose hydrolysis than that of *K. lactis* β-galactosidase at the acidic fermentation pH of 4.0. Whey permeate can be utilized for ethanol production in the wheat-to-ethanol fermentations of both STARGEN and jet cooking systems. However, the lactose-to-ethanol conversion was less efficient than that of the starch-to-ethanol conversion. Co-fermentation by efficient galactose-assimilating *S. cerevisiae* strains with other

commercial *S. cerevisiae* strains will most likely enhance galactose conversion to ethanol. In addition, future attempts of reusing β-galactosidase through enzyme immobilization and evaluating the nutritional value of DDGS are necessary and critical for a comprehensive assessment of whey/whey permeate utilization as a co-substrate for ethanol production.

4 Utilization of Immobilized β-Galactosidase for Ethanol Production during the Co-Fermentation of Wheat and Whey Permeate

4.1 Introduction

The co-fermentation of wheat and whey permeate by *S. cerevisiae* for ethanol production was investigated in Chapter 3. Soluble *A. oryzae* β-galactosidase was added at the onset of the fermentation to conduct lactose hydrolysis simultaneously with ethanol production. However, the expense of soluble β-galactosidase is a major and potential concern associated with this approach. The additional enzyme cost has the potential to increase the total ethanol production cost. Enzyme immobilization is one approach of great interest for the reduction of the high enzyme cost, which allows reuse of enzymes for multiple runs (Tanaka and Kawamoto, 1999). To understand the potential in this application, the immobilization of β-galactosidase was investigated.

β-Galactosidase is widely used by the food industry for lactose hydrolysis in milk due to the fact that a significant proportion of the population suffers from lactose intolerance as a result of inability to metabolize lactose (Shukla and Wierzbicki, 1975). In addition to milk lactose, whey lactose is also commercially hydrolyzed by β-galactosidase. The hydrolyzed whey/whey permeate is used as a sweetener in food products such as canned fruit syrups or beverages (Marwaha and Kennedy, 1988; Shukla and Wierzbicki, 1975). So far, the immobilization of β-galactosidase has been extensively studied with a continuous interest for both lactose hydrolysis and production of galacto-oligosaccharides (Husain, 2010; Panesar *et al.*, 2010; Grosava *et al.*, 2008a). According to literature, immobilization is considered to provide protective effects to β-galactosidases against several denaturing factors such as extreme pH, temperature, and

high ionic strength (Grosova *et al*., 2008b; Torres and Batista-Viera, 2012; Husain, 2010; Kosseva *et al.*, 2009).

Adsorption, covalent attachment, and entrapment are the three most popular methods used for enzyme immobilization. Among them, entrapment is considered as a simple and efficient method, for which natural polymers such as alginate, carrageenan, and chitosan are commonly used. Non-toxicity, biocompatibility, safe disposal, and low cost for the materials are the major advantages with enzyme entrapment (Kosseva *et al.*, 2009). Polyvinyl alcohol (PVA) hydrogel marketed as Lentkats Biocatalyst by LentiKat's Biotechnologies is one of the recently used matrices for enzyme entrapment, demonstrating mild conditions of preparation, good mechanical properties, low biodegradability, easy separation, and high stability, *etc*. (Rebros *et al*., 2006; Husain, 2010; LentiKat's Technologies, 2014). The easy separation of immobilized β-galactosidase from fermentation broth is one of the most important factors. The lens-shaped Lentikats Biocatalyst with a diameter of 3-4 mm is larger than the milled wheat particle of 0.5 mm, which can substantially facilitate the biocatalyst recycling as well as the recovery of fermentation broth for preparing DDGS which is the key by-product of grain-to-ethanol fermentation.

In this study, *A. oryzae* β-galactosidase was immobilized in PVA hydrogel for the co-fermentation of wheat and whey permeate. The focus of this chapter was to investigate the effectiveness of the immobilized β-galactosidase on the co-fermentation of wheat and whey permeate, as well as its reusability in the 7-cycle co-fermentations. The effectiveness of the immobilized β-galactosidase on the lactose hydrolysis percentage and ethanol yield efficiency was compared to that of the soluble enzyme. The reusability was

evaluated based on the retention of enzyme activity, lactose hydrolysis percentage, and ethanol yield efficiency. Immobilized β-galactosidase has been used for lactose hydrolysis in the simple matrices such as milk, cheese whey, and whey permeate (Husain, 2010; Kosseva *et al.*, 2009). A review of the current literature has revealed that this study is the first attempt to use the immobilized β-galactosidase in such complex matrix of wheat and whey permeate for lactose hydrolysis and ethanol production.

4.2 Materials and Methods

4.2.1 Grain, enzymes, whey permeate, and others

Spring wheat (AC Andrew) was ground by the laboratory hammer mill with a sieve size of 0.5 mm diameter. The moisture and starch contents of wheat flour were determined as described in section 3.2.1. Whey permeate was shipped on ice and stored at 4 °C after receipt. Lactose concentration in whey permeate was measured by HPLC as stated in section 3.2.3.1.

STARGEN™ 002, Optimash™ TBG, GC 626, and Fermgen from Genencor International (Palo Alto, CA, USA) were used for mash pre-treatment and starch granular hydrolysis. *A. oryzae* β-galactosidase was purchased from Sigma-Aldrich (St. Louis, MO, USA) to conduct whey lactose hydrolysis during fermentation. PVA and polyethylene glycol (PEG) were provided by LentiKat's Biotechnologies (Straz pod Ralskem, Czech) for the preparation of Lentikats Biocatalyst in which *A. oryzae* β-galactosidase was entrapped. D-Glucose Assay Kit and Lactose/D-Galactose Assay Kit were obtained from Megazyme (Country Wicklow, Ireland). SuperStart distiller's yeast was supplied from Lallemand Ethanol Technology (Milwaukee, WI, USA).

4.2.2 Enzyme immobilization

The immobilization of β-galactosidase in PVA hydrogel is a simple entrapment method carried out at mild conditions without any additional treatments such as cross-linking. Lentikats Biocatalyst was a porous matrix made of PVA and PEG in biconvex lens shape with a diameter of 3-4 mm and a center thickness of 200-400 nm. *A. oryzae* β-galactosidase was entrapped within the lattice of PVA-PEG matrix. The pore size on the surface of Lentikats Biocatalyst was estimated to be approximate 0.12 μm which allows lactose molecules to go through and interact with entrapped enzymes (Pikus, 2010).

The immobilized β-galactosidase was prepared according to the procedure recommended by LentiKat's Biotechnologies (2012). Briefly, *A. oryzae* β-galactosidase powder was weighed and dissolved in filtered 100 mM sodium acetate buffer (pH 4.5) and used as the enzyme stock solution containing 775 U/mL. PVA of 10% (wt/wt), PEG of 6% (wt/wt), and distilled water of 79% (wt/wt) which was compiled into a 250 mL Corning™ Pyrex™ storage bottle for preparation of the gel stock solution. Melting of PVA and PEG was carried out by heating the bottle in boiling water for around one and half hours until the gel stock solution became transparent. The bottle was capped loosely to allow water evaporation during heating. The gel stock solution was well mixed by a vortex mixer at speed 10 (Model 945404, Fisher Scientific, Dubuque, IA, USA) every 15 min during heating to prevent overheating. After the gel stock solution was thoroughly melted, water loss during heating was compensated by adding sterile distilled water. The bottle was subsequently capped tightly and kept in a water bath at 35 °C for 30 min. The gel stock solution was mixed by the vortex mixer every 10 min to ensure even cooling. The enzyme stock solution was added to the gel stock solution with a concentration of

5% (wt/wt); mixed thoroughly; and used as the final stock mixture for preparing immobilized β-galactosidase.

Immobilized β-galactosidase was prepared in a biosafety cabinet to ensure the sterile condition of the immobilized enzyme and avoid contamination of the subsequent fermentation. The stock mixture was poured into a sterile Petri dish (150 mm × 15 mm, Fisher Scientific, Dubuque, IA, USA). A lab device LentiPrinter was sunken into the stock mixture; then the LentiPrinter was used to transfer drops of catalyst to new weighed Petri dishes. The total weight of drops and the Petri dishes were recorded immediately after printing. All the drops printed were kept in the biosafety cabinet for 1-h drying at room temperature until 70-75% weight loss of the initial weight was achieved. A stabilizing solution of 0.1 M Na_2SO_4 was added to the Petri dishes for 2-h re-swelling of the dried drops to obtain the optimal mechanical properties. Then all the drops were collected and washed by filtered distilled water to remove Na_2SO_4 and unbound β-galactosidase.

4.2.3 Standard and whey permeate-blended fermentations

All the fermentations were carried out at 250 g shake-flask scale. Mash of 30% was used for the standard fermentation; while 25% wheat blended with whey permeate containing the same amount of fermentable carbon as 30% wheat was employed for the application of immobilized *A. oryzae* β-galactosidase. The mashes were prepared as previously described in section 3.2.2.1.

