RECOVERY OF PROTEIN AND ORGANIC COMPOUNDS FROM SECONDARY-FERMENTED THIN STILLAGE

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Food and Bioproduct Sciences

University of Saskatchewan

Saskatoon

By

Kornsulee Ratanapariyanuch

PERMISSION TO USE

In presenting this thesis, in partial fulfillment of the requirements for a degree of Doctor of Philosophy from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis, or parts thereof, for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or in part should be addressed to:

Head of the Department of Food and Bioproduct Sciences
University of Saskatchewan
Saskatoon, Saskatchewan Canada
S7N 5A8

ABSTRACT

Wheat-based thin stillage (W-TS) is liquid by-product of wheat ethanol production and contains valuable chemical intermediates such as 1,3-propanediol (1,3-PD), acetic acid, and glycerophosphorylcholine. Unfortunately, these compounds cannot be recovered/extracted easily due to the presence of high boiling point and hygroscopic solutes and unfermented particles from ethanol fermentation. Fermentation improvement study using endemic bacteria augmented with *Lactobacillus panis* PM1B discovered that glucose, incubation temperature, micronutrients, and pH adjustment affected two-stage fermentation (TSF). Importantly, fermentation could be scaled to a 210 L fermenter where 2% (w/v) 1,3-PD was produced. Unfermented particles should be removed prior to compound recovery. TSF was effective in producing solutions that were virtually free of colloids. Bacteria present in TSF system produced anoxic gas and exopolysaccharides and the combined action produced substantially clear solution. On the other hand, recovered particles, rich in lactobacilli, had a high protein content (50%, w/w, dry basis), which might be useful as an animal feed ingredient. Washing processes could lower moisture content and recover a high protein slurry (60% w/w, dry basis). Practical processes that incorporated fermentation using Lactobacilli could add substantial value to thin stillage and increase the value of products from ethanol production. These processes are scalable and readily implemented.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor, Dr. Martin J.T. Reaney for academic advice, guidance, patience and understanding, his support with correction of this thesis, and the opportunity to pursue this degree. I would also like to acknowledge the guidance and encouragement offered by the members of my advisory committee, Drs. Robert T. Tyler, Takuji Tanaka, Supratim Ghosh, Monique Haakensen, and Yen-Han Lin.

I would like to thank Saskatchewan Agriculture Development Fund for supporting this research. I thank my lab mates and my friends for their help, sharing of experiences, advice, technical input, and encouragement during my studies. Also special thanks to Dr. Youn Young Shim for her help preparing this thesis and the manuscripts from it.

Lastly, I would like to thank my family for their support throughout my studies at the University of Saskatchewan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1,3-PD	1,3-propanediol
3-HPA	3-hydroxypropionaldehyde
AChE	Acetylcholinesterase
AGF	Anoxic gas flotation
ATP	Adenosine triphosphate
a_w	Water activity
DAF	Dissolved air flotation
db	Dry basis
DDG	Dried distillers' grain
DDGS	Dried distillers' grain with solubles
DG	Distillers' grain
DhaB	Glycerol dehydratase
DHAP	Dihydroxyacetone phosphate
DhaT	1,3-PD dehydrogenase
DPFGSE	Double pulse field gradient spin echo
DS	Distillers' solubles
EPS	Exopolysaccharide
GPC	Glycerophosphorylcholine
HPLC	High performance liquid chromatography
MRS	de Man, Rogosa and Sharpe
MWCO	Molecular weight cut-off
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide plus hydrogen
NDF	Neutral detergent fibre
NMR	Nuclear magnetic resonance
RO	Reverse osmosis
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TS	Thin stillage
TSF	Two-stage fermentation
W-B	Wheat-based beer
W-DS	Wheat-based distillers' solubles
W-DWG	Wheat-based distillers' wet grains
W-TS	Wheat-based thin stillage
wb	Wet basis

CHAPTER 1
INTRODUCTION

Wheat-based thin stillage (W-TS) is a solution of organic and inorganic solutes containing suspended particles and protein (Ratanapariyanuch, 2009; Ratanapariyanuch *et al*., 2011). Some W-TS organic solutes, including 1,3-propanediol (1,3-PD), acetic acid, and glycerophosphorylcholine (GPC), are potentially valuable and might be worth recovering. However, these compounds are not readily recovered due to the present of high boiling point and hygroscopic solutes (glycerol and lactic acid) and particles. The complexity of this mixture could impede compound recovery increase equipment fouling, and limit heat transfer. Furthermore, the unfermented particles present in W-TS have a high protein content that could be recovered and utilized as a protein source for animal feed. Therefore, this research focuses on developing a simple biorefinery process for recovery of particles and valuable compounds from W-TS (Figure 1.1).

Global ethanol production increased from 70 billion L in 2007 to approximately 135 billion L in 2016 (OECD/Food and Agriculture Organization of the United Nations, 2014). It is predicted that production will expand to nearly 160 billion L in 2023. The conversion of grain, sugar and other biomass using yeast generates predictably large amounts of coproducts. In a laboratory setting, very high gravity fermentation starting with 300 g of fermentable sugar per liter yields aqueous ethanol solutions of approximately 20% (v/v) (Thomas and Ingledew, 1992). These solutions are heavily laden with water-soluble compounds and suspended particulate matter. In an industrial setting, the final concentration of ethanol solutions from fermentation can be lower ranging from 12 to 14%. After ethanol is separated from the fermentation media used in commercial ethanol production, a portion of this solution is often added to incoming grain and another portion is entrained with wet grains (Kwiatkowski *et al*., 2006; Soto *et al.*, 2005). The mass of water processed in an ethanol plant might be as much as 7 to 8 times the ethanol volume. Much of this solution/suspension is recovered as a dilute solution called thin stillage (TS). Normally, W-TS is evaporated to produce wheat-based distillers' solubles (W-DS) and this is sold as an animal feed ingredient (Ingledew *et al*., 2009). For instance, Pound-Maker

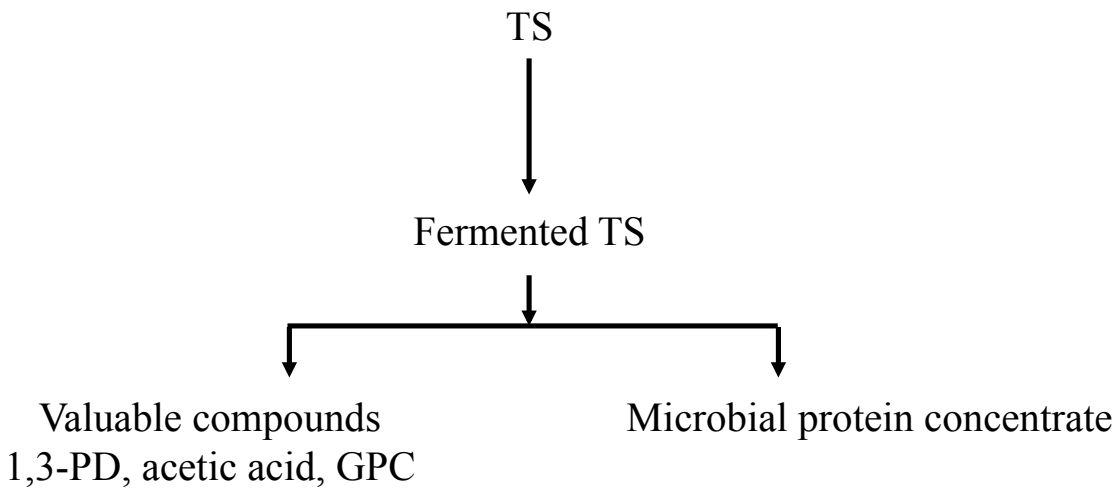

Figure 1.1 Research plan

Agventures Ltd. (Lanigan, SK, Canada), utilizes W-TS as a nutrient rich water source for animals. There may be greater value for the solutes present in TS as the particulate matter and solution coproducts of ethanol production are rich in potentially valuable materials. These fractions have been the subject of considerable bioprospecting research (Ingledew *et al*., 2009).

W-TS contains a range of potentially valuable solutes (Ratanapariyanuch *et al*., 2011) including organic solutes that are potentially valuable intermediates. As examples, 1,3-PD, acetic acid, and succinic acid, all constituents of W-TS, are commodity chemicals while GPC is a nutraceutical. W-TS is an excellent source for these organic solutes. Therefore, these potentially valuable compounds might be recovered rather than fed to animals. However, due to the complex nature of W-TS, there is no commercial production of commodity chemicals from it. W-TS solutes have many different properties. Some have high boiling points and/or are hygroscopic and/or are ionic and/or are zwitterionic. In addition, W-TS contains colloidals and suspended. Consequently, industrial scale compound extraction and recovery from W-TS will require careful process design to sequentially separate all materials efficiently. In our previous research, we discovered a W-TS sample that had high an elevated concentration of 1,3-PD and depleted glycerol. It was hypothesized that metabolism by endemic microorganisms present in W-TS converted the glycerol to 1,3-PD. Subsequent observations of W-TS incubated at 37 °C revealed similar that this conversion was typical of all samples analyzed. In addition, lactic acid also decreased in the W-TS samples while acetic acid accumulated. Although W-TS is a product of yeast fermentation it has been suggested that a second stage of fermentation or two-stage fermentation (TSF) process with endemic flora especially *Lactobacillus panis* strain PM1B might be employed to increase the value of W-TS. In addition, high boiling point and hygroscopic solutes such as glycerol and lactic acid can be converted to 1,3-PD and acetic acid, in that order (Reaney *et al*., 2013) that are more easily separated from W-TS while not affecting GPC. In spite of the simplicity of this fermentation process, this discovery has never been commercialized. One problem is the presence of undesirable particulate solids in W-TS that make processing difficult.

Particle removal is an essential initial process in many processes to limit equipment fouling while enabling mass and heat transfer during compound recovery and extraction processes. Several strategies might be used to clarify W-TS including; centrifugation (Kelsall and Piggot, 2009; LoCascio and Dunbar, 2014), clarifying agents (Castellari *et al*., 2001; Menkhaus *et al*., 2010; Scheimann, 2009), dissolved air flotation (Chadwick and Schroeder, 1973; Van Leeuwen

et al., 2008) and anoxic gas flotation (AGF) (Burke, 1997b, 2000a), filtration (Arora *et al*., 2010; Arora *et al*., 2009), and size exclusion (Kalbfuss *et al*., 2007; Zheng and Yu, 2007). Ideally preparation of a particle free solution can aid in compound recovery and extraction from complex mixtures. In addition, particles from fermented W-TS might have additional value if they are removed from the stillage solution.

Particles recovered from clarification of fermented W-TS could be rich in protein and, therefore, could be used as a protein source in animal feed and, thereby, add to the value of ethanol production. Furthermore, lactobacilli present in W-TS might be utilized as probiotic microorganisms in livestock and poultry feeds (Gaggìa *et al*., 2010). However, particles should be dried in order to extend storage life and reduce transportation costs. Several methods can be used to dry particles including drum drying (Kwiatkowski *et al*., 2006; Kingsly *et al*., 2010), spray drying (Yang, 1998; Prevost and Hammomd, 2004), and thin film evaporation (Moghaddam and Ohadi, 2005; Van Gansbeghe *et al*., 2002).

1.1 Hypothesis

Additional value may be realized from grain ethanol production from wheat if macromolecules, organic molecules, and inorganic molecules present in W-TS are recovered for use as a source of commodity platform chemicals and utilized as an animal feed ingredient. Novel processing methods that recover valuable compounds and macromolecules from W-TS will add value to this ethanol industry coproduct.

1.2 Objectives

The objective of this research is to develop methods to isolate compounds from W-TS by:

1) Breaking colloidal suspensions in W-TS using either additives or processes.
2) Determine the effect of culture conditions including agitation, micronutrients, pH, substrates, product concentration and intermediate concentration on accumulation of W-TS compounds 1,3-PD and acetic acid during TSF.
3) Determine the effect of bacterial growth and metabolism, especially anoxic gas (CO_2) and exopolysaccharides, on W-TS colloid stability.
4) Develop methods to separate W-TS particles including bacteria from solution and recover potentially valuable compounds from solution.

CHAPTER 2
LITERATURE REVIEW

2.1 Coproducts of Ethanol Production from Grain

Ethanol production from grain utilizes yeast species *Saccharomyces cerevisiae* to convert fermentable sugars to ethanol. The ethanol production produces by-products, which are TS and wet grain, which could be further processed by drying to distillers' solubles (DS) and dried distillers' grain with solubles (DDGS) (Ingledew *et al*., 2009). This review focuses on fermentation of grains, grain ethanol production, and composition and value of by-products from grain ethanol production.

2.1.1 Fermentation of Grains

In most commercial ethanol fermentation, the yeast species *S. cerevisiae* is employed as a microorganism that converts glucose and fructose to ethanol and CO_2 under anaerobic conditions (Ingledew, 2009b; Pretorius, 2000) (Figure 2.1). Where starch is the carbohydrate for ethanol production, it is typically hydrolyzed to form fermentable sugars using enzymes. One enzyme is a temperature stable α-amylase that hydrolyzes starch grains to produce glucose and dextrin. The other enzyme, glucoamylase, then converts dextrin to fermentable glucose (Ingledew, 2009b). Glucose released from starch is able to enter yeast cells through a membrane hexose transporter. In yeast cytoplasm, glucokinase, and hexokinase enzymes convert glucose to glucose-6-phosphate. In subsequent reactions, phosphoglucose isomerase converts glucose-6-phosphate to fructose-6-phosphate then phosphofructokinase adds phosphate derived from adenosine triphosphate (ATP) to produce fructose 1,6-diphosphate. The aldolase enzyme then cleaves the carbon bond producing 3 carbon products of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate. The DHAP is isomerized to a second glyceraldehyde-3-phosphate by triosephosphate isomerase. In a series of ensuing reactions glyceraldehyde-3-phosphate is phosphorylated to 1,3-diphosphoglycerate then isomerized to 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate, and dephosphorylated to pyruvate through reactions with glyceraldehyde-3-phosphate dehydrogenase,

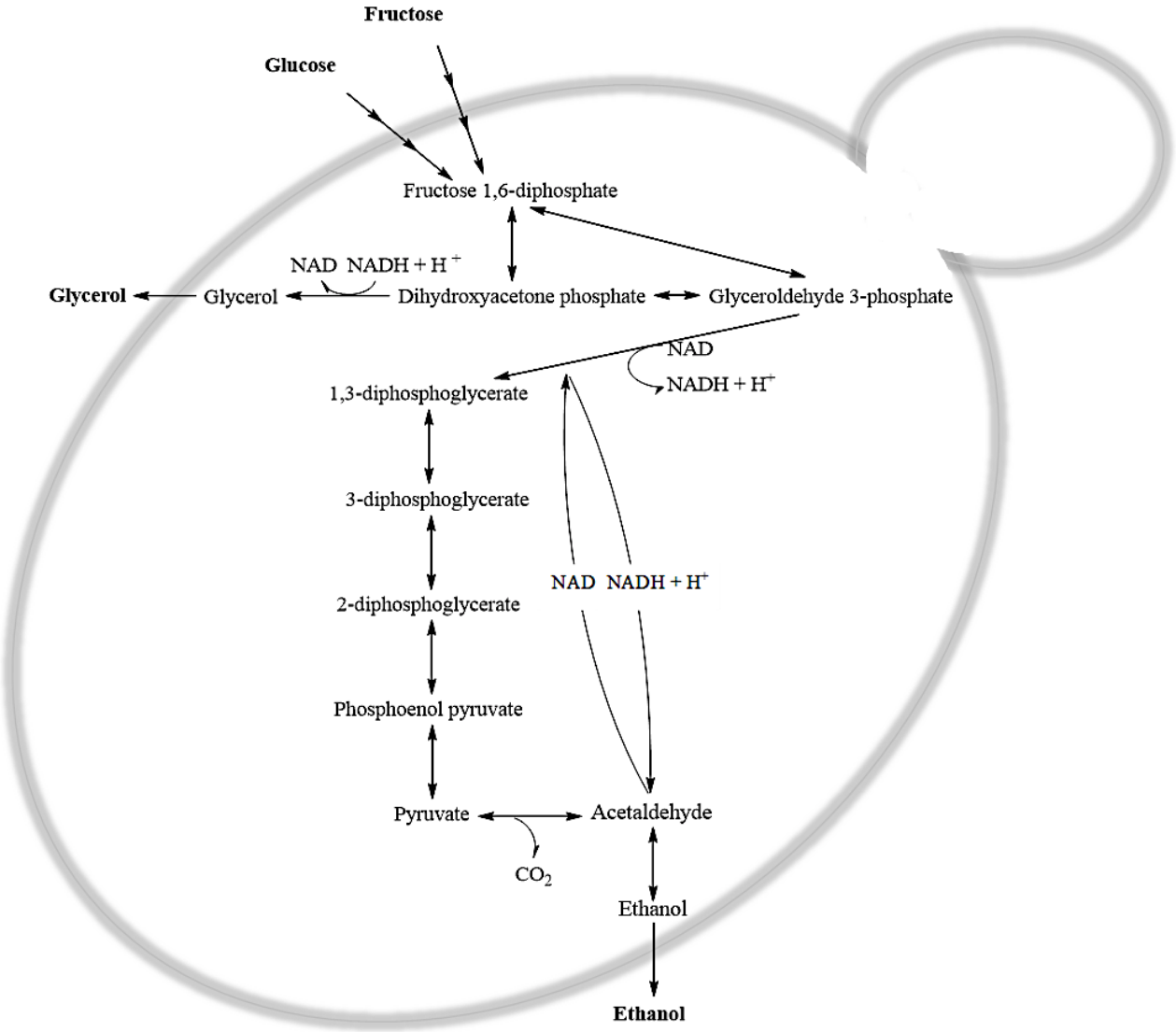

Figure 2.1 Yeast glycolysis pathways in sugar-containing mash

phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase, respectively. Pyruvate is then converted to acetaldehyde and ethanol by pyruvate decarboxylase and alcohol dehydrogenase, in that order (Bisson, 1993). Glycerol is commonly present in ethanol fermentation by *S. cerevisiae* as it allows yeast to generate nicotinamide adenine dinucleotide (NAD^+) required for glycolysis. Without glycerol accumulation during ethanol fermentation, $NADH + H^+$ will accumulate. Glycerol is produced during yeast growth or conditions where insufficient amounts of pyruvate or acetaldehyde are available to recycle $NADH + H^+$. Yeast produces glycerol from DHAP to convert $NADH + H^+$ to NAD^+. Stress (e.g. acid accumulation) could also induce glycerol accumulation due to proton accumulation and conversion of DHAP to glycerol (Ingledew, 2009b). After glycerol synthesis by yeast it is excreted (Figure 2.1).

2.1.2 Grain Ethanol Processing

A typical sequence of unit operations for processing grain to produce ethanol is presented as Figure 2.2. Grains quality is determined on sub-samples prior to fermentation. Starch, protein, and moisture content are measured and grain is tested for microbial contaminants including mold and mycotoxins. Suitable grain may then be dry milled to enable efficient contact with water and reaction with enzymes. Hammer mills are widely used in industry for this application. Milled grain particles are then mixed with water and/or recycled water (backset) (Kelsall and Piggot, 2009).

Backset contains beneficial nutrients for yeast growth but also bacterial metabolites such as acetic and lactic acid (Ingledew, 2009a; Ratanapariyanuch *et al*., 2011). These acids slow fermentation and induce sluggish yeast growth or cause yeast death. For example, at concentrations over 0.05% (w/v) acetic acid or 0.8% (w/v) lactic acid lead to yeast stress or death (Ingledew, 2009a).

Starch grain sugars are unavailable to enzymes and must be hydrated and gelatinized by a cooking/liquefaction processes before addition of hydrolytic enzymes. Cooking/liquefaction can be achieved by both: 1) using high temperatures (90–120 °C), or 2) cooler processing with α-amylase and glucoamylase enzymes that hydrolyze starch at fermentation temperatures. During high temperature gelatinization starch absorbs water and swells. Therefore, crystalline structures of starch granules hydrate gradually until they become swollen and subject to mechanical disruption. The enzyme α-amylase hydrolyses α-1,4 glucosidic linkages randomly at the beginning of liquefaction. Subsequently glucoamylase enzymes release glucose by hydrolysis of

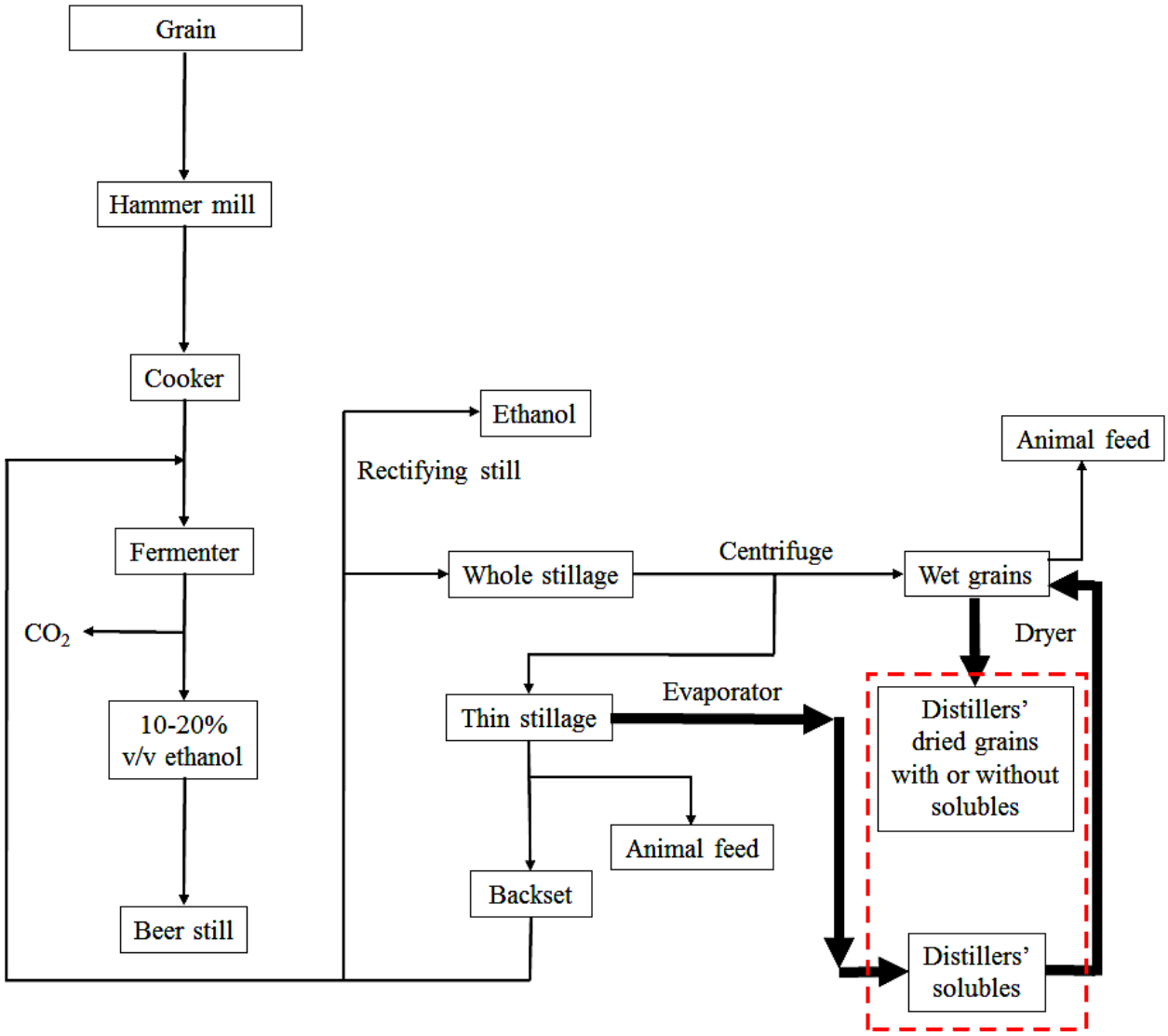

Figure 2.2 Process flow in a dry-grind ethanol plant (Modified from Ingledew *et al*., 2009)

α-1,4 and α-1,6 glucosidic linkages (Kelsall and Piggot, 2009). The combined reactions of the two enzymes are called saccharification. The slurry arising from saccharification of grain is called mash. The mash is cooled to the fermentation temperature (Kohl, 2009). Nutrients and yeast are then added to the mash and fermentation proceeds until the concentration of ethanol is approximately 10–20% (v/v). The fermented mash is called beer (Ingledew *et al*., 2009). Alcohol in the beer is distilled to produce two fractions: Lower boiling point components that are rich in alcohol (overhead product) and higher boiling point components that are rich in solids and soluble components (bottom product). Multiple steps of distillation produce ethanol solutions of up to 95%. The 95% ethanol is then dried to remove water (Madson, 2009). Molecular sieves have been utilized to remove water remaining in ethanol vapours during distillation. Anhydrous ethanol is obtained after this drying process (Swain, 2009). Anhydrous ethanol is also produced by using dried grain as a molecular sieve or by pervaporation.

The distillation bottoms produced by beer distillation are called either stillage or whole stillage. Stillage contains grain particles as well as suspended solids and solutes. Larger solids may be removed by centrifugation or screening the beer, e.g. belt presses, vibrating screens, or wedge wire screens have been used to remove coarse solids. Pressurized filters such as leaf filters have been used to separate finer suspended solids from whole stillage as well. Continuous centrifuges such as decanter or solid-bowl centrifuges are also utilized to remove coarse particles from stillage. Centrifuged solids, called wet cake or wet grains, can be utilized as animal feed. The liquid waste after solid removal is called TS (Monceaux and Kuehner, 2009). Approximately 15–50% of TS may be recycled by addition to the mash (Corrigan and Mass, 2009). When TS is used this way it is called backset. Occasionally TS is fed to animals as their water source (Ingledew *et al*., 2009) though, due to its low energy density, it must be used near to the fermentation plant. Pound-Maker Agventures (Lanigan, SK, Canada) uses W-TS as a water and nutrient source for cattle. Where feeding TS solution to animals directly is not possible, it may be evaporated to produce a concentrate. Evaporation increases the concentration of TS from 5–10% solids to approximately 30–50% solids syrup called distiller's solubles or DS (Monceaux and Kuehner, 2009). DS is either sold directly as a feed or mixed with wet grains and dried to produce DDGS. Coarse wet grains can be dried to make a dried distillers' grain (DDG) product. DDG and DDGS are excellent nutrient sources, e.g. fibre and protein, for animals (Mustafa *et al*., 2000).

In conclusion, 190-proof alcohol and 200-proof alcohol, DDG, DDGS, DS, wet grains,

aldehydes/ketones, CO_2, and fusel oils are saleable products of industrial fuel ethanol production (Ingledew *et al*., 2009). Nevertheless, some ethanol manufacturers e.g. Pound-Maker Agventures do not concentrate W-TS to produce syrup and DDGS.

2.1.3 TS, Compounds Present in TS, and Their Value

TS is a liquid coproduct of ethanol production that is recovered after ethanol distillation from beer and particulate removal (Section 2.1.2). W-TS contains a number of organic solutes that arise from the metabolic action of microorganisms on grain compounds. For example, glycerol arises from yeast fermentation of glucose by yeast while 1,3-PD is present due to the action of lactobacilli on glycerol (Ratanapariyanuch, 2009; Ratanapariyanuch *et al*., 2011). Particles that resist enzymatic and microbial degradation remain in the fermentation solution (Monceaux and Kuehner, 2009). Organic solutes have considerable promise for use as sources of commodity intermediate chemicals. In addition, W-TS yielded a product with approximately 36.6% (w/w, dry basis, db) protein (Mustafa *et al*., 2000). Therefore, value could be added to W-TS by recovery of both valuable compounds and protein.

The presence of organic solutes in stillage has been studied and reported several times and the components differ in these studies. Ethanol stillage from corn contained alanine, a small amount of ethanol, glycerol, lactic acid, various non-nitrogenous and nitrogenous acids, polyhydroxyl alcohols, sugars and glucosides (Dowd *et al*.,1993) while acetic acid, ethanol, glycerol, and lactic acid were identified in cane stillage using gas chromatography/mass spectroscopy and high performance liquid chromatography (HPLC) analysis. Acetic acid, glycerol, *chiro*-inositol, *myo*-inositol, lactic acid, and proline were found in citrus stillage; acetic acid, arabinitol, glycerol, glucose, lactic acid, lactose, and ribitol were detected in whey stillage (Dowd *et al*., 1994). Glycerol and lactic acid were found in all stillage materials. Proximate analysis of TS from wheat-based ethanol production indicated dry matter (8.4 ± 1.4%), ether extract (9.6 ± 1.6%), acid detergent fibre (3.4 ± 0.4%), neutral detergent fibre (NDF) (34.5 ± 4.6%), and crude protein (48.5 ± 1.8%). In addition, calcium, cobalt, iron, magnesium, manganese, phosphorus, and zinc were found in W-TS (Ojowi *et al*., 1996).