4.2.4 Analytical assays

4.2.4.1 Glucose, galactose, lactose, lactic acid, and acetic acid

Glucose, galactose, lactose, lactic acid, and acetic acid were analyzed according to the methods previously used in section 3.2.3.1. Glucose, lactic acid, and acetic acid concentrations of the fermentation samples were determined by HPLC. D-Glucose Assay Kit was used for the samples with a glucose concentration lower than 1 g/L. Lactose and galactose were measured by Lactose/D-Galactose Assay Kit. The lactose hydrolysis percentage obtained by the fermentations was calculated as Equation (3-1).

4.2.4.2 Ethanol

Ethanol was detected and measured by GC as previously described in section 3.2.3.2. Ethanol yield efficiency was calculated as the ratio of the actual ethanol yield to the theoretical ethanol yield. The actual ethanol yield was the amount of ethanol produced per g of fermentable carbon in practice. Carbon from wheat starch and whey lactose was accounted as the fermentable carbon.

4.2.4.3 Enzyme assay

The activity of immobilized β-galactosidase was measured towards lactose according to the methods used by Dwevedi and Kayastha (2009) and Pikus (2010) with a few modifications. The assay was carried out in 100 mL 4.7% (w/v) lactose solution (100 mM acetate buffer, pH 4.5). The samples were taken at the 30 min mark of the reaction and were heated immediately in a boiling water bath for 5 min to inactivate enzymes and stop lactose hydrolysis. Glucose released was determined by D-Glucose Assay Kit based on glucose oxidase-peroxidase (GOPOD) method. One unit of β-galactosidase activity in

this study was defined as the amount of enzyme that release 1 μmol glucose per min under the assay conditions.

4.2.5 Fermentations of using soluble and immobilized *A. oryzae* β-galactosidase

Mash of 25% wheat blended with whey permeate was prepared as section 3.2.2.1. The soluble and immobilized β-galactosidase were used individually in the whey permeate-blended fermentations with the same dosage of 20 U per g of lactose. Lactose, glucose, galactose, lactic acid, and acetic acid concentrations in 0-h and 72-h fermentation samples were measured. The initial and residual lactose concentrations were used to calculate the lactose hydrolysis percentage. Ethanol content was determined by GC for calculating ethanol yield efficiency. The comparisons of lactose hydrolysis percentage and ethanol yield efficiency between the fermentations using soluble and immobilized β-galactosidase were carried out to evaluate the effectiveness of the immobilized β-galactosidase on the whey permeate-blended fermentation.

4.2.6 Reusability of immobilized *A. oryzae* β-galactosidase

The reuse protocol for immobilized *A. oryzae* β-galactosidase is illustrated in Fig. 4-1. The fermentation substrate consisted of 25% wheat and whey permeate. The initial enzyme activity of immobilized β-galactosidase was determined as stated in section 4.2.4.3 prior to the application in the co-fermentations. After 72-h fermentation by *S. cerevisiae*, the immobilized β-galactosidase was collected using an autoclaved porcelain Buchner funnel with fixed perforated plate (Fisher Scientific, Dubuque, IA, USA) and washed with sterile distilled water. The enzyme activity after the first use was measured as section 4.2.4.3. Then the immobilized β-galactosidase was loaded for the second run.

As shown in Fig. 4-1, the immobilized β-galactosidase was recycled, measured, and reused for up to 7 cycles.

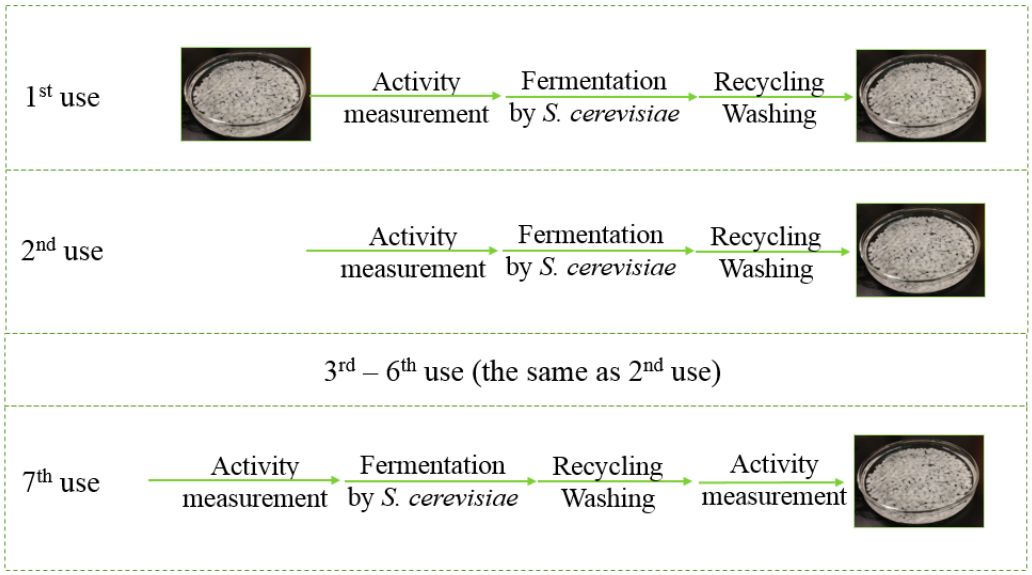


Figure 4-1 Scheme of reusing the immobilized *A. oryzae* β-galactosidase in the co-fermentations of wheat and whey permeate for 7 cycles

The enzyme activities were monitored to determine the activity loss during the 7-cycles of co-fermentation. The measured enzyme activity was used for calculating the retention of enzyme activity (%) which was also known as relative activity and residual activity, as the following Equation (4-1):

$$\text{Retention of enzyme activity \%} = \frac{\text{Enzyme activity after each use}}{\text{Initial enzyme activity}} \times 100$$

The samples at 0 h and 72 h of each fermentation were taken and used for the analyses of glucose, galactose, lactose, lactic acid, and acetic acid. The ethanol concentration in 72-h fermentation samples was measured for calculating ethanol yield efficiency.

4.2.7 Statistical analysis

All the fermentations were carried out in triplicate. The differences of lactose hydrolysis percentage and ethanol yield efficiency between the fermentations using the soluble and immobilized β-galactosidase, as well as the ethanol yield efficiencies obtained by the 7-cycle co-fermentations, were assessed by ANOVA followed by Fisher's Least Significant Difference test with 95% confidence ($\alpha = 0.05$). The regression equations and co-efficiency of determinations (R^2) of the correlations among the cycle number of reusing, retention of enzyme activity, and lactose hydrolysis percentage were derived using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

4.3 Results and Discussion

4.3.1 Comparison between the co-fermentations using soluble and immobilized *A. oryzae* β-galactosidases

There was no significant difference in the lactose hydrolysis percentage observed between the fermentations using the soluble and immobilized β-galactosidase, as shown in Fig. 4-2 A. In addition, the ethanol yield efficiency derived by the fermentation using the immobilized β-galactosidase was comparable to that of the soluble β-galactosidase (Fig. 4-2 B), indicating that immobilized β-galactosidase can be effectively used for the co-fermentation of wheat and whey permeate as the soluble β-galactosidase.

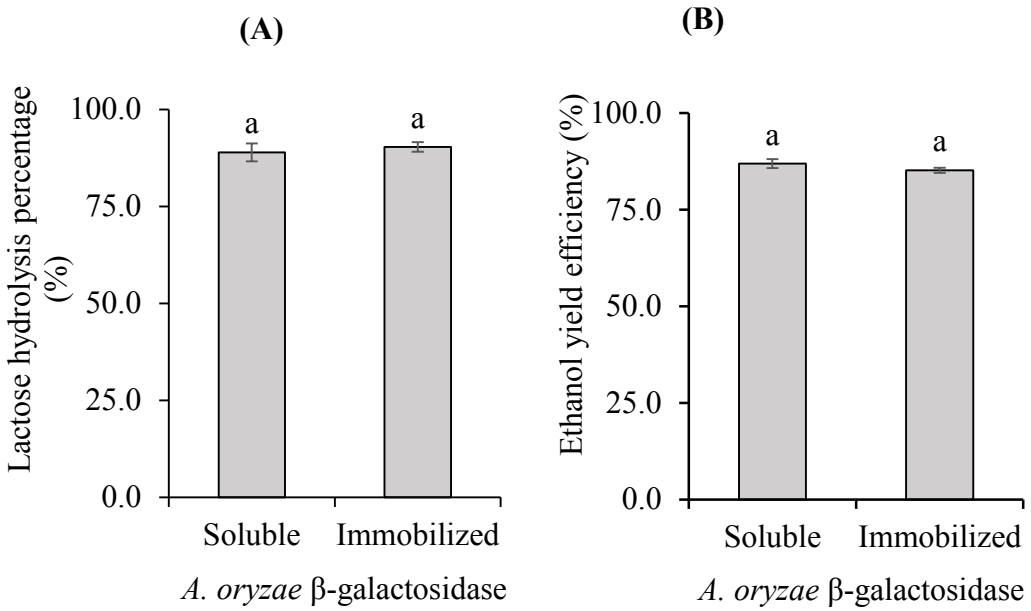


Figure 4-2 Lactose hydrolysis efficiency (A) and ethanol yield efficiency (B) of the co-fermentations of wheat and whey permeate using soluble and immobilized *A. oryzae* β-galactosidase

The substrate of 25% wheat blended with whey permeate, containing the same amount of total fermentable carbon as 30% wheat, was used for the co-fermentations. (Means with the same letter are not significantly different, $p<0.05$.)