Waste from sugarcane distilleries is commonly applied as fertilizer to fields. Benke *et al*. (1998) studied compounds present in sugarcane distillery waste using cross polarization/magic angle sample spinning ^{13}C nuclear magnetic resonance (NMR), diffuse reflectance Fourier

transform infrared spectroscopy, and gas chromatography. They identified amino acids, cellulose, lipids, and mono-, oligo- and/or polysaccharides as well as fumaric acid, lactic acid, malonic acid, oxalic acid, and succinic acid in this material.

Acetic acid, propionic acid, and other higher acids were present during anaerobic fermentation of cane sugar distillery waste (Pandiyan *et al*., 1999). Compounds present in TS derived from barley, rye, triticale, and wheat are shown in Table 2.1 (Mustafa *et al*., 2000). Davis *et al*. (2005) studied the chemical composition of W-TS produced by recombinant *Zymomonas mobilis* and found that TS solutes included arabinose (10.6 ± 0.8% w/w, db), galactose (trace), glucose (18.0 ± 2.1% w/w, db), mannose (trace), and xylose (21.5 ± 1.8% w/w, db), as well as acid-insoluble lignin (≤ 1.0% w/w, db), acid-soluble lignin (≤ 1.0% w/w, db), ash (1.3 ± 0.4% w/w, db), lipid (19.3 ± 1.5% w/w, db), and protein (31.5 ± 3.4% w/w, db). The composition of corn-based TS is included in Table 2.2 (Kim *et al*., 2008). W-TS contained protein and a number of organic solutes from bacteria, grain, and yeast metabolites including 1,3-PD (1.22 g/L), acetic acid (1.27 g/L), betaine, ethanol, glycerol (5.85 g/L), GPC, isopropanol, lactic acid (5.55 g/L), phenylethanol, and succinic acid. Furthermore, the zwitterionic solutes, betaine and GPC, and ionic solutes, calcium, carbonate, chloride, magnesium, nitrate, potassium, sodium, and sulfate, were also identified and quantified (Ratanapariyanuch *et al*., 2011).

2.1.4 DDG and DDGS: Their Utilization and Feed Value

Global livestock feed demand is increasing (International Feed Industry Federation, 2015b) and compound feed production is predicted to reach 1 billion tonnes annually. More than US $370 billion is spent on commercial feed manufacturing annually (International Feed Industry Federation, 2015a). Due to their protein and energy content, fishmeal, DDG, DDGS, canola meal, and soybean meal are widely utilized as animal feed ingredients. The protein content of DDG, DDGS, canola meal, soybean meal, and fishmeal have been reported as 31 (MacDonald *et al*., 2007), 37 (Beliveau and McKinnon, 2008), 38 (Higgs *et al*., 2014), 48 (Cordero, 2014), and 66% (Feedipedia, 2015), respectively. The ingredients are sold for prices that reflect protein content (Table 2.3). DDG is sold at the lowest price following by wheat-based DDGS, canola meal, soybean meal, and fishmeal in order of protein content. This reflects that higher protein content feed ingredients are sold at higher prices.

Table 2.1 Chemical compositions of TS derived from different cereal grains

Chemical compositions (g/kg)	TS			
	Wheat	Rye	Triticale	Barley
Ash (db)	64	69	87	77
Ether extract (db)	59	22	61	60
Carbohydrate composition				
Total carbohydrate (db)	522	634	455	555
Non-structural carbohydrate (db)	341	520	312	394
Neutral detergent fibre (db)	352	232	316	367
Acid detergent fibre (db)	85	81	72	87
Acid detergent lignin (db)	16	12	8	28
Starch	30	20	13	13
Protein composition				
Crude protein (CP, db)	366	275	397	308
Soluble protein of CP	237	358	321	174
Non-protein nitrogen of CP	200	341	277	160
Neutral detergent insoluble of CP	483	428	437	669
Acid detergent insoluble of CP	61	55	62	143

All values are % dry basis (db) except where otherwise noted.
Source: Adapted with permission by Mustafa *et al.* (2000) from John Wiley and Sons.

Table 2.2 Composition of corn-based TS by (A) cellulosic biomass compositional (analysis average of 2 batches) and (B) forage/feed nutritional analysis

(A) Cellulosic biomass compositional analysis	Quantity
Dry matter	7.7
Glucose (g/L)	0.9
Glucan (oligosaccharide, g/L)	12.4
Xylose (g/L)	0.7
Xylan (g/L)	3.7
Arabinose (g/L)	0.4
Arabinan (g/L)	0.5
Lactic acid (g/L)	16.8
Glycerol (g/L)	14.4
Acetic acid (g/L)	0.3
Butanediol (g/L)	1.9
Ethanol (g/L)	0.6
(B) Forage/feed nutrition composition analyses	**Quantity**
Compositional analysis	
Dry matter	6.2
Crude protein	1.3
Crude fat	1.3
Carbohydrates	2.8
Ash	0.8
Forage analysis	
Gross calories (kcal/kg)	28.0
Acid detergent fibre (ADF)	0.1
Cellulose	0.1
Starch	0.5
Mineral analysis	
Calcium (ppm)	31.0
Phosphorus	0.1
Potassium	0.2
Magnesium	0.1
Sulfur	0.1
Sodium	0.1
Chloride	0.0
Iron (ppm)	8.0
Manganese (ppm)	2.0

All values are % dry basis except where otherwise noted.
Source: Adapted with permission by Kim *et al.* (2008) from Elsevier.

Table 2.3 Meal price per metric ton

Animal feed	Price ($/metric ton)
DDG	173.38[1]
Wheat-based DDGS	370.71[2]
Canola meal	410.70[3]
Soybean meal	751.17[2]
Fishmeal	2,287.00[4]

The information of price and protein content retrieved from [1]Capehart *et al*., 2015, [2]Cordero, 2014 (calculate from short ton to long ton and conversion rate US $1 = CAD $1.18), [3]Canola Council of Canada, 2015, and [4]Indexmundi, 2015.

2.1.4.1 DDG

DDG use in animal feed is studied intensively. For example, DDG use as a protein supplement in the diet of crossbred heifers was investigated. A linear increase in average daily gain and decrease in forage intake were observed with increases in dietary DDG (MacDonald *et al*., 2007). Moreover, DDG is also used as an energy source. Dietary DDG can affect other aspects of animal performance. For example, feeding corn-based DDG to heifers was associated with higher artificial insemination pregnancy rate. Artificial insemination conception and pregnancy rates were improved even though diets were isocaloric (Martin *et al*., 2007). DDG had greater NDF digestibility than alfalfa silage, corn silage, small grain silage, ryegrass silage, and straw (Hoffman, 2008). Therefore, the heifers likely obtained more energy from NDF when supplementing DDG compared to NDF from forages.

2.1.4.2 DDGS

2.1.4.2.1 Cattle/ruminant

DDGS is employed as a protein source in cattle/ruminant feeds. Calves fed corn-based DDGS with 5.9, 13.9, and 14.8% of acid detergent insoluble nitrogen gained more weight and exhibited more efficient feed conversion than control animals fed dried-rolled corn (Ham *et al*., 1994). Buckner *et al*. (2007) studied the optimum levels of DDGS for finishing beef steers in corn-based diets with 0, 10, 20, 30, 40, and 50% (db) inclusion of DDGS. Increasing the inclusion level of DDGS did not affect dry matter intake while average daily gain and final body weight were changed. The data were well fitted with a quadratic model observed where 20% DDGS inclusion was optimal for animal performance. The feed to gain ratio was improved compared to control diets where 20% DDGS was included in the diet. In addition, the energy levels of 10-40% DDGS diets were more than that of the control diet. The energy value, however, declined when DDGS inclusion was increased above 20% inclusion in the diet. The optimum average daily gain and feed to gain ratio was observed at 20% DDGS inclusion. These results were supported by the report of Klopfenstein *et al*. (2008). In this study increased DDGS with forage was associated with a cubic response in feed to gain ratio and a quadratic response in average daily gain. The maximum average daily gain and feed to gain ratio were found at 20–30% and 10–20% DDGS inclusion, respectively. Diets containing DDGS reduced acidosis in cattle fed grain due to high levels of fat, fibre, and protein and low levels of fermentable carbohydrates (U.S. Grains Council, 2012).

Anderson *et al*. (2006) fed DDGS to lactating dairy cows at 10 and 20% inclusion and discovered that DDGS increased milk yield. DDGS inclusion of 20% of diet provided greater milk yield compared to control diets and those with just 10% DDGS inclusion. Similarly, Kleinschmit *et al*. (2006) included up to 20% of corn-based DDGS in diets of lactating dairy cows. Energy-corrected milk, feed diet efficiency, milk fat, and milk yield were higher in cows fed a DDGS diet compared to controls. Milk protein yield also tended to be greater when feeding DDGS. Based on the large amount of data showing positive effects of feeding DDGS, the U.S. Grains Council (2012) approved inclusion of up to 20% DDGS with forage feeds as long as dietary nutrients are balanced. Furthermore, greater milk production was possible feeding diets containing up to 30% DDGS when compared with diets without DDGS. Moreover, due to its fibre content DDGS replaced high-starch feed ingredients such as corn. Therefore, acidosis problems associated with starchy diets could be minimized. Higher inclusion of dietary DDGS provided a mechanism to increase dry matter intake where the greatest dry matter intake was achieved at 20–30% DDGS inclusion.

2.1.4.2.2 Poultry

DDGS is also an acceptable ingredient for poultry diets (Świątkiewicz and Koreleski, 2008). Thacker and Widyaratne (2007) study the performance of male broiler chicks when feeding soybean meal containing diets where wheat-based DDGS were substituted at 5, 15, and 20% DDGS inclusion. As inclusion levels increased digestibility coefficient for dry matter, energy, and phosphorus decreased. No significant differences in feed conversion, feed intake, or weight gain were observed even though feed conversion and weight gain tended to decline with 20% dietary DDGS. DDGS can be included up to 15% in broiler diets without detrimental effects on performance (Thacker and Widyaratne, 2007). DDGS was included in diets at 0, 5, 10, 15, 20, and 25%. As DDGS inclusion level increased, a reduction of weight per volume of feed was observed. When broiler chicks were provided diets including DDGS there was no significant effect of inclusion level on body weight gain. DDGS inclusion at 25% was associated with greater feed intake and poor feed conversion when compared with controls. Additionally, with 15 and 25% DDGS inclusion in diets, a significantly lower dressing percentage was observed. Breast weight was significantly decreased when expressed as percent of live weight but was not affected when expresses as percentage of carcass weight when DDGS were fed at 25% inclusion compared to the control. When feeding a diet with 15% inclusion of DDGS wing weight, as

percent of live weight and carcass weight, was greater than controls. DDGS may be fed to broilers at levels 15–20% DDGS inclusion with little negative effect on life performance but a loss of dressing percentage and breast weight may occur (Wang *et al*., 2007). Shim *et al*. (2011) stated that broilers fed diets with up to 24% DDGS performed well if the feed was properly balanced.

2.1.4.2.3 Swine

DDGS is considered as an attractive energy source and replacement for soymeal in swine diets due to its high energy, moderate lysine, moderate protein, and high phosphorus contents as well as its digestibility. However, there are limitations when DDGS inclusion in diets exceeded 20%. Pork fat firmness decreased while manure volume, nitrogen, and phosphorus excretion increased with this diet. In addition, it is necessary to supplement diets with amino acids. However, if proper diet formulation is practised, these limitations are largely overcome. The inclusion of DDGS in piglet starter diets improved gain efficiency without affecting pig mortality. However, no improvement in pig performance is observed in most studies where corn-based DDGS was included in grower-finisher diets (U.S. Grains Council, 2012).

2.1.4.2.4 Aquatic Animal

Fishmeal is a nutrient-rich ingredient for animal feed manufactured by processing fish. It is normally produced from wild-caught small marine fish, which usually are not consumed by humans due to high percentage of bone and oil. It is also produced from by-catch from other fisheries and by-products or trimmings from seafood processing (Miles and Chapman, 2005). Although it is a good quality ingredient for animal feed, its prices have been rising while availability is declining (U.S. Grains Council, 2012). Fibre is not desired in most aquaculture diets and there is little fibre in fishmeal, thus, it is a preferred source of protein (Naylor *et al*., 2009). Fibre is especially deleterious in fish (Lovell, 1998) and shrimp (Akiyama, *et al*., 1992) performance. It is desirable to replace dwindling fishmeal protein sources with more abundant plant-based protein. DDGS was investigated for its efficacy as a candidate for inclusion in fishmeal due to its moderately high protein content and low phosphorus content compared to fishmeal. DDGS does not contain anti-nutrient factors such as gossypol, erucic acid and glucosinolate, and trypsin inhibitor found in cottonseed, rapeseed, and soybean meal, respectively. Furthermore, the price of DDGS was significantly lower than the price of fishmeal.

DDGS inclusion at 10% of diet was associated with suitable performance. In addition, performance was maintained at higher inclusion levels of DDGS (up to 30%) when amino acids (e.g. lysine, methionine, tryptophan) were balanced by addition of supplements or inclusion of complementary protein sources which have suitable amino acid balance (U.S. Grains Council, 2012).

DDGS is a suitable dietary ingredient for aquaculture, cattle, dairy cattle, poultry, and swine feeds due to its high fat, fiber, and protein, and low fermentable carbohydrates. This composition could reduce acidosis in ruminant and improve animal performance when partially replacing other protein sources in animal feed. However, it may contain mycotoxins from mould and produce negative effects on animal performance. DDGS should be tested for mycotoxins before it is included in feed. Moreover, DDGS sources typically contain substantial amounts of sulfate. Feeding excessive amounts of sulphur to ruminants can cause polioencephalomalacia, a degradation of the nervous system (U.S. Grains Council, 2012). Both mycotoxins and sulphur content must be considered when determining the concentration of DDGS incorporated into animal feed.

The price of protein sources feed for animals is increasing but plant-based protein sources are less expensive than those derived from animals. DDG and DDGS, by-products from ethanol production, contain substantial fiber as well as protein. Due to their protein content these resources have been studied and utilized as a protein source in animal feed e.g. aquatic animal, cattle/ruminant, poultry, and swine feed. In most reports of DDG and DDGS inclusion in diets, low inclusion levels produced small improvements or no effect on animal performance. However, as inclusion of DDG and DDGS increases, fibre and imbalanced amino acids affect animal performance. This is more pronounced in monogastric animals. Therefore, DDG and DDGS cannot replace other protein sources. Moreover, nutrition especially amino acid nutrition should be balanced when using these protein sources in feed.

2.2 Valorization of TS

W-TS includes a number of organic solutes (Ratanapariyanuch *et al*., 2011). 1,3-PD, acetic acid, and GPC are potentially valuable commodity intermediate chemicals and may be worth recovering. Unfortunately, high boiling point and hygroscopic solutes present in W-TS are problematic for compound extraction and recovery. Therefore, elimination or reduction of

undesirable solutes should precede extraction of valuable compounds. W-TS is known to host a range of endemic flora including bacteria, fungi, and yeast (Ratanapariyanuch, 2009). *L. panis* strain PM1B, a strain of bacteria present in W-TS, has the ability to convert high boiling point and hygroscopic solutes (glycerol and lactic acid) to more valuable compounds (1,3-PD and acetic acid) through its fermentation pathways (Reaney *et al*., 2013). Consequently, TSF using endemic flora is a novel method to overcome problems associated with glycerol and lactic acid during compound separation. In order to achieve the maximum compound conversion, the important parameters for fermentation, e.g. co-factors, nutrients, pH, and temperature, have to be evaluated.

2.2.1 Potentially Valuable TS Compounds

While there are several organic solutes in W-TS that might be isolated from stillage, the goal of this research was to develop methods to recover 1,3-PD, acetic acid, and GPC from W-TS. 1,3-PD is an intermediate chemical used in polycondensation reaction synthesis of polyesters and polyurethanes from monomers (Biebl *et al*., 1999). In addition, 1,3-PD was employed as a monomer for polyamide and polyester production. It is suited for use in, biocides, biodegradable plastic, transparent ballistic plastic, and textiles (Saxena *et al*., 2009). Furthermore, other applications of 1,3-PD included cosmetics, foods, medicines, and transport fuels e.g. antifreeze, fuel additives, and lubricants (Xiu and Zeng, 2008). Global sales of 1,3-PD were 146 kilo tonnes in 2014 and sales are expected to reach 225.9 kilo tonnes by 2022 (Business Standard, 2015).

Acetic acid solutions with water are utilized as the food ingredient vinegar. In addition, it is a precursor for production of polyvinyl acetate (New World Encyclopedia, 2012), which can be used for synthetic fibres (Manning and Hutten, 1992). Moreover, household-cleaning products also contained acetic acid. It is utilized as a solvent e.g. in production of terephthalic acid which is a raw material for polyethylene terephthalate production. Furthermore, acetic acid is widely used in producing metal acetates e.g. aluminum acetate and iron (II) acetate (mordant for dyes), copper (II) acetate (pigment and fungicide), palladium (II) acetate (a catalyst for organic coupling reaction for example the Heck reaction), and sodium acetate (textile industry and a food preservative), (New World Encyclopedia, 2012). Global demand for acetic acid is expected to grow to approximately 18,000 kilo tons and $13.31 billion dollars by 2022 (Grand View Research, 2015).

GPC is known as a precursor in the biosynthesis of membrane phospholipids and the neurotransmitter acetylcholine (ACh) (Sangiorgi *et al*., 1994). The impact of various dietary cholinergic compounds including GPC on cognitive function has been studied. Oral GPC significantly improved cognitive symptoms. Other cholinergic precursors such as choline or lecithin did not provide comparable effects (Parmetti *et al*., 2007). GPC could be considered as a cholinergic that acts like a parasympathomimetic drug in dementia disorder and in acute cerebrovascular disease (Parnetti *et al*., 2001). When treating malignant human mammary epithelial cells with nonsteroidal anti-inflammatory agent indomethacin, an increase in GPC and decrease in phosphorylcholine level were observed. Moreover, in several breast cancer cell lines, anti-microtubule drugs e.g. anticancer drug paclitaxel induced a rise in GPC level. Furthermore, aberrant choline phospholipid metabolism was caused by inflammation-related pathologies and neurological disorders including Alzheimer's disease, Gaucher's disease, and multiple sclerosis (Glunde *et al*., 2004). Involvement of GPC in diverse cellular functions such as inhibition of lysophospholipase activity, inhibition of phosphatidyl inositol transfer protein alpha, and maintenance of renal osmolarity is known. GPC was also utilized in elasticizes, emollients, moisturizers, nutritive creams, pharmaceutical preparations, restitutives, and topical cosmetics. These preparations include drug combinations with acetylcholinesterase (AChE) inhibitors (such as donepexil and rivastigmine) to treat adult cognitive disorders, including Alzheimer's disease. This combination allowed a smaller dose of AChE inhibitor leading to reduced toxic effects caused by long-term AChE inhibitor treatments. In addition, GPC systematic toxicity is low as is its topical toxicity (Reaney *et al*., 2014). Lopez *et al*. (1991) discovered that GPC reversed scopolamine-induced amnesia. Furthermore, brain ACh levels could be partially enhanced by GPC after scopolamine administration to the cortex and hippocampus, but not the striatum. Rats treated with α-GPC were stimulated with potassium and it was observed that ACh released by the hippocampus increased. The effect of GPC and a reference drug (ST200) on neuropsychological parameters was tested with 126 patients who had probable senile dementia (Alzheimer's type). It was observed that most neuropsychological parameters were improved significantly when treating the patients with GPC and its effect lasted longer than ST200 (Parnetti *et al*., 1993). Glyceryl-phosphorylcholine diesterase degraded α-GPC to choline and a glycerol-1-phosphate. The choline and glycerol-1-phosphate utilized for ACh synthesis and reconstitution of phospholipid products including nerve cell membrane components. It was also

suggested that incorporation and storage of cholinergic precursors into brain phospholipids is important for ACh biosynthesis (Amenta *et al*., 2001). GPC is increasingly being marketed as a cognitive drug as the number of people who are suffering from Alzheimer's disease is increasing. AC immune (2015) reported that approximately 44 million people currently suffer from Alzheimer's disease in the US and the number of sufferers is estimated to increase to 135 million people in 2050. The market for Alzheimer's disease treatments is $5 billion and expected to grow to $20 billion by 2020.

2.2.2 Production of 1,3-PD

1,3-PD can be synthesized by hydration of acrolein followed by hydrogenation, hydroformylation, and hydrogenation of ethylene oxide. Unfortunately these chemical reactions require expensive catalysts and severe reaction conditions (high pressures and temperatures). Therefore, expensive reactors and substantial energy consumption result in high production costs for this compound (Ferreira *et al*., 2014). Consequently, 1,3-PD production using living organisms is preferred (Table 2.4) (Saxena *et al*., 2009). 1,3-PD may be produced from biodiesel glycerol by the use of *Citrobacter freundii,* strain FMCC-B 294 (VK-19), in sterile and non-sterile reactors (Metsoviti *et al*., 2013). Tryptic Soy Broth was utilized as a medium and cultures were maintained at 30 °C with agitation 150 ± 5 rpm. Fermentation media pH was maintained at 7.0 using 5 M NaOH. Cultures were provided glycerol in fed-batch fermentation with 40 g/L at the beginning of fermentation. When glycerol concentration was below 5 g/L, raw glycerol (60%, v/v) and yeast extract (1%, w/w) were added to the fermentation medium. The *C. freundii* strain [FMCC-B 294 (VK-19)] could tolerate up to 170 g/L glycerol. The maximum concentration of 1,3-PD 45.9 g/L was achieved when approximately 100 g/L glycerol was provided. A peak concentration of 68.1 g/L 1,3-PD was obtained in fed-batch fermentation with 1,3-PD yields per glycerol consumption approximately 0.4 g/g and volumetric production 0.79 g/L/h. For non-sterile fed-batch fermentation, 66.3 g/L of 1,3-PD was obtained when supplementing the fermentation medium with 176 g/L of raw glycerol.

Clostridium butyricum NCIMB 8082 cultures were grown in N_2 purged media containing glycerol at 35 °C based on media developed by Chatzifragkou *et al*. (2011) and Wilkens *et al*. (2012). Conversion of glycerol to 1,3-PD and productivity were similar for both media: 50% and 0.32 g/L/h for the medium of Chatzifragkou *et al*. (2011) and 56% and 0.21 g/L/h for the

Table 2.4 1,3-PD production by fermentation

Fermentation type	Organism	1,3-PD (g/L)	YPD (mol/mol)	QPD (g/L/h)	Reference
Batch	*K. pneumoniae* ATCC 15380	57.5	0.56	2.4	Tag (1990)
	K. pneumoniae M 5al	58.8	0.53	0.92	Cheng *et al*. (2007)
	C. butyricum DSM 5431	56.0	0.62	2.2	Biebl *et al*. (1992)
	C. butyricum VPI 3266	35.0	0.65	0.6	Saint-Amans *et al*. (1994)
	C. butyricum CNCM 1211	67.0	0.63	–	Himmi *et al*. (1999)
Fed-batch	*K. pneumoniae* ATCC 25955	58.1	0.44	1.0	Held (1996)
	K. pneumoniae ATCC 25955	73.3	0.48	1.5	Held (1996)
	K. pneumoniae ATCC 25955	73.3	0.48	0.92	Cameron *et al*. (1998)
	K. pneumoniae M 5al	53.0	0.41	0.94	Cheng *et al*. (2006)
	K. pneumoniae DSM 2026	53.0	0.47	1.7	Mu *et al*. (2006)
	K. pneumoniae DSM 2026	75.0	0.61	2.2	Liu *et al*. (2007)
	K. oxytoca LDH 3	83.5	0.62	1.39	Yang *et al*. (2007)
	K. pneumoniae AC 15	71.0	0.64	2.37	Zheng *et al*. (2008)
	K. pneumoniae DSM 2026	61.1	0.51	2.0	Mu *et al*. (2008)
	C. butyricum DSM 5431	58.0	0.68	2.7	Günzel *et al*. (1991)
	C. butyricum VPI 3266	65.0	0.69	1.0	Saint-Amans *et al*. (1994)
	C. butyricum DSM 5431	70.3	0.68	1.5	Abbad-Andaloussi *et al*. (1995)
	C. butyricum E5	65.6	0.65	1.2	Petitdemange *et al*. (1995)
	C. butyricum DSM 5431	70.4	0.68	1.4	Reimann and Biebl (1996)
	C. butyricum mutant 2/2	70.5	0.66	0.9	Reimann and Biebl (1996)
Fed-batch two-stage	*K. pneumoniae* AC 15	74.07	0.62	3.08	Zheng *et al*. (2007)
Continuous	*K. pneumoniae* DSM 2026	48.5	0.61	4.9	Menzel *et al*. (1997)
Continuous two-stage	*C. butyricum* F 2b	46.0	0.53	3.4	Papanikolaou *et al*. (2000)
Continuous two-stage	*C. butyricum* F 2b	43.5	0.49	1.33	Papanikolaou *et al*. (2008)
	C. freundii DSM 30040	41.5	0.62	1.38	Boenigk *et al*. (1993)
Micro-aerobic	*K. pneumoniae* DSM 2026	59.50	–	1.57	Chen *et al*. (2003)
Micro-aerobic fed-batch	*K. pneumoniae* DSM 2026	72.0	0.57	2.1	Liu *et al*. (2007)
	K. pneumoniae M5al	83.56	0.62	1.61	Yang *et al*. (2007)
Aerobic fed-batch	*K. pneumoniae* XJPD-Li	65.26	0.56	3.16	Ma *et al*. (2009)
	K. pneumoniae TUAC01	70.6	0.56	1.05	Hao *et al*. (2008)
Immobilized	*K. pneumoniae* ZJU 5205	4.1	0.30	16.4	Zhao *et al*. (2006)
	C. freundii DSM 30040	16.4	0.57	8.20	Pflugmacher and Gottschalk (1994)

Source: Adapted with permission by Saxena *et al.* (2009) from Elsevier.

medium of Wilkens *et al*. (2012). Furthermore, the results from 1 L fed-batch fermentation achieved productivity and yield of 1.29 g/L/h and 0.56 g 1,3-PD/g glycerol, respectively (Ferreira *et al*., 2014).

While other bacteria could be utilized for 1,3-PD production, some of them are possibly pathogenic and toxins may be produced from their metabolic pathways. In addition, these rganisms may require expensive nutrients and/or strictly anaerobic conditions which creates difficulties for handling and increases the cost of raw materials. Reaney *et al*. (2013) discovered *L. panis* PM1B from fermented W-TS that has ability to convert glycerol and lactic acid to 1,3-PD and acetic acid, respectively, which are valuable compounds. Moreover, *L. panis* PM1B is not pathogenic and does not require strict anaerobic conditions.

2.2.3 *L. panis* Strain PM1B and Its Fermentation Pathways

When grown in either TS or de Man, Rogosa and Sharpe (MRS) media with added glycerol, *L. panis* PM1B depletes glycerol while 1,3-PD and acetic acid accumulate. Conversion pathways in MRS based media are well studied but very little is known of the behaviour of this organism when grown on TS.

In one of the few studies of bacterial metabolism of TS Skinner-Nemec *et al*. (2007) inoculated biofilm reactors with fermenter samples from the ethanol industry. The bacteria present in biofilms were purified and identified. The predominant bacteria were lactic acid bacteria. In addition, the results from ribosomal RNA gene sequencing showed that *L. panis* was found in production isolates among contaminant bacteria.

W-TS contains a range of potentially valuable solutes, but their recovery in a commercial process is problematic. W-TS is a complex mixture of hygroscopic compounds (glycerol, polysaccharides, salts etc.) and high boiling solutes (glycerol, GPC, lactic acid, succinic acid, ionic and zwitterionic solutes, polysaccharide, and protein). The complexity of the mixture creates problems for compound extraction and recovery. Fermentation of TSF with *L. panis* PM1B might reduce high boiling and hygroscopic solutes and produce a solution that is more amenable to processing for solute recovery.

L. panis strain PM1B isolated from W-TS from wheat-based ethanol industry converts glycerol and lactic acid to 1,3-PD and acetic acid, respectively (Reaney *et al*., 2013). *L. panis* strain PM1B expresses the enzyme glycerol dehydratase (DhaB) that converts glycerol first to 3-

hydroxypropionaldehyde (3-HPA). Subsequently, 1,3-PD dehydrogenase (DhaT) and NADH from other metabolic pathways reduces 3-HPA to 1,3-PD (Kang *et al*., 2013b). This pathway occurred in absence of oxygen and presence of various carbon sources e.g. glucose (Khan *et al*., 2013). In addition, lactic acid could be converted to acetic acid using anaerobic lactic acid degradation (Kang *et al*., 2014b). The pathways of glycerol conversion and lactic acid degradation are shown below (Figure. 2.3).