4.3.2 Reusability of immobilized *A. oryzae* β-galactosidase

4.3.2.1 Retention of enzyme activity

The residual enzyme activity of immobilized β-galactosidase was presented in the format of retention of enzyme activity, which was calculated as a percentage of the residual activity to the initial activity as shown as Equation (4-1). Before the first use, the residual enzyme activity was considered as the same as the initial value, resulting in 100% retention of enzyme activity. As shown in Fig. 4-3, a negative correlation between the two variables (cycle number and retention of enzyme activity) was observed, indicating an inverse relationship. The retention of enzyme activity decreased with the

increased cycle number of reusing the immobilized β-galactosidase. A coefficient of determination (R^2) of 0.98 was achieved, showing that 98% of the total variation of enzyme activity retention could be accounted for the variation of the cycle number (Taylor, 1990). In addition, the absolute value of the correlation coefficient (|R|) at 0.99 can be calculated from R^2. According to Mason *et al*. (1983) and Taylor (1990), when |R| is ≤ 0.35, the correlation is normally considered to be weak. Modest or moderate correlation is obtained with a |R| in the range of 0.36 to 0.67. Strong or high correlation is considered when 0.68 ≤ |R| < 1.0. The |R| of 0.99 achieved in this study was higher than 0.9, therefore, the linear relationship between cycle number of reusing immobilized β-galactosidase and retention of enzyme activity was considered as a very high correlation. The |R| of 0.99 was even considerably close to 1 which presents a perfect linear correlation, indicating that the actual data fitted the linear pattern defined by the regression equation well.

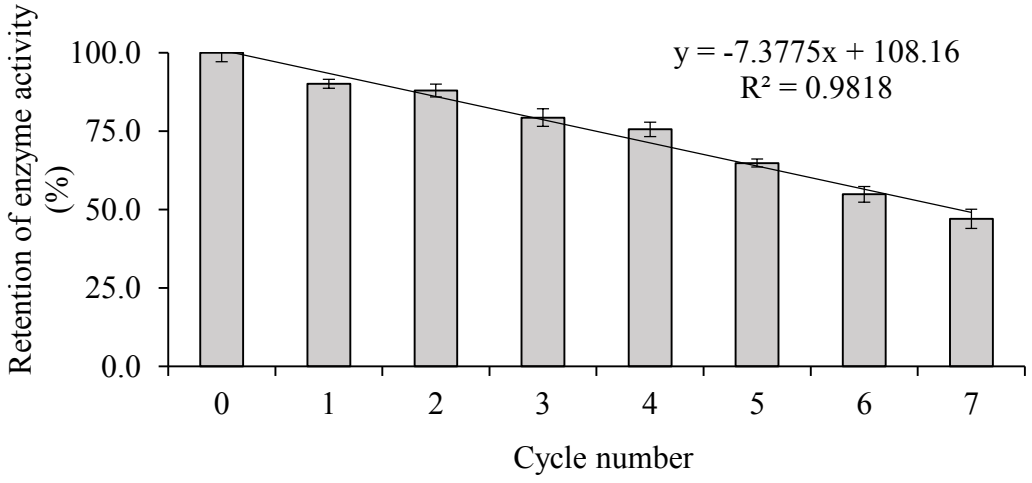


Figure 4-3 Enzyme activity retention of the immobilized β-galactosidase during the 7-cycle co-fermentations and its correlation with the cycle number of reusing immobilized β-galactosidase

The regression equation of y = - 7.3775x + 108.16 (where x = cycle number of reusing and y = retention of enzyme activity) clearly showed that the immobilized enzyme would lose about 7.38% each time when it was reused for the co-fermentation of wheat and whey permeate. After the 7-cycle reuse in the fermentation, 47.0% enzyme activity was retained, showing 53% enzyme activity loss after the applications for 504 h. The enzyme activity retention of the immobilized β-galactosidase obtained in this study was highly competitive to the results reported by other researchers. For instance, Haider and Husain (2009) immobilized *A. oryzae* β-galactosidase onto cellulose support by immunoaffinity binding. The enzyme activity retention of 46% was obtained after 120-h successive use. PVA hydrogel was used by Neri *et al*. (2008) for the immobilization of *Kluyveromyces lactis* β-galactosidase. Only 47% of the initial activity was observed after 24-h incubation at 35 °C.

4.3.2.2 Lactose hydrolysis percentage

The lactose hydrolysis percentages achieved by the co-fermentations with 7-cycle reuse of the immobilized β-galactosidase were presented in Fig. 4-4. Similar to the retention of enzyme activity, a negative correlation between lactose hydrolysis percentage and cycle number of reusing immobilized β-galactosidase was observed. The lactose hydrolysis percentage decreased when the cycle number increased, which represented an inverse relationship.

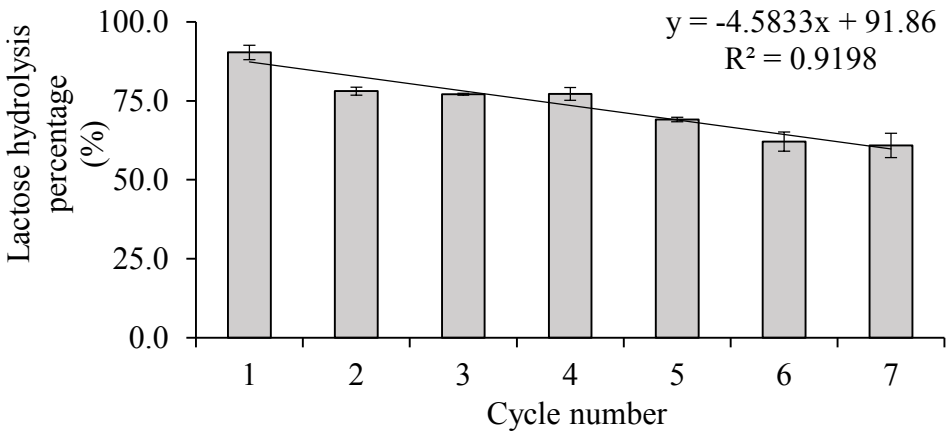


Figure 4-4 Lactose hydrolysis percentages of the fermentations using the immobilized β-galactosidase during the 7-cycle reuse and its correlation with the cycle number of reusing immobilized β-galactosidase

A coefficient of determination (R^2) of 0.92 was achieved, showing that 92% of the total variation in lactose hydrolysis percentage can be explained by the variation of the cycle number. The absolute value of the correlation coefficient (|R|) of 0.96 illustrated a very high correlation between the lactose hydrolysis percentage and the cycle number of reusing immobilized β-galactosidase. The regression equation of y = - 4.5833x + 91.86 was given based on the actual values, where x = cycle number of reusing and y = lactose hydrolysis percentage. The lactose hydrolysis lessened by around 4.58% each time when the immobilized β-galactosidase was reused for simultaneous lactose hydrolysis and co-fermentation with wheat. An approximate 31.5% reduction in lactose hydrolysis percentage was observed after the 7-cycle reuse with a total running time of 504 h. The lactose hydrolysis by the immobilized β-galactosidase in PVA hydrogel was also monitored by Batsalova *et al*. (1987). The lactose hydrolysis percentage decreased from 75% to 50% after a total running time of 150-180 h, showing 33.3% reduction.

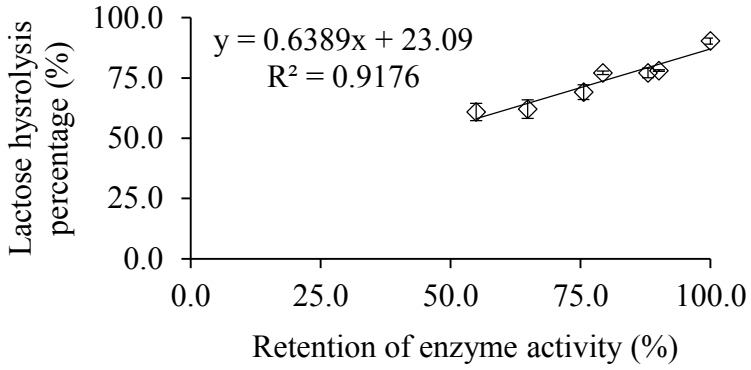


Figure 4-5 Correlation between retention of enzyme activity and lactose hydrolysis percentage during the 7-cycle co-fermentations of wheat and whey permeate using the immobilized β-galactosidase

Lactose hydrolysis depends on the enzyme activity of the immobilized β-galactosidase. Both the lactose hydrolysis percentage and the retention of enzyme activity were negatively correlated to the cycle number of reusing immobilized β-galactosidase. Hence, the correlation between lactose hydrolysis percentage and retention of enzyme activity was also investigated. As illustrated in Fig. 4-5, these two variables had a positive correlation with the coefficient of determination (R^2) of 0.92, showing that 92% variation of the lactose hydrolysis percentage was due to the variation of the retention of enzyme activity. The strength of the linear correlation was very high according to the |R| of 0.96. In addition, the regression equation of y = 0.6389x + 23.09 exhibited that every 1% decrease of the retention of enzyme activity would result in the lactose hydrolysis percentage reduction of about 0.64%.