An aerotolerant acidophilic anaerobe *L. panis* PM1B strain isolated from stillage was able to grow over a wide range of temperatures and tolerant to high concentrations of acetic acid, ethanol, lactic acid, and sodium chloride. In addition, *L. panis* PM1B was resistant to several common antibiotics. Moreover, arabinose, galactose, glucose, lactose, maltose, and xylose could be utilized, as carbon sources for growth while fructose and sucrose did not sustain *L. panis* PM1B. Based on its growth on these sugars *L. panis* PM1B may be characterized as a group III heterofermentative lactic acid bacterium. In the absence of oxygen with glucose as the carbon source *L. panis* PM1B produces 1,3-PD. This reaction indicates that the 1,3-PD production pathway provided NADH recycling needed for glucose metabolism. This metabolism was prevalent in MRS medium (Khan *et al*., 2013).

1,3-PD production and NAD^+ regeneration in *L. panis* PM1B cultured in modified MRS media is affected by glycerol concentration and environmental factors such as initial glycerol content, pH, and temperature. Under these conditions the expression level of enzymes and shifting the NAD^+ regeneration pathways affected the production of 1,3-PD in *L. panis* PM1B. The NADH flux from ethanol to 1,3-PD occurred when glycerol was supplemented at 13.81 g/L. However, a negative effect on regeneration of NAD^+ *via* 1,3-PD production was found when concentration of glycerol was 37.6 g/L. This led to the decrease of transcriptional levels and activity of DhaT resulting in the shutdown of 1,3-PD production. In addition, acidic conditions activated the expression of genes for DhaB and DhaT. Furthermore, glycerol reduction could not be restarted when glucose was added after its depletion. After glycerol depletion, enhanced ethanol production was a normal response to added glucose (Kang *et al*., 2013b).

When culturing *L. panis* PM1B in modified MRS in the presence of glycerol under anaerobic conditions, the major route of NADH reoxidation was 1,3-PD formation. Whereas under aerobic condition H_2O_2 was produced through NADH recycling via NADH:H_2O_2 oxidase activity. H_2O_2 accumulated in the presence of oxygen resulting decreased 1,3-PD production and

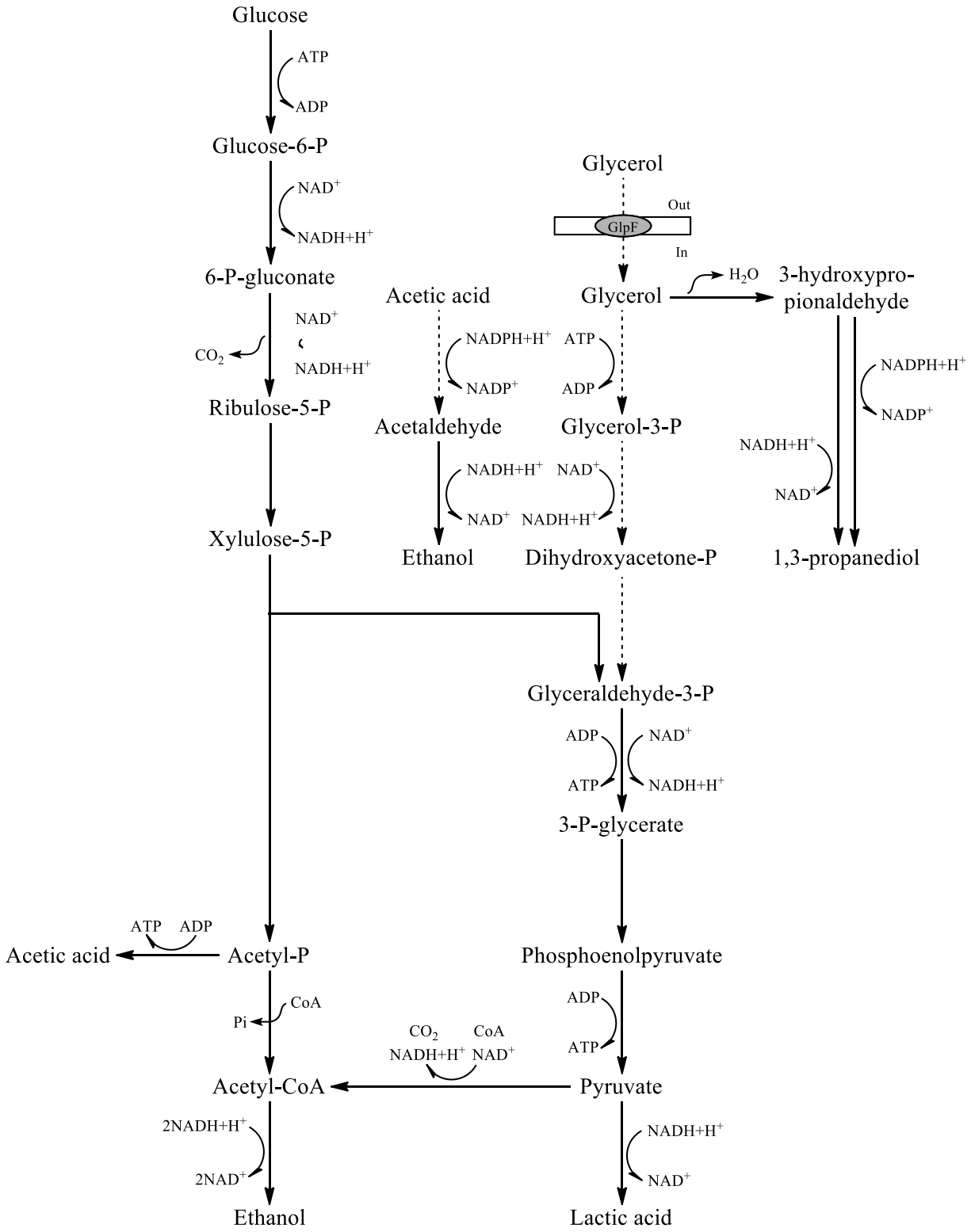

Figure 2.3 Schematic diagram of the glycerol metabolic pathway in the engineered PM1 strain. Dashed lines represent metabolically engineered pathways. Source: Adapted with permission by Kang *et al*. (2014b) from American Society for Microbiology.

growth inhibition. When *L. panis* PM1B was cultured in modified MRS medium containing citric acid (4.6 g/L) and glycerol (13.8 g/L) as electron receptors under microaerobic conditions, 6.7 g/L of 1,3-PD was produced. However, when cultured under aerobic conditions, only 0.5 g/L of 1,3-PD accumulated (Kang *et al*., 2013c).

Grahame *et al*. (2013) studied the effects of glucose and glycerol concentrations, inoculum size, temperature, aeration, pH, and carbon source on fermentation with *L. panis* PM1B grown in modified MRS medium (50 mg $MnSO_4$, 100 mg $MgSO_4.7H_2O$, 2 g ammonium citrate, 2 g K_2HPO_4, 5 g sodium acetate, 5 g yeast extract, 10 g meat extract, 10 g peptone). Inoculum size and temperature (27–37 °C) had little influence on 1,3-PD production but adjusting media to an alkaline pH at the beginning of the culture and adding glucose and glycerol enhanced 1,3-PD production. Moreover, the shift of metabolic end products from ethanol to lactic acid was associated with increased 1,3-PD production as this pathway increased NADH for conversion of glycerol to 1,3-PD. The optimum conditions in MRS media were 0.9 g/L glucose, 13.8–18.4 g/L glycerol, and pH 9–10. Under these conditions 16.3 g/L of 1,3-PD was achieved from 27.6 g/L glycerol (71% conversion efficiency). Additionally, when fermentation was initiated at pH 10, 17.0 g/L of 1,3-PD was produced (75% conversion efficiency) when growing *L. panis* PM1B in modified MRS medium containing 18.0 g/L glucose and 27.6 g/L glycerol.

The effect citrate on redox potential, ATP production, and metabolic pathways in *L. panis* PM1B was reported. When citrate is provided to *L. panis* PM1B in modified MRS medium the NAD^+ regeneration route is enhanced and additional ATP is produced (Kang *et al*., 2013a).

Typically *L. panis* PM1B cannot grow with fructose as a sole carbon source but a heterofermentative *L. panis* PM1B strain was identified. This organism produced different end products than group III heterofermentative lactobacilli using 6-phosphogluconate/phosphoketolase pathway for instance, more lactate, less acetate and no mannitol (Kang *et al*., 2013d).

The transcriptional repressor PocR (annotated as a transcription factor of AraC family) also played a role in regulating the 1,3-PD biosynthetic pathway in *L. panis* PM1B. PocR is the key transcription repressor of 1,3-PD biosynthesis by *L. panis* PM1B. When PocR gene was over expressed, it led to 81% suppression of *pduC* (PduCDE large subunit which involved in 1,3-PD and 3-HPA production) transcription resulting the 22% activity decrease of PduCDE. Subsequently, the production of 1,3-PD and 3-HPA was reduced 40% compared to the control

strain, thus confirming the role of this regulator in the pathway from glycerol to 1,3-PD (Kang *et al*., 2014c).

2.2.3.1 Effects of Glycerol to Glucose Ratio on Lactobacilli Fermentation

Glucose is an important nutrient for bacterial growth and supplies reducing power for 3-HPA reduction. Studies of the impacts of glucose: glycerol ratios are important in improving 1,3-PD accumulation. Addition of glucose to resting *L. reuteri* cells in modified MRS medium (no added sodium acetate, glucose, and tween 80) increased glycerol conversion to 3-HPA by regulating NAD/NADH ratio (Lüthi-Peng *et al*., 2002). The reduction of 3-HPA to 1,3-PD reoxidized NADH produced from glucose metabolism. The highest content of 1,3-PD in media (2.3 g/L) was found with 18.0 g/L glucose and 5.5 g/L glycerol compared to just 0.1 g/L when no glucose was added to a medium with 5.5 g/L glycerol (Lüthi-Peng *et al*., 2002).

Pflügl *et al*. (2012) also studied the effect of glucose: glycerol ratios on 1,3-PD accumulation by *L. diolivorans* in fed batch cultures grown in modified MRS medium containing 33 g/L of glucose. At higher ratios of glucose to glycerol, more ethanol and lactic acid accumulated while acetic acid remained constant. In addition, the highest concentration of 1,3-PD (73.7 g/L) was found in fed-batch fermentation with co-feeding of glucose: glycerol 0.1:1 M.

2.2.3.2 Effects of Vitamin B12 on Lactobacilli Fermentation

Bacterial glycerol and related diol dehydratase enzymes require coenzyme B12 as a co-factor (Poznanskaja *et al*., 1979; Schneider *et al*., 1970). Biebl *et al*. (1999) stated that vitamin B12 is involved in dehydration of glycerol to 3-HPA and reduction of 3-HPA to 1,3-PD in the biochemical pathways for glycerol fermentations with 1,3-PD as the end product. In addition, Pflügl *et al*. (2012) studied the effects of vitamins (nicotinic, riboflavin, or vitamin B12) on the fermentation of *L. diolivorans*. Vitamin B12 enhanced 1,3-PD accumulation to approximately 85 g/L in MRS medium supplemented with 3% (w/v) glucose and 1% (w/v) glycerol followed by supplementing with a glucose/glycerol solution with a molar ratio 0.1:1 and feeding rate of 1.5 mL/h.

2.2.3.3 Effects of Temperature and pH on Lactobacilli Fermentation

L. panis strain PM1 grew best at pH 4.5 in modified MRS culture media while slow growth was observed at alkaline pH. During fermentation *L. panis* strain PM1 produces acids that accumulate and lower pH. Furthermore, maximum growth occurs at 37 °C with decreased

growth at higher temperatures. At 50 °C or above, no growth of *L. panis* strain PM1B was observed even though cultures survived these conditions. In addition, growth was very slow at 4 °C and cultures survived for more than 1 month at this temperature (Khan *et al*., 2013).

Grahame *et al*. (2013) stated that inoculum size, temperature (27–37 °C) did not significantly affect 1,3-PD accumulation. In addition, the optimum pH for 1,3-PD accumulation was 9–10 in the present of 9.0 g/L glucose and 13.8–18.4 g/L glycerol and *L. panis* strain PM1. The pH was adjusted at inoculation but tended to decrease during fermentation in this study.

2.2.3.4 W-TS Fermentation using Lactobacilli

The effect of fermentation parameters including pH, strain of *L. panis* PM1B [wild type and a recombinant strain expressing a NADPH-dependent aldehyde reductase gene], and temperature on glycerol disappearance and 1,3-PD accumulation in W-TS media was studied. *L. panis* PM1B was grown in either modified MRS or clarified W-TS. Batch fermentation in clarified W-TS using the NADPH-dependent aldehyde reductase (YqhD) recombinant strain at pH 4.5 and temperature 30 °C provided the highest 1,3-PD accumulation, 12.85 g/L, and yield, 0.84 g/g. The molar ratio of glucose to glycerol was 0.37 when cultures were grown in MRS medium and 0.61 in clarified W-TS medium. Greater consumption of glucose in clarified W-TS medium could be the result of limited availability of this nutrient after 48 h of fermentation. In pH-controlled (pH = 4.5) batch fermentation using clarified W-TS as media, 1,3-PD accumulation was 16.23 g/L and yield was 0.72 g/g while fermentation time decreased. Interestingly, the yield of 1,3-PD increased to 0.74 g/g in clarified W-TS without the accumulation of 3-HPA when adding Mg^{2+} and Mn^{2+} (Kang *et al*., 2014a).

Khan (2013) and Kang *et al*. (2013b) demonstrated that conversion of glycerol to 1,3-PD required $NADH+H^{+}$ from glucose metabolism. Subsequently, Kang *et al*. (2014b) genetically engineered *L. panis* PM1B to utilized glycerol present in W-TS directly to produce platform chemicals and energy for growth without supplementing with exogenous nutrients e.g. fermentable sugars and nitrogen sources. They introduced glycerol diffusion facilitator, glycerol kinase, glycerol-3-phosphate dehydrogenase, NADPH-dependent aldehyde reductase, and triosephosphate isomerase genes of *Escherichia coli* into *L. panis* PM1B. The metabolic pathway of modified *L. panis* PM1B is presented in Figure 2.3. Modified cultures accumulated 4.6 g/L of 1,3-PD at pH 7.5.

2.3 Processing Dilute Solutions

Chemical and biotechnological processes may produce dilute streams of useful compounds. Combinations of processes including absorption, clarification, distillation, and drying are required to concentrate and extract compounds from such dilute streams. Compounds produced by fermentation are often dilute solutions. For example, the concentration of 1,3-PD in media after fermentation ranges from 30–130 g/L (Hao *et al*., 2006). Hao *et al*. (2006) reacted butyraldehyde with dilute solutions of 1,3-PD to produce low polarity acetals that were readily extracted from solution. Most other extraction methods utilize evaporation to remove water from fermentation media before recovering 1,3-PD. However, large amounts of energy are required and removal of salts prior to evaporation/distillation should be considered.

2.3.1 Processing for Drying Dilute Solutions in Water

Evaporation is often used to concentrate dilute aqueous solutions. Evaporation requires efficient heat transfer to vaporize water and, thereby, concentrate non-volatile solutes or suspension (Monceaux and Kuehner, 2009). Effective heat transfer involves optimization of conduction, convention, and radiation. However, drying solutions relies primarily on conduction and convection. Conduction involves transfer of heat energy between adjacent objects. For example, conduction occurs through the walls of heat exchangers. Convection occurs where heat is transferred by bulk transport and mixing. It is typical that convection occurs when energy is transferred in fluids and gasses. Hotter gasses and liquids have lower density than cooler fluids of the same composition. The hot low-density gas or fluid will rise from a hot surface and carry heat with it (Geankoplis, 2003d). A range of devices including thin film evaporators and multi-effect evaporators, drum dryers, spray dryers are available. The evaporator selected for any process depends on the nature of the process.

Evaporative processes often require steam as a source of energy. Addition of heat is required to raise the solution temperature to the vapour saturation point. Additional energy (heat of vaporization) leads to evaporation. When solvent is evaporated non-volatile solutes are concentrated. A simple evaporator is typically inefficient and is not cost effective for bulk solvent removal. Vapour from such evaporators is typically passed through a separator for removing entrained liquids then it is condensed. The heat of the vapours is transferred to cooling water in the condenser. The concentrate is pumped to storage. The steam condensate is often

returned to a boiler for reuse (Monceaux and Kuehner, 2009) and vapour generated from the evaporator is exhausted to the atmosphere (Geankoplis, 2003a). A thin film evaporator is an example of single-effect evaporator. Thin film evaporators are preferred for drying liquids that are heat sensitive or have low thermal conductivity or are viscous liquids or have a combination of these properties. Falling film evaporators are an example of a thin film evaporator that use gravity to create a thin film that covers evaporation surfaces (Najder, 1964). Heat is transferred from heated surfaces through the thin film to the liquid-vapour surface where evaporation occurs (Moghaddam and Ohadi, 2005). Van Gansbeghe *et al*. (2002) studied the recovery of lactic acid from fermentation media. Ion exchange or related principles were utilized as pre-treatments to eliminate ions that might catalyze polylactate formation. Pre-treated solutions were prepared with between 1 and 20% lactate w/w. A mechanically shaken thin film evaporator was utilized to concentrate solutions at pressures between 0.1 Pa and 10 kPa absolute with temperatures between 110–150 °C. Lactic acid solutions were concentrated twice to obtain 50–90% w/w lactic acid. The lactic acid was further concentrated to 100% at a pressure between 1 and 50 kPa absolute and temperature between 50–160 °C. Ranghavan *et al*. (2004) utilized a thin film evaporator to manufacture a ready-dilute sugarcane juice beverage powder. The sugarcane juice was prepared from sugarcane. Sugarcane juice was filtered and emulsified prior to concentration. Filtered emulsified sugarcane juice was concentrated to a solid content 40–50 °Brix using a thin film evaporator. The concentrated sugarcane juice was homogenized and dried under vacuum to obtain solid sugarcane cake that was ground and anti-caking agent was added at the final step (Raghavan *et al*., 2004). However, single-effect evaporators have significant disadvantages. The major cost of single-effect evaporation is for steam production as the latent heat of vapour exits the single-effect evaporator without reuse. Multi-effect evaporation recovers the latent heat of vaporization. Raw steam is utilized to heat the feed in the first stage of a multi-effect evaporator. The vapour leaves from the first evaporator stage then goes to the second stage where it is used to heating incoming media. The boiling temperature in the evaporator second stage is lower than the vapour condensation temperature. Consequently, the pressure of the evaporator second stage must be lower than that of the first evaporator stage. Vapour from the second evaporator stage may be transferred to a third evaporator stage in a similar manner (Geankoplis, 2003a). Multi-effect evaporation is used to conserve energy used in food processing, pulp and paper manufacture, petroleum refining, and desalination (El-Dessouky *et al*., 2000). Bremford and

Müller-Steinhagen (1996, 1999) studied multi-effect evaporator performance for concentrating paper and pulp waste called "black liquor". They concluded that increasing steam temperature and reducing feed liquor flow rate led to higher outlet concentration compared to increasing heat transfer area and recycling a fraction of product liquor, respectively. In addition, the maximum possible pressure and temperature produced the maximum outlet liquor concentration. When constant steam temperature was maintained, fouling led to decrease evaporator capacity, outlet liquor concentration, steam economy, and supply steam flow rate. Multi-effect evaporators are used to produce DS from TS (Monceaux and Kuehner, 2009).

Drum dryers are typically hollow cylinders with a diameter ranging from 0.5–6.0 m and a length of 1–6 m. The inner surface of the drum is heated, often with steam at temperatures up to 200 °C. Moist material may be applied to the drum's outer surface as a thin layer (0.5–2.0 mm). The majority of moisture in a material may be removed providing a dried product, which is scraped from the drum. Drum drying heating times range from a few second to minutes. Typically a moisture content of less than 5% is achieved after drying. A drum dryer, depending on the configuration and nature of dried material, may consume between 1.1–1.6 kg steam/kg evaporated water. This indicates that energy efficiency is 60–90%. The capacity of drum driers is up to 80 kg water/h × m^2 with the rate of production of dried product of 5–50 kg/h × m^2 the latter number is largely dependent on the moisture content, type of the feed and other operating conditions (Tang *et al*., 2003). Drum dryers are utilized to evaporate water from mixtures of corn-based DS and wet grains (Kwiatkowski *et al*., 2006). The moisture of the mixture is reduced from 63.7 to 9.9%. The dried product is called DDGS. Kingsly *et al*. (2010) studied the effect of process variables on the physical and chemical characteristics of corn-based DDGS. A series of 2 rotary drum dryers was used as two-stage drying process apparatus. When the quantity of DS added decreased, moisture content and the particle size of DDGS decreased. In addition, a narrow size distribution (< 1500 μm) was observed in 80% of particles. Furthermore, color, fat, glycerol, total soluble sugars, and true density reduced while amino acid, insoluble fiber, and protein increased when less DS was added.

Production of dilute aqueous sprays is a hydrodynamic process used in spray drying (Deegan, 2000). Rapid drying is induced by the combination action of the elevated solvent evaporation rate at the boundary of the droplet due to high surface area and deposition of solutes at the boundary leading to contact line pinning. Solutes migrate to droplet boundaries creating

ring-like deposit after drying (Deegan, 2000; Deegan *et al*., 2000). Yang (1998) utilized spray dryer to dry TS from a rice spirit distillery. TS was dried by passing it through a mini spray dryer (Büchi 191) at an air flow rate 600 L/h, aspirator 70%, inlet temperature 180 °C, and pump 10%. Addition of gelling agents like gum arabic or maltodextrin improved spray drier capacity. Moreover, dried TS could be employed as culture medium for *S. cerevisiae*. Prevost and Hammond (2004) discovered that corn-based wet distillers' grain (DG) could be dried using a spray dryer. The dried protein rich product contained less than 15% (w/w) water when the hot air exhaust temperature for drying was maintained between 60−77 °C. In addition, under normal humidity condition, operating with contacting time less than 3 minutes and inlet hot air (233 °C), the drying temperature of wet DG should not exceed the temperature required to evaporate volatile organic solutes present in wet DG. Therefore, volatile organic solutes remain in the protein rich product. These compounds enhanced product palatability.

2.3.2 Processes for Clarification and Particulate Recovery

Where possible solids should be removed from aqueous streams prior to processing dilute solutions, for example concentration, extraction etc. Solid removal can mitigate equipment fouling and enhance heat and mass transfer. For example, in the dairy industry heat exchanger fouling is caused by particulates attaching to equipment surfaces (Visser and Jeurnink, 1997). In addition, the particles obtained from clarification processes might be useful. Examples of using recovered particles can be found in the ethanol industry. Unfermented particles present in stillage after ethanol distillation are recovered by centrifugation and sold as animal feed (Monceaux and Kuehner, 2009). There are several clarification methods that can be applied to large volumes of solution encountered in industrial production including coagulant, centrifugation, filtration, size exclusion, and dissolved gas flotation and AGF.

2.3.2.1 Coagulants

Coagulants are often used to reduce colloids in turbid solutions. Colloids are defined as suspensions of small (1−1,000 nm) particles that remain dispersed and do not readily sediment. Colloid particles frequently collide with solution molecules and remain in continuous random movement (Brownian motion) that is sufficient to overcome sedimentation due to gravity. In addition, colloidal particles are typically charged (usually in unequal quantity) due to absorption of polar groups from gas or ionic solutes on their surfaces. Colloidal particles that carry the same

charge repel each other limiting collisions and aggregation. Aggregation can allow the particles to attain sufficiently large size so that settling occurs (Rand, 1959). Where aggregation is not possible the colloid remains stable (Spellman, 2014a). Coagulation processes can destabilize suspensions of colloidal particulates (Rand, 1959). Coagulation requires compression of the electric double layer that surrounds each particle. Absorption and charge neutralization can occur when the colloid particles absorb a coagulant with the opposite charge. This would lower particle net surface charge. For some zwitterionic colloid particles, adjusting the pH minimizes charge. Coagulation is also possible with interparticle bridging with a coagulant (polymer). Coagulants absorb on available sites of adjacent colloidal particles creating a bridge between them. Particles may also be removed through enmeshment using coagulants that sweep through the solution and entangle colloids as they settle (Crittended *et al*., 2005).

Solutions fermented by yeast, particularly wine are often clarified by the addition of filtration aids called fining agents. Castellari *et al*. (2001) studied commercial fining agents in red wines to remove ochratoxin A. Both inorganic (bentonite, carbons A−C, celite, kaolinite, KFeCN, PVPP, silica gels A−C, and zeolite) and organic solutes (egg albumin, carrageenan, cellulose, gelatin, pectin, and potassium caseinate) were tested. Hydrophobic and positive charge agents (activated carbons, egg albumin, gelatin, potassium caseinate, and silica gel positively charged) were effective for reducing ochratoxin A.

Corn-based stillage solids were dewatered with an anionic polymer of acrylamide-acrylic acid sodium salt copolymer and acrylamide-2-acrylamido-2-methyl-1-propanesulfonic acid sodium salt. Dewatering devices (belt filter press, centrifuge, plate and frame filter press, vacuum filtration unit, recessed chamber press, and screw press) were employed to dewater grain stillage solids (Scheimann, 2009).

Menkhaus *et al*. (2010) studied methods to clarify corn-based stillage and recover water from bioethanol production. Polyelectrolytes [A-1883, A-130, A-140, C-1592, C-4512, C-4516, and N-1986 obtained from Kemira North America (Mobile, AL)] were utilized in this study. The polyelectrolytes were diluted to 50 g/L in water. For each test stillage (10 mL) was mixed with the diluted polyelectrolytes. Concentrations of polyelectrolyte solutions were between 0 and 15 mg polymer/g total soluble solid. After adding, the polyelectrolyte solutions were gently mixed for 5 min and centrifuged at 277 × *g* for 5 min. Supernatant mass and total dissolved and suspended solids were quantified. A-140 treated stillage had the lowest concentration of

suspended solids in the supernatant and highest solid concentration in the cake [0.15% (w/w) of suspended solid and more than 40% (w/w) solids in the cake].

2.3.2.2 Centrifugation

The separation of particles by gravity may be too slow because the particle densities and fluid density are often too similar. In addition, some particles might aggregate by association forces. Centrifugation increases particle sedimentation rate many fold. Centrifugation force has little effect on small particle settling velocity. However, centrifugation can coagulate particles and settle larger particles as the disturbing effects of Brownian motion and free convection currents are less as size increases. Therefore, particles can be sedimented when centrifugation force is applied. The higher the centrifugation force, the smaller the particles that can be precipitated (Geankoplis, 2003c).

Centrifugation can separate unfermented particles (wet cake) from whole stillage to produce wet cake and TS. Wet cake can be utilized as animal feed (Monceaux and Kuehner, 2009). A portion of TS can be returned to the next fermentation batch where it acts as a source of process water and nutrients called backset (Kelsall and Piggot, 2009). TS can be concentrated by evaporation to produce DS (Ingledew *et al*., 2009). Centrifugation of whole stillage has an advantage over filtration, as it is less sensitive to blocking or fouling.

LoCascio and Dunbar (2014) studied corn/other grain TS clarification. TS was mixed with a high molecular weight, charged, anionic GRAS-designated polymer (20-40 ppm) to produce clarified TS and flocculated solids. The clarified TS and flocculated solids were discharged to a gravity filter belt. Clarified TS contained spent yeast. A solid-liquid separator, for example a disc stack centrifuge, can recover spent yeast fractions.

2.3.2.3 Filtration

Filtration can remove solid particles from slurries. The passage of the solid particles is prevented by the filter and allows small particles and solution to pass (Geankoplis, 2003c). The use of microfiltration and ultrafiltration membranes to treat wastewater has been reported by several researchers (Ratanapariyanuch *et al*., 2014). Bento and Fleming (1993) utilized two-step membrane recovery process to recover glycerol and lactic acid from corn-based TS. A portion of the insoluble suspended solids present in TS were smaller than 10 μm. TS was passed through ultrafiltration membranes (pore size no greater than 0.05 μm) at 30–100 °C and 101–507 kPa.

The ultrafiltration permeate (contained soluble solids having molecular weight less than 1 kDa) was passed through a nanofiltration membrane at 30–100 °C and 2.5–7.1 MPa yielding a nanofiltration permeate containing dissolved solids with molecular weights less than 200 Daltons. At least 70% (w/w) of the glycerol and lactic acid passed this membrane. The nanofiltration permeate was then passed through reverse osmosis (RO) membranes (30–60 °C and pressure 4.1–6.1 MPa) with a pore size of less than 50 Daltons. The RO permeate was substantially water while the RO concentrate contained 90% of the lactic acid and glycerol from the nanofiltration permeate.