4.3.2.3 Ethanol yield efficiency

The ethanol yield efficiencies of the co-fermentations were presented in Fig. 2-6. There was no significant difference among the ethanol yield efficiencies of the fermentations using the immobilized β-galactosidase for the first, second, and third times, indicating that the immobilized β-galactosidase can be reused for at least three times without any significant compromise of ethanol yield. The whey permeate-blended fermentations using the immobilized β-galactosidase possessed the significant lower ethanol yield efficiencies from 86.9% to 79.5%, compared to 92.9% efficiency of the control. That probably stemmed from the incomplete lactose hydrolysis and residual galactose (Hahn-Hagerdal, 1985).

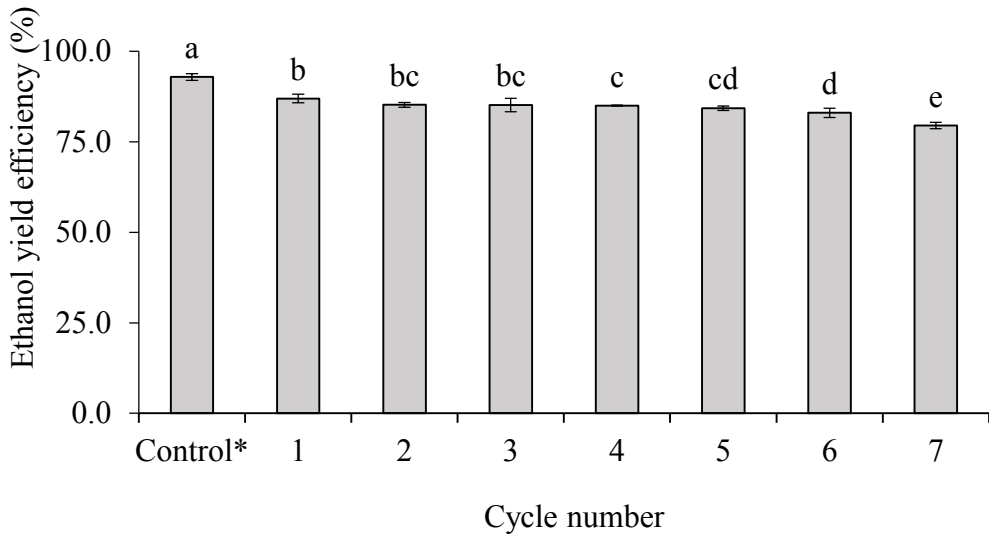


Figure 4-6 Ethanol yield efficiency of the control and the 7-cycle co-fermentations of wheat and whey permeate using the immobilized β-galactosidase

*Wheat of 30% was used for the control. 25% wheat blended with whey permeate, containing the same amount of total fermentable carbon as 30 % wheat, was used for the fermentations with the immobilized β-galactosidase. (Means with the same letter are not significantly different, $p<0.05$.)

With the 7-cycle reuse of the immobilized β-galactosidase in the co-fermentations, the ethanol yield efficiency decreased from 86.9% to 79.5%, which was mainly attributed to the reduced enzyme activity of the immobilized β-galactosidase resulting in less lactose hydrolyzed for ethanol production as shown in Fig. 4-2 and 4-3. Lewandowska and Kujawski (2007) co-immobilized *S. cerevisiae* together with β-galactosidase in calcium alginate for ethanol production from milk permeate containing 12% (w/v) lactose. It was observed that the ethanol yield efficiency decreased during the reuse of co-immobilized biocatalysts. Interestingly, the ethanol yield efficiency increased in the first a few runs, then it decreased by 49.7% in the following four cycles.

4.3.3 Sugars and organic acids

The galactose, glucose, lactic acid, and acetic acid concentrations in 0-h and 72-h samples of the fermentations using the soluble and immobilized β-galactosidases were shown in Table 4-1. Similar to the results obtained in Chapter 3, the residual galactose was detected which was mainly attributed to glucose repression and low galactose consumption rate (Ramakrishnana and Hartley, 1993; Staniszewski *et al*., 2009). The glucose present in 0-h fermentation samples was sourced from the pre-saccharification of starch by STARGEN enzymes. The variation of the initial glucose contents from 60.2 g/L to 69.4 g/L was due to the random error caused by several parameters involved in the handling of pre-saccharification step, for instance, heating/cooling time, location in the incubator shaker for mash heating, and handling time to initiate fermentations. The presence of lactic and acetic acids in 0-h samples was originated from the whey permeate. Natural microorganisms present in whey permeate would consume lactose and

produce lactic and acetic acids during the shipment and storage, which has been discussed by Wongso (1993).

Table 4-1 Lactose, galactose, glucose, lactic acid, and acetic acid concentrations in samples of the co-fermentations of wheat and whey permeate using soluble and immobilized *A. oryzae* β-galactosidase

Cycle number*	Galactose (g/L)		Glucose (g/L)		Lactic acid (g/L)		Acetic acid (g/L)	
	0 h	72 h	0 h	72 h	0 h	72 h	0 h	72 h
Soluble	2.5 ± 0.2	2.9 ± 0.5	64.0 ± 1.8	0.07 ± 0.00	1.2 ± 0.1	1.8 ± 0.0	-	-
1	2.6 ± 0.1	2.3 ± 0.2	62.1 ± 0.3	0.09 ± 0.00	1.1 ± 0.2	1.6 ± 0.1	-	-
2	2.0 ± 0.1	6.3 ± 0.2	69.4 ± 0.6	0.11 ± 0.02	1.3 ± 0.0	1.4 ± 0.0	0.7 ± 0.0	-
3	1.8 ± 0.2	4.8 ± 0.5	60.2 ± 1.2	0.11 ± 0.00	1.3 ± 0.1	1.2 ± 0.2	0.6 ± 0.3	0.1 ± 0.0
4	1.9 ± 0.0	3.5 ± 0.4	67.8 ± 0.6	0.11 ± 0.00	1.4 ± 0.2	1.2 ± 0.3	0.6 ± 0.1	0.1 ± 0.1
5	1.6 ± 0.0	6.1 ± 0.3	62.0 ± 0.9	0.11 ± 0.00	1.2 ± 0.1	1.6 ± 0.1	0.6 ± 0.3	0.1 ± 0.0
6	1.9 ± 0.1	6.4 ± 0.4	64.0 ± 1.8	0.07 ± 0.00	1.5 ± 0.2	1.5 ± 0.2	0.7 ± 0.1	0.2 ± 0.0
7	1.8 ± 0.1	5.9 ± 0.2	62.1 ± 0.3	0.09 ± 0.00	1.8 ± 0.0	2.0 ± 0.0	0.6 ± 0.0	0.4 ± 0.0

* Cycle number was referred to the fermentations using the immobilized β-galactosidase.

4.4 Conclusions

The application of immobilized β-galactosidase for simultaneous lactose hydrolysis during the co-fermentation of wheat and whey permeate is an interesting solution of great practical importance to reuse β-galactosidase, which can significantly reduce the production cost that associates with enzyme usage. The immobilized β-galactosidase in PVA hydrogel can be effectively used for the co-fermentation with wheat and whey permeate, according to the comparison of lactose hydrolysis percentage and ethanol yield efficiency between the co-fermentations using the soluble and immobilized β-galactosidases. The immobilized β-galactosidase can be reused at least three times in the 72-h fermentations without significant reduction in ethanol yield efficiency. During the 7-

cycle reuse in the co-fermentations, the enzyme activity of immobilized β-galactosidase and lactose hydrolysis percentage decreased by 7.38% and 4.58% respectively each time when the immobilized β-galactosidase was reused, which was competitive to the results derived by other studies on β-galactosidase immobilization.

5 Chemical Characteristics of Dried Distillers' Grains with Solubles from the Co-Fermentation of Wheat and Whey Permeate

5.1 Introduction

The industrial production of fuel ethanol has seen a dramatic increase during the last two decades, which was mainly promoted by the high demand of environment-friendly energy and the enhanced awareness of gradual depletion of fossil fuels. Global ethanol production has been projected to reach approximately 168 billion liters in 2022 based on the data obtained during 2010-2012 (OECD-FAO, 2013). The United States, one of the major ethanol producers, has shown an extreme boost in ethanol production from 175 millions of gallons in 1980 to 13,300 millions of gallons in 2013 (RFA, 2014d). With the increase of ethanol-blending minimum from 10% to 15%, the growing production of ethanol is confidently predicted to continue. E15 is available in 12 states with a price of 10-15 cents cheaper than the gasoline without ethanol (RFA, 2014a). The fuel ethanol production of Canada enormously increased from 211.3 millions of gallons in 2007 to 449 millions of gallons in 2012, showing more than 100% increase in five years (RFA, 2014b). All gasoline vehicle manufactured since 1980's can utilize the gasoline containing 10% ethanol which is widely available at gas stations across Canada (NRC, 2013).