Arora *et al*. (2009) studied fractionation of corn-based TS using ultrafiltration. Regenerated cellulose membranes with molecular weight cut-offs (MWCO) 10 or 100 kDa were chosen for filtering TS in a stirred cell. The effects of temperature (25 and 75 °C) and transmembrane pressure on permeate flux rate and resistant were evaluated (Arora *et al*., 2010; Arora *et al*., 2009). Larger pore size membranes achieved higher fluxes. Flux increased threefold and fivefold when temperature increased from 25 to 75 °C for membranes of 10 and 100 kDa, respectively. The optimum transmembrane pressures were 207 and 69 kPa, respectively. During filtration, particles accumulated on the membranes and flux decreased. As particle deposits flux decreased. Fouling could be mitigated by seeking optimum operating conditions e.g. flow rate, pressure, temperature etc. (Arora *et al*., 2010).

2.3.2.4 Size Exclusion

Size exclusion media have been utilized to remove suspended colloids or particles (Ginn, 2000). The size exclusion media prevent diffusion of particles larger than a specific pore size between 2 compartments (Kee *et al*., 2012). Only particles that are smaller than the pore size can enter the pore while larger particles cannot access the pore interior (Fallah *et al*., 2012). Therefore size exclusion media retain small particles while larger particles are excluded.

Kalbfuss *et al*. (2007) utilized size exclusion and anion-exchange chromatography in downstream processes to separate human influenza A virus from cell culture constituents. The virus passed through the size exclusion (Sepharose 4 FF packed column) more quickly than smaller impurities present (protein and host cell DNA). Protein was reduced 35% and host cell DNA was reduced 34%. Fractions containing virus from size exclusion column were then passed through an anion exchange column (Sepharose Q XL) eluted with 38.0 g/L of NaCl to remove host cell DNA. It was found that host cell DNA was reduced 67 fold. Overall, a 52% product

yield was attained with a 19 fold reduction of protein and 500 fold host cell reduction. Zheng and Yu (2007) evaluated the distribution of pore size and porosity of aerobic granules utilizing size exclusion chromatography. An aerobic granule from bench-scale sequential batch reactor fed with medium containing glucose was packed in a glass column (2.0 × 40 cm). Acetate (82 Da) and polyethylene glycols (400 Da) were utilized as internal standards for evaluating total porosity and blue dextran (2,000 kDa) was utilized to determined void volume. Polyethylene glycols ranging from 0.4−40 kDa were utilized to test the pore size distribution and exclusion limit. They discovered that granule porosity varied from 68−93%. In addition, polyethylene glycol molecules with molecular weight more than 137 kDa could not penetrate through small-size diameter granules (0.2−0.6 mm). Moreover, polyethylene glycols with molecular weights of 76 kDa and 29 kDa could not pass through the middle-size (0.6−0.9 mm) and large-size (0.9−1.5 mm) granules, respectively. Furthermore, the pores in the granules may clog due to extracellular polymeric substances and this leads to a decrease in porosity.

2.3.2.5 Dissolved Air Flotation and Anoxic Gas Flotation

Dissolved air flotation (DAF) is a scalable method that can remove suspended solids, oil, and other contaminants from a liquid by introducing microscopic air bubbles. Air bubbles in water have negatively charged surfaces and bubbles are repelled by electrostatic forces between them (Edzwald, 2010), similar to electrostatic repulsion of colloids. The repulsive charge may be reduced by interactions between bubbles and positively charged particles. Additionally, other forces include van der Waals, hydrodynamic retardation, and hydrophobic also involve in bubble and particle interaction (Edzwald, 2010). Bubbles enhance coagulation of colloidal materials (Rubio *et al*., 2002) as bubbles attach to contaminants, oil drops, and solids. This attachment increases particulates buoyancy leading the particles to float to the surface when the bubbles are released from the waste stream. They can be mechanically skimmed and taken from the bulk solution. Clarification rates can be up to 97% or more (Pan America Environmental Inc., 2015). Furthermore, energy and maintenance of DAF equipment are lower when compared with centrifuges and filters (Macfarlane *et al*., 2009).

Chadwick and Schroeder (1973) used DAF for removing solids from winery stillage. Suspended solids were reduced 29% with 33% effluent recycle. When recycling ratio increased 50%, it led to 56.5% suspended solid removal. Moreover, 45.2% suspended solid removal without recycling was achieved when adding flocculants (1 mg/L). Van Leeuwen *et al*. (2008)

utilized DAF to recover fungal biomass with corn solid from TS from dry-grind corn milling with fungi. They discovered that corn solids and fungal biomass might be separated easily using DAF. In addition, fungi biomass had high protein and could be utilized in animal feed supplement and pet food. It also improves flavor and feed nutrient value by blending with DDG.

AGF process is similar to DAF. AGF can be used for treating waste containing substantial amounts of grease, oil, and suspended solids (Burke, 1997b). The AGF process uses anoxic gas, which is gas without oxygen. Anoxic gases include CO_2, methane, and nitrogen produced from anaerobic digestion, to concentrate, float, and return bacteria, enzyme, organic acids, protein, and undigested substrate to an anaerobic digester (Burke, 2000b). The advantages of using AGF are 1) Gas flotation is less disruptive for bacterial consortia when compared to centrifugation and cross flow membranes; 2) The operational costs associated with AGF are less when compared to centrifuges or gravity belts. Part of the lower cost is due to decreased polymer use; 3) It does not disturb the bacteria community; and 4) AGF does not introduce toxins to the anaerobic bacteria (Burke, 2000b). In addition, the particles recovered from AGF could be centrifuged or belt pressed for the use of animal feed (Burke, 1997b).

Burke (1997b) reported that when treating French fry processing waste and potato dehydration waste with AGF using pilot plant scale equipment, AGF reduced total chemical oxygen demand and particulate chemical oxygen demand more than 95% with contact reactor volumetric loading rates of 15 kg/m^3/day and processing volume rate 5 kg/m^3/day without chemicals required for influent pH adjustment. Nonetheless, when flow in the AGF system was doubled, the chemical oxygen demand reduction decreased from 95 to 80%. Burke (2000a) utilized anoxic gas to remove inorganic material in anaerobic treatment process. He reported that a portion of organic material was converted into gas by anaerobic bacteria in the anaerobic reactor followed by separation of a portion of anaerobic bacteria and a portion of soluble products. Separated anaerobic bacteria and dissolved inorganic material were recycled into anaerobic reactor. Undissolved inorganic material was separated from anaerobic bacteria, dissolved inorganic solutes, and organic solutes, by differential density separators and hydrocyclones with utilization of dilution water. Therefore, the majority of anaerobic bacteria and organic material were not removed by the AGF system. In addition, anaerobic bacteria, and wholly or partially undigested organic material from soluble products were returned to the second anaerobic reactor for further conversion. Anaerobic bacteria from the second anaerobic

reactor were recycled to the first anaerobic reactor.

2.3.3 Processes for Solute Recovery

Solutes can be recovered using several methods including evaporative concentration and membrane separation (Luo *et al*., 2014).

2.3.3.1 Evaporation/Distillation

Evaporation is normally utilized to separate compounds based on vapour pressure (Geankoplis, 2003e). For evaporation, liquid is heated until it boils. Solvent or volatile solutes are vaporized. Solvent vapour passes by diffusion to a condenser where it is cooled and liquefied. The solute remaining in the heating unit is more concentrated due to solvent removal by evaporation (Geankoplis, 2003a). Distillation may be used to separate various volatile components of a solution. Distribution of these components between aqueous phase and vapour phase is the key for compound separation. The evaporation of liquid phases at their boiling points creates the vapor phase. The separation of components by distillation requires differences in the composition of the aqueous phase and the vapor phase, which is in equilibrium at the boiling point of the liquid (Geankoplis, 2003e).

Xiu and Zeng (2008) stated that 1,3-PD present in fermentation media could be recovered using distillation. The fermentation medium was dewatered using a falling film evaporator to remove volatile acids and water. 1,3-PD recovery could be accomplished using two vacuum rectification columns. The distillation temperature of 1,3-PD is 214 °C at standard pressure but this is reduced to 139 °C under vacuum pressure of 0.095 MPa. However, deproteinization and desalination are required prior to 1,3-PD distillation. After grain fermentation by yeast in the production of ethanol (Sections 2.1.1 and 2.1.2), beer is distilled to recover ethanol in a distillation tower, which has a number of internal components called trays. The tower temperature decreases from the bottom to the top. The ethanol vapor rises and condensed liquid flows down the tower. However, when the mole % of ethanol reaches the azeotrope concentration (95% ethanol in water) the concentration of ethanol cannot be further increased by distillation (Madson, 2009). Consequently, molecular sieves are used to remove water remaining in 95% ethanol to obtain anhydrous ethanol (Swain, 2009).

2.3.3.2 Membrane Separation

The important of membrane separation has increased in chemical technology and broad ranges of membranes are available for various applications (Baker, 2012). Membranes are semipermeable barriers that control the movement of molecules between two phases. The membranes may separate two gas phases, two liquid phases, or a liquid and gas phase (Geankoplis, 2003b). Therefore, one or more components of a mixture is allowed to permeate through membrane freely while other components are rejected (Baker, 2012). Several types of membranes are available and these are largely characterized by the size of the molecules they reject. Reversed osmosis membranes have smaller pores than nanofiltration membranes, which have smaller pores than ultrafiltration membrane while microfiltration membranes have still larger pores (Geankoplis, 2003b). The separation target, within the limitations of a specific application, is the primary consideration in membrane selection (Jirjis and Luque, 2010).

Wu (1988) utilized high pressure RO combined with ultrafiltration to recover clarified TS (supernatant after centrifuging TS) solid from dry-milled corn fractions and ground corn. Cellulose acetate and SEPA-O polysulfone membranes were compared for their efficiency in producing clarified TS from corn. Ultrafiltration was conducted with 1 kDa molecular MWCO membrane at a pressure of 680 kPa. Flow rate of permeate from ultrafiltration of clarified TS from dry-milled corn fractions and clarified TS from ground corn were 12−18 $L/m^2 \times h$ (using cellulose acetate membrane) and 15 $L/m^2 \times h$ (using polysulfone membrane) at ambient temperature, in that order. The permeate from ultrafiltration was passed through an RO system equipped with an SW 30-2521 module with 1.1 m^2 polyamide membrane with 100 Da MWCO. The pressure of RO system was 6.8 MPa and flowrate of RO permeate was 18−26 $L/m^2 \times h$ at ambient temperature. Ultrafiltration permeates had 23−60% of the total nitrogen content and 38−65% of the total solids of clarified TS. In addition, RO permeate contained 0.32−1.3, 0.13−0.41, and 77− 86% of the total nitrogen content, the total solid content, and the total volume of ultrafiltration permeate, respectively. Therefore, 99.6− 99.9 and 99.8− 99.9% of nitrogen and solids, respectively from clarified TS could be recovered. Wu (1990) recovered protein-rich by products from oat-based TS after ethanol distillation. Clarified oat-based TS was fractionated after centrifuging using ultrafiltration and RO. A cellulose acetate membrane with MWCO 1 kDa operated at 680 kPa was utilized for ultrafiltration and a polyamide membrane with MWCO 100 Da operated with 6.8 MPa was used for RO. The flow rate of ultrafiltration and RO were 8.6 and

19 L/m^2 × h, respectively. It was found that 34% of nitrogen and 44% of solid in clarified oat-based TS could pass through the ultrafiltration membrane. Permeate from RO was 75% of total volume and contained 0.22% of total nitrogen, 0.61% of total solid, and 0.072% of total ash of ultrafiltration permeate.

2.3.3.3 Extraction

Extraction processes involve transferring components between 2 liquid phases or absorption on solids or stripping of liquids or solids with a vapour phase (Khoury, 2015). Extraction occurs by differential solubility or interfacial chemical reactions (Pereira *et al*., 2007). Normally a counter current flow column is utilized. In each stage, the mixture of 2 inlet liquid streams occurs in order to reach the equilibrium. The mixture is finally separated into 2 outlet liquid streams. The extraction may require a number of equilibrium stages. Therefore, the mixing vessels may be connected in series in a counter current system. Gravity induced mixing occurs in the extraction column where a heavier liquid flows downward and lighter liquid flows upward when the density of one liquid is higher than the other. Extraction requires mass transfer but can also involve compound separation. After extraction or distillation, other separations might be required for compound recovery. Furthermore, choice of any ancillary apparatus, solvent, the number of stages, and the solvent-to-feed ratio have to be considered in order to design the complete extraction protocol (Khoury, 2015).

Li *et al*. (2009) extracted 1,3-PD from glycerol-based fermentation medium using an aqueous two-phase extraction system composed of hydrophilic solvents and inorganic salts. Extraction with ethanol (46%, v/v) and saturated ammonium sulfate recovered 93.7% of the 1,3-PD with a 4.77 partition coefficient. However, 2,3-butanediol was extracted along with 1,3-PD and acetoin. A maximum selectivity coefficient of 1,3-PD to glycerol (6.0) was achieved and 99.7% of cell mass and 79.0% of protein were removed. Li *et al*. (2011) utilized methanol/phosphate aqueous two-phase system to extract 1,3-PD from a glycerol-based fermentation medium. They found that using 35% (v/v) methanol with a saturated phosphate buffer at pH 10.7 achieved a 38.3% partition coefficient and 98.1% of 1,3-PD was recovered. In addition, 94.7% of the phosphate in lower phase could be recovered when adding 1.5 volume of methanol to the salt rich phase and adjusting pH to 4.5. Moreover, 99.75% of cell mass and 92.4% of protein could be removed from the fermentation medium.

2.4 Brief Introduction to Chapter 3

As described in Chapter 2, TS is a dilute waste stream from ethanol production after ethanol distillation. Normally, ethanol industries evaporate water in TS to produce DS or utilize TS directly as water source for animal feed. W-TS contains a number of organic solutes of which some are valuable compounds e.g. 1,3-PD, acetic acid, and GPC are useful chemicals and intermediate chemicals. These compounds could be recovered/extracted from W-TS or DS. Unfortunately, W-DS and W-TS contain particles remaining after ethanol fermentation. These particles could interfere with mass transfer, foul equipment and interfere with processing. Therefore, a method is required to remove particles from W-TS prior to compound recovery to limit interference.