DDGS, a valuable animal feed, was the co-product generated from ethanol production using dry-grind process which is the major approach used by the fuel ethanol industry (RFA, 2014a). In this process, all grain kernel components go through grinding, cooking, liquefaction, saccharification, fermentation, distillation. After ethanol is recovered by distillation, the leftover fermentation mash is further processed and dried as

DDGS (Liu, 2011). The DDGS production is positively related to the ethanol production. Additional grain input is required to supply starch for more ethanol production, leading to proportionally increased amount of the leftover being processed for DDGS. Thus, the DDGS production has been substantially increased in response to the rapid growth of ethanol production. Around 35.5 million metric tons of feed was contributed by the ethanol industry of the United States in the 2012/2013 marketing year, though the ethanol production was affected by the drought (RFA, 2014a). With the large amount of DDGS available on the market, the chemical composition of DDGS is of great importance to both academic studies and industrial applications. Currently, the major challenge of using DDGS as animal feed is high variation of nutrient contents compared to other traditional feed, such as soy meal (Belyea *et al*., 2004; Rosentrater, 2007; Liu, 2011).

Utilization of DDGS as animal feed can significantly offset the production cost of ethanol (Jacques *et al*., 2003). The income of marketing DDGS is one of the important factors which influence the profits for ethanol producers. It is reported that 27% of the gross revenue can be earned by a dry-grind ethanol plant from the sale of the co-products generated from the ethanol production in 2013 (RFA, 2014a). The annual wheat DDGS production of approximate 0.26 million tons was achieved by the Canadian ethanol plants in 2009/2010, which valued at around 51 million dollars (FOBI, 2011).

So far, a few studies on co-fermenting grains with whey/whey permeate for ethanol production have been reported with goals to reduce the cost of ethanol production due to the low cost of whey/whey permeate (Friend *et al*., 1982; Gibbons and Westby, 1988; Kadar *et al*., 2011). Dry-grind processes were used in these studies; however, little attention has been placed on recovery of the resulting DDGS, much less nutritional

evaluation. Recently, solid residue from ethanol fermentation has been studied by Oleskowicz-Popiel *et al*. (2012) in the follow-up study for the work done by Kadar *et al*. (2011). The solid residue was referred to distillers' grains (DG) to which the soluble-containing thin stillage was not blended. The crude protein content of the solid residue was measured to be only 9.3% which was significantly lower than the reported average value of about 30% in traditional DDGS.

In Chapter 3, both the granular starch hydrolysis (STARGEN-based) and the conventional jet cooking (jet cooking-based) approaches were adopted for the fermentations of wheat only and whey permeate-blended wheat. Eventually, four different types of DDGS were prepared from the fermentations conducted in 5-L bioreactors. The goals of this chapter were to provide in-depth nutritional profile of the DDGS produced from the co-fermentation of wheat and whey permeate, as well as to evaluate the change of chemical characteristics caused by the supplementation of whey permeate for partial wheat replacement and the different fermentation approaches. To our best knowledge, this is the first study to demonstrate a comprehensive nutritional analysis of the DDGS form ethanol production using grains and whey/whey permeate.

5.2 Materials and Methods

5.2.1 Chemicals

Petroleum ether, methanol (≥ 99.8%), and n-hexane (≥ 98.5%) of certified ACS reagent grade, as well as sodium carbonate (≥ 99.5%) of certified ACS were purchased from Fisher Scientific (Dubuque, IA, USA). Gallic acid (97.5-102.5%), 3 N methanolic HCl (Supelco), methyl heptadecanoate (≥ 99%), caffeine (ReagentPlus®), and Folin-Ciocalteu's phenol reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Anhydrous sodium sulfate of ACS grade was sourced from EMD Millipore (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) containing a nitrogen content of 9.57 ± 0.04% and benzoic acid (C 723, pelletized) were derived from Leco Corporation (St Joseph, MI, USA) and IKA®-Werke GmbH & Co. KG (Staufen, Germany), respectively. Standard 463, a mixture of fatty acid methyl esters (FAMEs), was purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). Total starch kit was obtained from Megazyme (Country Wicklow, Ireland).

5.2.2 Preparation of DDGS

Beers of both the STARGEN- and jet cooking-based fermentations using wheat only and whey permeate-blended wheat in 5-L bioreactors were used for preparing DDGS, which were processed through three main steps-ethanol evaporation, freeze-drying, and milling. Ethanol and part of water in the beers were evaporated by a rotary evaporator at 72 °C for around 1 h with a constant rotation speed at 3 (vacuum V-500, vacuum controller V-800, rotavapor R-200, heating bath B-490, BÜCHI Labortechnik AG, Switzerland). Then the concentrated beers were dehydrated in a freeze-drier (VirTis Ultra 35L, SP Scientific, Stone Ridge, NY, USA) at -60 °C for three days. All the DDGS were stored at -20 °C for future analyses.

5.2.2 Proximate analysis

Moisture content of the DDGS was analyzed by AOAC method 934.15 (AOAC, 2006). Crude protein was determined by combustion using a Leco C/N analyzer (TruSpec Micro CHNS, Leco Corporation, St Joseph, MI, USA) with caffeine and EDTA as standards. Crude fat was extracted by petroleum ether for 5 h using the Goldfisch fat extractor (LABCONCO Corporation, Kansas City, MO, USA) and dried for weighing the

amount of crude fat extracted from the known amount of DDGS samples (AOAC method 920.39). AOAC method 942.05 was adopted to determine ash content of the DDGS. Crude fiber was analyzed in F57 filter bags by an Ankom fiber analyzer (A200/220, Ankom Technology, Fairport, NY, USA). Carbohydrate content was calculated following the Equation (5-1):

$$\text{Carbohydrate\%} = 100\ \% - [\ \text{Moisture\%} + \text{Ash\%} + \text{Crude fat\%} + \text{Crude protein\%}]$$

5.2.3 Gross energy determination

Gross caloric energy of the DDGS was determined under adiabatic mode using an IKA® oxygen bomb calorimeter (C5000, IKA®-Werke GmbH & Co. KG, Staufen, Germany) equipped with a cooling system (C5001 S1, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The bomb calorimeter was calibrated by benzoic acid as a caloric standard.

5.2.4 Residual starch analysis

Residual starch content in DDGS samples was determined using Megazyme Total Starch Kit. Starch was hydrolyzed by α-amylase and amyloglucosidase to D-glucose which was measured by a colorimetric reaction catalyzed by glucose oxidase and peroxidase.

5.2.5 Analysis of total free phenolics

Folin-Ciocalteu assay was used for the measurement of total free phenolics. The free phenolic compounds were extracted in 80% (v/v) methanol by 1-h sonication (45 KHz, 80 W) (575DA, CREST Ultrasonics, Trenton, NJ, USA). After the extraction, the supernatant was reacted with Folin-Ciocalteu phenol reagent at room temperature for 1 h

at a basic condition of pH ~10 created by sodium carbonate (10% in water, w/v). The absorbance of the developed blue solution was read at 750 nm. Double distilled water was used instead of the sample supernatant in the reaction as the blank. A standard curve with a $R^2 \geq 0.999$ was prepared using gallic acid solutions with gradient concentrations (from 0 to 100 μg/mL). The content of total free phenolics was expressed as the equivalent concentration of gallic acid.

5.2.6 Analysis of minerals

Microwave-assisted HNO_3 digestion was adopted for the pre-treatment of DDGS samples using a laboratory microwave digestion system (MARS 5, CEM, Buckingham, UK). The program used was maximum power 1600 W, 100%, ramp 30 min, hold 10 min, 150 PSI, 205 °C. The solid residue was removed by filtration after the digestion. Then the filtrate was loaded for the analyses of Ca, K, Na, P, Mg, Fe, Zn, Mn, and Cu by inductively coupled plasma-optical emission spectrometry (ICP-OES, iCAP^{TM} 7600, Thermo Fisher Scientific, Waltham, MA, USA). The mineral contents were determined in the Natural Resources Analytical Laboratory, the Department of Renewable Resources, University of Alberta.

5.2.7 Analysis of fatty acids

The DDGS samples were processed through acid-catalyzed derivatization of fatty acids by methanol, leading to the formation of FAMEs which were analyzed by gas chromatography equipped with a frame ion detector (GC-FID, 7890A, Agilent Technologies, Mississauga, ON, Canada). Methanolic HCl was used to carry out the methylation of fatty acids for 20 min at 50 °C. Methyl heptadecanoate (methyl C17:0, 1 mg/mL in n-hexane) was added to the reaction solution as the internal standard, followed

by the extraction of FAMEs by n-hexane. The n-hexane layer containing FAMEs was collected after 10-min centrifugation at 1,500 g. A pitch of anhydrous sodium sulfate was mixed with the n-hexane layer to eliminate any possible moisture. Then, the n-hexane layer was loaded for GC-FID analysis with sodium sulfate solids removed by 5-min centrifugation at 1,500 g. The column of BP20 [0.25 μm × 30 m × 0.25 mm] was used with helium as the carrier gas at 1.0 mL/min. Sample volume of 1 μL was injected in splitless mode at 230 °C. The oven was programmed as the following: hold at 50 °C for 0.2 min; increase from 50 °C to 170 °C at 20 °C/min; hold at 170 °C for 5 min; increase from 170 °C to 230 °C at 10 °C/min; hold at 230 °C for 13 min. The peaks of FAMEs were identified by comparing their retention times to those of the standard mixture (Standard 463) containing 48 species of FAMEs. The response factor of 1.0 was used for the internal standard as well as all the fatty acids.

5.2.8 Analysis of amino acids

Available lysine content was determined by AOAC official method 975.44. Dinitrofluorobenzene (DNFB) was used to react with the free ε-amino groups in proteins. Those lysine units of which ε-amino groups have been bounded to other groups are nutritionally unavailable, which cannot react with DNFB. The DNFB-treated DDGS samples was then subject to acid hydrolysis by 6 M HCl, resulting in the release of unavailable lysine of which the amount was determined. The DNFB-untreated DDGS samples were processed directly by acid hydrolysis and loaded for the analysis of total lysine. The content of available lysine was calculated as the difference between the total and unavailable lysine contents.

Other amino acids were measured using AOAC official method 982.30. Acid hydrolysis by 6 M HCl was employed to prepare the hydrolysate for the determination of all amino acids except methionine, cysteine, and tryptophan. The hydrolysate for the analysis of methionine and cysteine was prepared by performic acid oxidation followed by acid hydrolysis. Alkaline hydrolysis by NaOH was used to obtain the hydrolysate for the determination of tryptophan. All the amino acids were measured by an amino acid analyzer (Model L-8900, Hitachi Ltd., Tokyo, Japan) which was operated based on cation-exchange chromatography coupled with post-column ninhydrin derivatization and quantitation. The analyses of amino acids were carried out in the Agricultural Experiment Station Chemical Laboratories, University of Missouri.

5.2.9 Statistical analysis

Three replicates for each type of DDGS were derived as each fermentation was conducted in triplicate (described in Chapter 3). All the analyses of DDGS samples were carried out in triplicate, except for the amino acid analysis which was done in duplicate. The differences of the same nutritional parameter among the four types of DDGS were assessed by ANOVA followed by Tukey's test with 95% confidence ($\alpha = 0.05$).

5.3 Results and Discussion

5.3.1 Proximate composition

The proximate compositions of the four types of DDGS were illustrated in Table 5-1. The DDGS produced from the co-fermentations of wheat and whey permeate had the lower contents of crude protein, fat, and fiber than the DDGS from the fermentation of wheat only in both STARGEN- and jet cooking-based systems. That is likely due to the

considerably lower protein and fat contents of whey permeate than those of wheat. Whey permeate contained 2.75% of crude protein and 0.07% crude fat (wt/wt, dry matter) (Parashar *et al*., manuscript in preparation), while the crude protein and fat contents in the wheat flour used for the fermentations were determined to be 11.6% and 1.9%, respectively. Whey permeate is also known to have no fiber present. In contrast, higher ash and carbohydrate contents were observed in the DDGS from the co-fermentations of wheat and whey permeate. It is probably attributed to the high mineral contents in whey permeate which was originated from milk. The higher carbonhydrate content was possibly contributed by the residual lactose and galactose in the 72-h fermentation broth which remained in DDGS, as discussed in Chapter 3.

With the same substrate being used, DDGS obtained from the jet cooking-based fermentations contained higher ash content than those from the STARGEN-based fermentations. One possible explanation can be the exogenous introduction of mineral compounds during the jet cooking process, for instance, more pH adjustment steps required for the mash preparation (Gibreel *et al*., 2009; Belyea *et al.,* 2006).

Table 5-1 Proximate compositions of the DDGS produced from STARGEN- and jet cooking-based fermentations using wheat only and whey permeate -blended wheat*

Parameter (%, wt/wt, dry matter)	Wheat only		Wheat + whey permeate	
	STARGEN	Jet cooking	STARGEN	Jet cooking
Crude protein	43.4 ± 0.3^{a}	43.3 ± 0.5^{a}	35.1 ± 0.8^{b}	35.9 ± 0.9^{b}
Crude fat	5.5 ± 0.4^{a}	4.3 ± 0.4^{b}	2.9 ± 0.2^{c}	2.7 ± 0.3^{c}
Ash	5.3 ± 0.3^{d}	5.8 ± 0.1^{c}	6.7 ± 0.3^{b}	8.7 ± 0.2^{a}
Carbohydrate	45.8 ± 0.5^{c}	46.7 ± 0.5^{c}	55.2 ± 1.0^{a}	52.8 ± 0.7^{b}
Crude fiber	7.5 ± 0.3^{b}	7.9 ± 0.3^{a}	5.4 ± 0.2^{d}	6.2 ± 0.3^{c}

*In the same row, means with different letters are significantly different (p<0.05).

Overall, the proximate compositions of these four types of DDGS were comparable to the results reported by other studies. The crude protein of 32.1-45.8% was observed in the wheat DDGS produced by Canadian ethanol plants (FOBI, 2011). Wheat DDGS containing 2.9% and 5.4% crude fat were reported by Widyaratne and Zijlstra (2007) and FOBI (2011). The range of 3.1-8.1% for ash content was determined by Cromwell *et al*. (1993). The crude fiber content in DDGS was measured to be within a range of 5.7-7.3% (Gibreel *et al*., 2011; Nade *et al*., 2012).

5.3.2 Gross energy, residual starch, and total free phenolics contents

As shown in Table 5-2, the gross energy results of these four types of DDGS were similar to each other. The overall mean of gross energy (5133.5 cal/g) was very close to 5178 cal/g reported for the wheat DDGS by FOBI (2011). The DDGS derived from the STARGEN-based fermentations contained more residual starch than those from the jet cooking-based fermentations, which was in agreement with the results obtained by Gibreel *et al*. (2011).

With the same fermentation approach being used, the concentration of total free phenolics in the DDGS produced from wheat only was significantly higher than that from wheat and whey permeate. This is because cereal grains are the major source of phenolic compounds (such as phenolic acids, flavonoids, and tannins etc.) for DDGS. The comparison among the DDGS derived from the two fermentation approaches showed that the lower total free phenolics contents in DDGS were obtained by the jet cooking-based fermentations. It is likely due to the loss of phenolic compounds during the jet cooking process, which has been demonstrated by Kandil *et al.* (2012). Phenolic acids are capable of binding enzymes and starch with their carboxyl and hydroxyl groups. The interactions

were significantly enhanced by boiling. Therefore, the intense heating of 110-120 °C involved in the jet cooking process could possibly facilitated the interactions of phenolic acid-enzyme and phenolic acid-starch.

Table 5-2 Residual starch, gross energy, and total free phenolics contents in DDGS produced from STARGEN and jet cooking-based fermentations using wheat only and wheat blended with whey permeate*

Parameter (dry matter)	Wheat only		Wheat + whey permeate	
	STARGEN	Jet cooking	STARGEN	Jet cooking
Residual starch (%)	1.3 ± 0.2^{b}	0.3 ± 0.0^{c}	4.0 ± 0.8^{a}	0.3 ± 0.0^{c}
Gross energy (cal/g)	5296.5 ± 13.9^{a}	5112.4 ± 74.7^{b}	5058.4 ± 42.4^{b}	5066.6 ± 228.4^{b}
Total free phenolics (mg GAE**/g)	11.3 ± 0.5^{a}	6.6 ± 0.2^{c}	8.9 ± 0.4^{b}	5.7 ± 0.2^{d}

*In the same row, means with different letters are significantly different ($p<0.05$); **GAE: gallic acid equivalent.

5.3.3 Mineral concentrations

The mineral concentrations of the DDGS were shown in Table 5-3. The DDGS derived from the co-fermentations of wheat and whey permeate possessed significantly higher concentrations of Na, K, and Ca than those from the fermentations of wheat only in both STARGEN and jet cooking-based systems. The lower Fe, Zn, and Mn concentrations were observed in the DDGS from the co-fermentations of wheat and whey permeate. They are likely attributed to relatively higher Na, K, and Ca contents and lower Fe, Zn, and Mn contents in whey permeate, compared to those in wheat grains. For instance, 0.68% Ca was measured in whey permeate (Parashar *et al*., manuscript in preparation); while wheat grain only contains about 0.09% Ca or even less (FOBI, 2011; Erdman and Moul, 1982). As for Fe, a considerably higher content of 44 ppm was

reported for wheat (Lai *et al*., 1981), compared to 5.31 ppm determined in whey permeate (Parashar *et al*., manuscript in preparation).

The P and Mg contents were similar among the four types of DDGS no matter whether whey permeate was blended or which fermentation approach was used. Moreover, there was no significant difference of Cu contents observed for the DDGS produced from wheat only and whey permeate-blended wheat. However, the DDGS obtained from the jet cooking-based fermentations contained considerably higher Cu concentrations than those from the STARGEN-based. The significantly different Na concentrations were also detected for the DDGS produced from the fermentations using different approaches, which was presumably resulted from the additional pH adjustment step by NaOH involved in the jet cooking process (Gibreel *et al*., 2009).

Table 5-3 Mineral concentrations of DDGS produced from STARGEN and jet cooking-based fermentations using wheat only and wheat blended with whey permeate*

Minerals (dry matter)	Wheat only		Wheat + whey permeate	
	STARGEN	Jet cooking	STARGEN	Jet cooking
Na (mg/g)	1.8 ± 0.4^{c}	5.5 ± 0.4^{b}	5.1 ± 0.7^{b}	9.1 ± 0.7^{a}
K (mg/g)	12.6 ± 0.8^{c}	11.9 ± 0.8^{c}	15.9 ± 1.6^{b}	18.0 ± 0.3^{a}
Ca (mg/g)	1.4 ± 0.1^{c}	1.4 ± 0.1^{c}	2.7 ± 0.3^{b}	4.4 ± 0.2^{a}
P (mg/g)	9.5 ± 0.8^{ab}	9.1 ± 0.7^{ab}	8.7 ± 1.1^{b}	9.8 ± 0.7^{a}
Mg (mg/g)	4.0 ± 0.3^{a}	3.8 ± 0.3^{ab}	3.5 ± 0.4^{b}	3.7 ± 0.1^{ab}
Fe (μg/g)	210.0 ± 20.9^{a}	188.9 ± 10.1^{a}	122.0 ± 8.3^{c}	155.4 ± 8.1^{b}
Zn (μg/g)	78.8 ± 7.6^{a}	77.4 ± 6.2^{a}	60.3 ± 5.0^{b}	64.1 ± 2.1^{b}
Mn (μg/g)	132.9 ± 8.4^{a}	125.5 ± 6.2^{a}	98.1 ± 9.5^{b}	97.9 ± 2.5^{b}
Cu (μg/g)	15.7 ± 4.8^{b}	31.0 ± 7.8^{a}	14.7 ± 6.1^{b}	29.0 ± 9.6^{a}

*In the same row, means with different letters are significantly different ($p<0.05$).

Though there were variations of mineral compositions caused by the supplementation of whey permeate, the resulting mineral concentrations were still within

the range of the published values (Spiehs *et al*., 2002; Batal and Dale, 2003; Belyea *et al*., 2006; Liu and Han, 2011). Among these minerals, P is considered as the most important because of the following two reasons. First, it is one of the three most expensive nutrients in the diet. Second, if the feed with an elevated P concentration is used, the excessive P will be excreted and remain in the wastes which is a potential burden on the environment (Schmit *et al*., 2009). Thus, the P content in animal feed possesses significant implications in both animal nutrition balancing and manure management (Spiehs *et al*., 2002). The P concentrations of 0.87-0.98% obtained in this study were within the generally agreeable range of 0.5-1.0% for P concentration in DDGS (Liu, 2011). Moreover, the P in the DDGS would demonstrate substantially higher bioavailability than that in the native cereal grains. Since phytate P, the predominant presence of P in cereal grains, was degraded by yeast phytase during the fermentations, leading to the production of inorganic P (Cromwell, 1979; Liu and Han, 2011). Phytate P is nutritionally unavailable to monogastric animals; while inorganic P, also known as phosphate/free P, can be well utilized (He *et al*., 2009).

5.3.4 Fatty acid concentrations and compositions

As shown in Table 5-4, the comparison between the DDGS derived from the two types of substrates illustrated that lower concentrations of fatty acids were obtained by the DDGS from the co-fermentations of wheat and whey permeate. Since whey permeate used in this study contained scarcely any fat (Parashar *et al*., manuscript in preparation), indicating that wheat grain was the major source of fatty acids in the DDGS. As expected, the STARGEN-based fermentations provided the DDGS with higher fatty acid concentrations, compared to the fermentations using jet cooking. This trend was also

observed by Gibreel *et al*. (2011). The significant difference of fatty acid concentrations was found in the DDGS produced from Pronghorn triticale and four wheat species when different fermentation approaches were used.

Table 5-4 Fatty acid concentrations in DDGS produced from STARGEN and jet cooking-based fermentations using wheat only and wheat blended with whey permeate*

Fatty acid (mg/g, dry matter)	Wheat only		Wheat + whey permeate	
	STARGEN	Jet cooking	STARGEN	Jet cooking
Linoleic acid	33.5 ± 1.1^{a}	27.7 ± 1.2^{b}	25.8 ± 1.4^{c}	21.9 ± 1.3^{d}
Oleic acid	9.4 ± 0.3^{a}	8.5 ± 0.4^{b}	7.3 ± 0.4^{c}	6.8 ± 0.4^{d}
Palmitic acid	13.1 ± 0.5^{a}	11.5 ± 0.5^{b}	9.8 ± 0.5^{c}	9.0 ± 0.6^{d}
Others	4.9 ± 0.2^{a}	3.9 ± 0.2^{b}	3.5 ± 0.3^{c}	3.2 ± 0.2^{c}
Total	60.9 ± 2.1^{a}	51.6 ± 2.3^{b}	46.4 ± 2.5^{c}	40.9 ± 2.5^{d}

*In the same row, means with different letters are significantly different ($p<0.05$).

In addition to the concentration, fatty acid composition, which was calculated as the percentage of each fatty acid in the total fatty acid content, has been widely presented and evaluated by other researchers. The fatty acid compositions of the DDGS from wheat and whey permeate-blended wheat were shown in Fig. 5-1. There was no significant difference between the DDGS from the two types of substrates. Linoleic acid was the predominant fatty acid followed by palmitic acid and oleic acid, which was similar to the fatty acid composition reported in other studies. The DDGS adopted by Nade *et al.* (2012) for feeding beef cattle consisted of 55.69% linoleic acids, 25.89% Oleic acid, 13.91% palmitic acid, and 4.51% other fatty acids. The study of Moreau *et al*. (2011) has elucidated that the fatty acid composition was maintained constantly during dry grind ethanol production using corn with the final composition in DDGS of linoleic acid at

54.51%, oleic acid at 25.59%, and palmitic acid at 16.24%. The variation of palmitic and oleic acids compositions among the DDGS analyzed in this study and those reported was presumably due to the different grains used for ethanol production. As for wheat DDGS, the palmitic and oleic acids compositions of the DDGS derived in this study were comparable to 18-21% palmitic acid and 12-15% oleic acid which were determined by Gibreel *et al*. (2011).

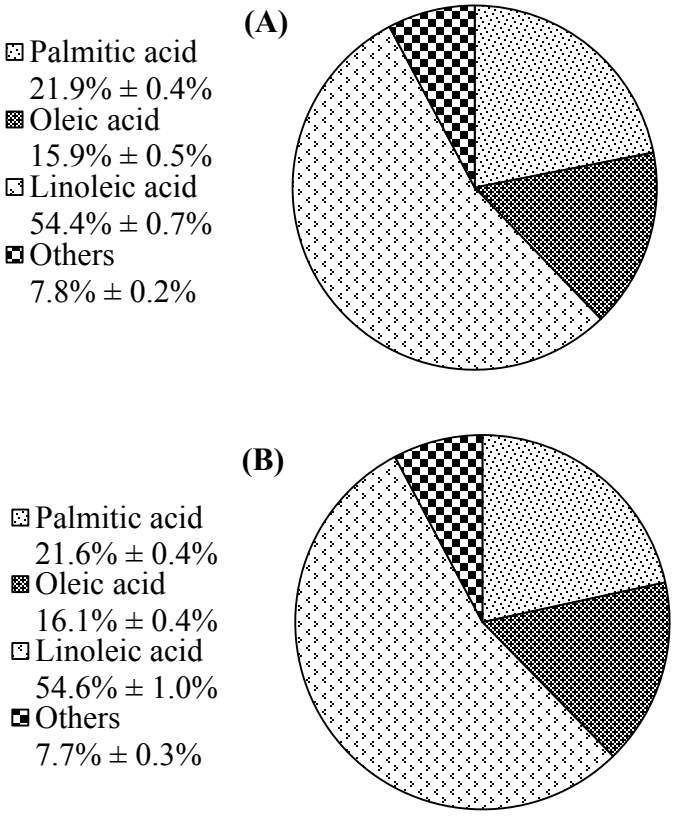


Figure 5-1 Fatty acid compositions* of the DDGS produced from wheat only and whey permeate-blended wheat in both the STARGEN and jet cooking-based fermentations. (A)**DDGS from wheat only; (B)**DDGS from wheat blended with whey permeate.

*The composition of each fatty acid was calculated as a percentage (wt/wt) in the total fatty acid content. **The results present in the pie charts were the average values of the DDGS from the STARGEN- and jet cooking-based fermentations.

5.3.5 Amino acid concentrations

The observed concentration of amino acids is presented in Table 5-5. Reduced amino acid concentrations in the DDGS from the co-fermentations of wheat and whey permeate was observed, compared to those from the fermentations of wheat only. It can be explained by the deproteinization of whey prior to its application in the fermentation. The protein fraction in whey is traditionally isolated and recovered for the production of protein supplement, resulting in the whey permeate containing only 2.75% protein. When the whey permeate was blended in the wheat mash, the remaining solids from whey permeate in the DDGS diluted the concentrations of amino acids from wheat grains and yeasts.

Interestingly, fermentations using jet cooking provided the DDGS with higher amino acid contents, compared to the STARGEN-based fermentations. That is probably due to heat-induced unfolding and dissociation of some proteins were involved in the heating process (Kwok *et al*., 1998). The change of essential amino acid contents in whole-grain wheat during various thermal processes was monitored and assessed by Hakansson *et al.* (1987). It was discovered that steam flaking (89 °C for 18 min; 79-81 °C for 25 min) and autoclaving (130 °C for 27 min; 150 °C for 25 min) did not reduce the amino acid contents analyzed using acid hydrolysis. Some amino acids were even demonstrated to have slight increases of their contents, such as leucine, phenylalanine, tyrosine, tryptophan, and valine. In the study of Kwok *et al*. (1998), the available lysine content in soymilk increased when the heat treatment at 120 °C was adopted for 20 min. The lysine content started to decrease gradually during the prolonged heating time from 20 to 50 min; however, the content was still higher than the initial value. The increase of total

lysine in the DDGS from jet cooking-based fermentations was also detected by Gibreel *et al.* (2011).

Generally, most amino acid concentrations of the DDGS analyzed in this study were comparable to the results published (Spiehs *et al*., 2002; Batal and Dale, 2003; Belyea *et al*., 2006; Liu and Han, 2011; Kim *et al*., 2010), except for a few amino acids with higher concentrations, such as tryptophan, cysteine, glutamic acid, glycine, and proline. Among all these amino acids, lysine content is of great interest for the application of DDGS as an animal feed additive due to its low resistance to heat damage. The minimum total and available lysine contents of 0.98% and 0.96% in the DDGS from the co-fermentation of wheat and whey permeate were considerably higher than 0.67% recommended by National Research Council (1998) and 0.65% by Feedstuffs Reference Issue (1999), indicating high quality of the resulting DDGS from the co-fermentation. In addition, the wheat DDGS analyzed in this study were nutritionally competitive to other wheat DDGS in Canada which were reported to have the total and available lysine contents of 0.89% (FOBI, 2011).

Table 5-5 Amino acid concentrations of DDGS produced from STARGEN and jet cooking-based fermentations using wheat only and wheat blended with whey permeate*

Amino acid (%, wt/wt, dry matter)	Wheat only		Wheat + whey permeate	
	STARGEN	Jet cooking	STARGEN	Jet cooking
Essential				
Total lysine	1.24 ± 0.01^{b}	1.55 ± 0.01^{a}	0.98 ± 0.02^{c}	1.26 ± 0.02^{b}
Available lysine	1.20 ± 0.01^{b}	1.42 ± 0.02^{a}	0.96 ± 0.01^{d}	1.17 ± 0.02^{c}
Arginine	1.60 ± 0.03^{b}	1.95 ± 0.01^{a}	1.27 ± 0.04^{d}	1.53 ± 0.01^{c}
Histidine	0.78 ± 0.00^{b}	0.87 ± 0.01^{a}	0.59 ± 0.01^{d}	0.68 ± 0.01^{c}
Isoleucine	1.27 ± 0.01^{b}	1.56 ± 0.01^{a}	0.99 ± 0.02^{d}	1.24 ± 0.02^{c}
Leucine	2.08 ± 0.01^{b}	2.66 ± 0.01^{a}	1.58 ± 0.03^{c}	2.10 ± 0.02^{b}
Methionine	0.44 ± 0.01^{b}	0.58 ± 0.01^{a}	0.32 ± 0.01^{c}	0.43 ± 0.01^{b}
Phenylalanine	1.65 ± 0.01^{b}	1.75 ± 0.01^{a}	1.23 ± 0.02^{d}	1.37 ± 0.02^{c}
Threonine	1.28 ± 0.01^{b}	1.43 ± 0.01^{a}	0.96 ± 0.02^{d}	1.13 ± 0.02^{c}
Tryptophan	0.52 ± 0.01^{a}	0.47 ± 0.01^{b}	0.41 ± 0.02^{c}	0.39 ± 0.03^{c}
Valine	1.74 ± 0.01^{b}	1.94 ± 0.01^{a}	1.29 ± 0.03^{d}	1.53 ± 0.02^{c}
Non-essential				
Alanine	1.76 ± 0.01^{a}	1.69 ± 0.01^{b}	1.28 ± 0.02^{d}	1.48 ± 0.01^{c}
Aspartic Acid	2.17 ± 0.02^{b}	2.25 ± 0.01^{a}	1.71 ± 0.03^{d}	1.82 ± 0.02^{c}
Cysteine	0.81 ± 0.01^{a}	0.79 ± 0.01^{b}	0.62 ± 0.02^{c}	0.63 ± 0.01^{c}
Glutamic Acid	8.10 ± 0.12^{a}	8.15 ± 0.13^{a}	6.60 ± 0.11^{b}	6.67 ± 0.10^{b}
Glycine	1.89 ± 0.02^{a}	1.85 ± 0.01^{b}	1.37 ± 0.03^{d}	1.45 ± 0.02^{c}
Proline	3.25 ± 0.08^{a}	3.15 ± 0.06^{a}	2.45 ± 0.08^{b}	2.52 ± 0.05^{b}
Serine	1.54 ± 0.02^{b}	1.79 ± 0.02^{a}	1.16 ± 0.03^{d}	1.39 ± 0.01^{c}
Tyrosine	1.17 ± 0.01^{a}	1.15 ± 0.00^{a}	0.89 ± 0.02^{c}	0.94 ± 0.01^{b}

*In the same row, means with different letters are significantly different ($p<0.05$).

5.4 Conclusions

DDGS, an important by-product of grain-to-ethanol fermentation, is closely related to the economical and sustainable operation of ethanol production. The chemical characteristics of DDGS are of great essence to the animal feed industry and the livestock farming. The variation of some nutrient contents in the DDGS from the two fermentation approaches (STARGEN- and jet cooking-based) was mainly attributed to the different heating patterns and enzyme treatments involved. The whey permeate supplementation

for partial wheat replacement has contributed to the enhancement of some nutrient contents, such as total carbohydrate, Ca, K, and Na. Meanwhile, the contents of other nutrients were reduced by the supplementation of whey permeate, such as crude protein, fat, fiber, Fe, Zn, Mn, phenolic compounds, fatty acids, and amino acids, since these nutrients were low or absent in whey permeate. The gross energy of the DDGS was maintained at a comparable level, as the reduced energy from protein and fat was made up by the increased carbohydrate content. To summarize, the supplementation of whey permeate for partial wheat replacement did change the nutritional profiles of the resulting DDGS. However, the nutritional values were comparable to the results reported by other studies. Therefore, the DDGS obtained from the co-fermentation of 25% wheat and whey permeate was considered as acceptable for its application as an animal feed additive.

6 Overall Conclusions and Recommendations

A. oryzae β-galactosidase is suitable for the simultaneous lactose hydrolysis at the conditions of the conventional grain-to-ethanol fermentation. The contribution of whey permeate to the ethanol production has been proved in the simultaneous lactose hydrolysis and co-fermentation of wheat and whey permeate. It was also demonstrated that the supplementation of whey permeate for partial wheat replacement was effective in both the STARGEN- and jet cooking-based fermentation systems. However, the considerably low consumption rate of galactose by *S. cerevisiae* is the major concern of the co-fermentation of wheat and whey permeate.

The attempt to reuse *A. oryzae* β-galactosidase in the co-fermentation was carried out by immobilizing β-galactosidase in PVA hydrogel. The enzyme activity and lactose hydrolysis percentage of the immobilized β-galactosidase decreased gradually during the reuse with a reusability profile competitive to those of other immobilized β-galactosidase published. In addition, no significant reduction of ethanol yield was observed for the first three reuses of the immobilized β-galactosidase. The enzyme immobilization in PVA hydrogel is a promising approach to reuse *A. oryzae* β-galactosidase in the co-fermentation, which can potentially reduce the enzyme cost associated with the co-fermentation of wheat and whey permeate.

The DDGS derived from the co-fermentation of 25% wheat and whey permeate possessed comparable chemical characteristics to the DDGS reported by other studies. Some nutrient contents were enhanced by the whey permeate supplementation. However, the reduction of some nutritional parameters was also observed, such as crude protein,

fat, fiber, Fe, Zn, Mn, phenolic compounds, fatty acids, and amino acids. These nutrients were low or even absent in whey permeate. Therefore, it can be reasonably predicted that these nutritional parameters will further decrease when more wheat is replaced by whey permeate.

Sustainable and economical utilization of whey/whey permeate is not only essential for the cheese industry but also promising for the ethanol industry. The results of this study can be used as a reference for the incorporation of whey permeate into the conventional grain-to-ethanol production. However, there are several recommendations for future work regarding the co-fermentation of grains and whey permeate in order to make the process more feasible and efficient. First, it would be interesting to use a mixed culture of a commercial *S. cerevisiae* strain and an efficient galactose-assimilating *S. cerevisiae* strain for the co-fermentation. As the galactose-assimilating *S. cerevisiae* strain can be used to substantially enhance galactose conversion to ethanol (Keating *et al*, 2004), thus minimizing carbon waste and the risk of severe Maillard reaction in the down-stream process. As for the immobilized *A. oryzae* β-galactosidase, more detailed information is necessary for its industrial application, such as the cost for matrix, space, and specific conditions involved in the preparation of the immobilized enzyme. A comprehensive economical evaluation is of great essence for its commercialization. Lastly, the influence of whey permeate supplementation on the co-product (DDGS) should be considered in addition to ethanol for the decision-making on the extent of whey permeate incorporation. As the trend of some nutrient reduction was observed in the DDGS from the co-fermentation in this study. Though it is stated that the amount of whey permeate used for ethanol production depends on the availability of whey permeate

and the production capacity of ethanol plants, the nutritional values of the resulting DDGS also serves as an important factor for choosing an appreciate percentage of wheat replacement by whey permeate.

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