CHAPTER 3
CLARIFICATION OF WHEAT-BASED DISTILLERS' SOLUBLES AND THIN STILLAGE

3.1 Abstract

Wheat-based thin stillage (W-TS) is a dilute aqueous solution that contains organic compounds and suspended particles. Wheat-based distillers' solubles (W-DS) is syrup produced by evaporation of W-TS. Organic compounds in W-TS such as 1,3-propanediol (1,3-PD), acetic acid, glycerol, glycerophosphorylcholine (GPC), and lactic acid, are materials of commerce and both W-DS and W-TS are potential commercial sources of these compounds. W-TS solutions are dilute and must be concentrated by evaporation or filtration prior to compound recovery. Clarification is an essential early step prior to concentration as solids often foul process equipment and interferes with heat and mass transfer for compound extraction/recovery. W-DS is significantly more stable than W-TS and, therefore, we used diluted W-DS as a starting material for clarification studies. Three clarification strategies were studied including addition of clarification agents, particle exclusion using size-exclusion medium prepared from wheat-based distillers' wet grains (W-DWG) and coagulation by fermentation. Bentonite (0.4%, w/v) was the most effective clarifying agent for diluted W-DS. W-DWG was used as compressible elastic size-exclusion medium to exclude solution particles. Solutions obtained by pressing the spongy solids had lower turbidity than W-TS. Fermentative coagulation employed endemic W-TS fermentation organisms that produced both CO_2 and exopolysaccharides. W-TS particles aggregated, adhered to CO_2 gas bubbles, and floated as TSF progressed. Centrifugation at high speeds did little to clarify diluted W-DS and W-TS and ultrafiltration rates were slow with beer solution and these methods were not investigated further. Turbidity reduction and yield of low turbidity solution by fermentation was much greater than achieved by the other clarification treatments. In addition, fermentation generated separable protein-rich (50%, db) slurries rich in glutamic acid but low in lysine, tryptophan, and sulfur containing amino acids. Tryptic fragments of slurry proteins indicated that *Lactobacillus sp.* contributed major proteins of 40 and 55 kDa.

3.2 Introduction

Wheat-based thin stillage (W-TS) is a coproduct of wheat fermentation for fuel ethanol production. W-TS contains a number of solutes and suspended solids (Ratanapariyanuch, 2009; Ratanapariyanuch *et al*., 2011). Several organic solutes are compounds sold as commercial products such as 1,3-propanediol (1,3-PD), acetic acid, glycerophosphorylcholine (GPC), and lactic acid. The predicted annual global markets for 1,3-PD, acetic acid, and GPC are approximately $776.3 million by 2022 (Business Standard, 2015), $13.31 billion by 2022 (Grand View Research, 2015), and more than $20 billion by 2020 (AC Immune, 2015), respectively. These compounds might be recovered from stillage by a series of unit operations but recovery of W-TS compounds might prove difficult due to hygroscopic solutes (e.g. glycerol and lactic acid) with high boiling points as well as suspended solids. High boiling point and hygroscopic solutes might be reduced or eliminated by two-stage fermentation (TSF) leaving suspended particles that must be managed during compound recovery operations.

Distillers' solubles (DS) and thin stillage (TS) contain solids, 25–28% and approximately 2.5%, respectively (Meredith, 2003). Solids present in corn-based TS or DS could foul process equipment during TS evaporation to produce DS and interfere with heat and mass transfer (Challa, 2015; Zheng, 2013). Moreover, particulates that attach to heat exchanger tube walls could decrease heat transfer efficiency (Putman, 2000; Müller-Steinhagen *et al*., 2011). Furthermore, bacterial biofilms might limit heat transfer (Putman, 2000) as evaporation efficiency decreases in the presence of particulates or viscous materials (Menkhaus *et al*., 2010).

Clarification of W-TS or wheat-based distillers' soluble (W-DS) is a necessary step prior to efficient compound extraction and recovery. TSF products and W-TS include suspended solids some of which are colloidal. Due to charge and particle size, colloids do not settle (Rand, 1959) but can be removed by microfiltration or ultrafiltration (Geankoplis, 2003b). However, colloids and high molecular weight substances can accumulate near membrane surfaces during ultrafiltration to form polarization layers that increase flow resistant (Porter, 1972). Processes or treatments are often applied to, or conducted on, solutions prior to ultrafiltration to prepare them for clarification (Spellman, 2014b). Industrial clarification processes include dissolved air flotation and size-exclusion clarification. The combination of processes used and treatments depends on the nature of material(s) being processed.

TSF and W-TS are turbid solutions that settle slowly. Processes or treatments that break or mitigate colloids are needed as preparative steps prior to separation of dissolved compounds. The objective of this study was to clarify W-TS and its TSF product using three methods: employing clarifying agents, size-exclusion using W-DWG, and fermentative coagulation using endemic bacteria. Centrifugation and ultrafiltration were tested as clarification approaches but were not promising.

3.3 Materials and Methods

3.3.1 Materials

W-DS samples were obtained from Terra Grain Fuels (Regina, SK, Canada) on July 14, 2011 and May 28, 2012; hereafter, called W-DS1 and W-DS2, respectively. W-TS samples collected from Pound-Maker Agventures Ltd. (Lanigan, SK, Canada) on April 2, 2012, October 22, 2012, and March 28, 2013; hereafter, called W-TS1, W-TS2, and W-TS3, respectively. A wheat-based beer (W-B) sample was obtained from Pound-Maker Agventures Ltd. on April 2, 2012. W-DS, W-TS, and W-B samples were stored in 10 L plastic containers at 4 °C until utilized. Clarifying agents utilized in this research followed Castellari *et al*. (2001). Clarifying agents included bentonite (Cat. No. B235-500), κ-carrageenan (Cat. No. 22048), celite 545 (Cat. No. CX0574-1), cellulose from cotton linters (Catalogue No. C6288), gelatin from bovine skin type B (Cat. No. G9391), gelatin from porcine skin type A (Cat. No. G2500), pectin from citrus fruit esterified ≥ 85% (Catalogue No. P9561-25G), pectin from citrus peel esterified ≤ 26% (Cat. No. P9135), GENU® pectin type D slow set z, silica gel 60 particle size 0.040-0.063 mm (Cat. No. 1.11567), and tannic acid (Cat. No. 36410).

3.3.2 Preparation of W-DWG from W-B

W-DWG was obtained from W-B for use as a size-exclusion medium. W-B (17 kg) was passed through a rotary 2-deck screen (LS18S333P3WC, Sweco, Florence, KY, USA) equipped with an upper screen (diameter 40.6 cm, sieve opening size, 234 μm) and a lower screen (diameter 40.6 cm, sieve opening size, 104 μm). The W-DWG (4.85 kg) was retained on the upper screen and W-B solution (11.1 kg) passed through the lower screen with 1.02 kg recovered between the upper and lower screens. The W-DWG was hand-pressed on a USA standard sieve

number 60 (diameter 30.5 cm, sieve opening size, 250 μm, St. Catharines, ON, Canada) to recover 3.38 kg of hand-pressed W-DWG. Approximately 450 g of W-DWG was washed ten times by mixing with reverse osmosis (RO) water, collected on a sieve with diameter 30.5 cm, sieve opening size of 850 μm, and pressed against the screen by hand between washings to remove smaller seed debris and solution.

3.3.3 Clarification using Clarifying Agents

W-DS1 was diluted with RO water (1:3) then centrifuged at 8,671 × *g* at 4 °C for 15 min (Avanti® J-E, Beckman Coulter Canada Inc., Mississauga, ON, Canada) followed by filtration of the supernatant through cotton. The clarifying agent concentrations were varied (0.1, 0.2, 0.3, 0.4, and 0.5%, w/v). Centrifuged cotton-filtered diluted W-DS1 solution (40 mL) and clarifying agent were mixed in 50 mL centrifuge tubes using a Mini Vortexer™ (MV1, IKA-works Inc., Wilmington, NC, USA) then stored at 4 °C for 72 h to allow for particle aggregation and settling. After 72 h, solution from the top of each tube was withdrawn and optical density at 600 nm (OD_{600}) was measured in duplicate with RO water as a blank using a UV-Vis spectrometer (G 10S UV-Vis, Thermo Scientific, Waltham, MA, USA) (Klassen *et al*., 2011). After holding at 4 °C for 72 h, the OD_{600} of centrifuged cotton-filtered diluted W-DS1 solution without treating with clarifying agent was compared with the OD_{600} of diluted W-DS1 solution, without centrifugation and filtration through cotton. Treatments with clarifying agents that produced decreased turbidity were retested with diluted W-DS1 solution without centrifugation and cotton filtration steps. These mixtures were stored at 25 °C for 24 h. Solution turbidity was determined as previously described.

3.3.4 Clarification of W-TS using W-DWG

W-DWG was used as a size-exclusion medium to clarify W-TS. It was hypothesized that the pores in W-DWG could exclude W-TS particles. Small particles could enter the W-DWG pore structure while larger particles would be excluded. W-DWG is sponge-like and it was further hypothesized that pressing the W-DWG bed could release less turbid particle depleted solution from pores.

W-DWG (100 g), obtained from W-B, was mixed with W-TS1 (100 g) in a coffee press that was modified to act as a piston screen press (Figure 3.1). The apparatus was used to apply

Figure 3.1 Piston screen press

uniform pressure to a W-DWG bed. Pressure (0.7 kPa) was applied for 10 min to the W-DWG and solution released was removed with a syringe while pressure was applied. Pressure was increased to 1.0 kPa for 10 min to release additional free solution. This was also recovered by syringe. W-DWG mass was recorded after the second pressing and removal of free solution. Solid remaining after pressing was air-dried and weighed. Solution recovered from pressing was weighed and turbidity was measured as follows. Recovered solutions or W-TS1 (0.05 mL) were diluted with RO water (0.95 mL) and turbidity was measured at 600 nm. Reuse of W-DWG was also tested. W-DWG was prepared and pressing treatments were conducted as previously described. After pressing material with 1.0 kPa, W-TS1 was added to solids in the press to bring the total mass to the original mass (200 g) of W-TS and W-DWG. The sample was then pressed sequentially with 0.7 and 1.0 kPa. Free solution was removed after each pressing. This cycle was repeated three times. Sample masses after pressing and recovered solution masses were recorded and turbidity of solution recovered by pressing was determined as previously described.

3.3.5 Clarification of W-TS using Fermentative Coagulation

TSF produces gas, likely CO_2, based on organisms present in W-TS, e.g. *Lactobacillus panis*. It was observed that particles in W-TS floated in the fermentation media (Figure 3.2) and a portion of the media became less turbid during fermentation. Therefore, it is possible to clarify a portion of the W-TS during TSF by skimming floating material from the fermenter. Fermentation medium was prepared from glycerol (1,600 g), corn sugar (360 g), freeze-dried W-TS2 (200 g) and W-TS3 to produce 20 L of fermentation medium, which was then inoculated with 3 L of starting culture (made from W-DS2), as described in Section 4.3.2.3. Cultures were maintained under anaerobic conditions in 25 L polyethylene fermenters equipped with gas traps. Floating materials, middle layers or bottom layers in fermentation media were subsampled (middle layer and bottom layer were taken 24 h after fermentation ceased without stirring). Floating material was recovered daily to determine effects of slurry removal on W-TS turbidity. Medium pH was adjusted to 5.0 using 1 M NaOH or 1 M HCl with stirring by a Polytron homogenizer (45TE, Bronwill Scientific, Rochester, NY, USA). After adjusting the pH, a sample (50 mL) was collected for analysis of dry matter and nitrogen contents.

Figure 3.2 Floating particles from fermentation

In addition, skimmed particles were analyzed to determine amino acid profile, protein and dry matter contents, and protein molecular weight. Fermentation medium turbidity was determined at 600 nm after skimming solids and adjusting pH as previously described in Section 3.3.4.

3.3.6 Protein Content

Protein content was determined using the Kjeldahl method (AOAC 981.10, 2006) as modified from Ratanapariyanuch *et al*. (2011). Non-protein nitrogen content, contributed by betaine and GPC, was subtracted from total nitrogen content prior to protein content calculations using Equation 3.1. A factor of 5.7 was used to convert nitrogen to protein content (Sosulski and Imafidon, 1990).

$$\%N = (\text{mL of 0.05 N HCl sample} - \text{mL HCl blank}) \times \text{normallity of HCl} \times 14.007 \times \frac{1}{\text{sample weight}} \times 100 \quad (3.1)$$

3.3.7 Moisture Content

Moisture content was determined according to AOAC 950.46 (2006) by oven drying samples to a constant mass at 100 ± 2 °C for 16–18 h as modified from Ratanapariyanuch (2009). The moisture content was calculated using Equation 3.2.

$$\%\text{Moisture} = \frac{\text{weight of sample} - \text{weght of dried sample}}{\text{weight of sample}} \times 100 \quad (3.2)$$

3.3.8 Double Pulse Field Gradient Spin Echo-NMR Spectroscopy

Double pulse field gradient spin echo proton (DPFGSE)-NMR was conducted according to a modified method based on Ratanapariyanuch *et al*. (2011). Samples were centrifuged (Spectrafuge™ 24D, Labnet International, Inc., Edison, NJ, USA) at 9,200 × *g* for 10 min prior to analysis. After centrifugation, supernatant samples were filtered through syringe filters (25 mm syringe filter with 0.45 µm PTFE membrane, VWR International, West Chester, PA, USA). Proton NMR spectra were recorded at 500 MHz (AMX 500-MHz, NMR Bruker, Mississauga, ON, Canada) with 16 scans per spectrum using a DPFGSE-NMR pulse sequence. NMR data collection and analysis were conducted with TopSpin™ 3.2 software (Bruker BioSpin GmbH, Billerica, MA, USA). Deuterium oxide (Cambridge Isotope Laboratories Inc., Andover, MA,

USA) and dimethylformamide (EMD Chemicals Inc., Gibbstown, NJ, USA) were used as solvent and internal standard, respectively.

3.3.9 Electrophoresis and Peptide Mass Fingerprinting

Floating material from TSF from replicate 1 at 148 h fermentation was pressed once through a hydraulic lab press (Carver, Inc. Wabash, IN, USA) at 163 MPa to disrupt bacteria and yeast cells (French and Milner, 1955). The pressed and non-pressed samples were centrifuged at 9,200 × *g* for 10 min (Spectrafuge™ 24D, Labnet International, Inc., Edison, NJ, USA). Supernatant protein contents were determined by a modified Bradford method (1976) using bovine serum albumin as a standard prior to electrophoresis. The identity of supernatant proteins was confirmed by electrophoresis separation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gels were prepared using a 15% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel. Supernatant samples from floating material (20 µg protein) and PageRuler™ Prestained Protein Ladder (12 µL; Fisher Scientific, Fair Lawn, NJ, USA) with a range of 10–170 kDa were applied to separate polyacrylamide gel lanes. Gels were subjected to electrical current in a Mini-Protein Tetra Cell system (BioRad, Richmond, CA, USA) as modified from Laemmli (1970). Electrophoresis operating conditions, gel staining, and destaining followed Ratanapariyanuch *et al*. (2012). SDS-PAGE followed by peptide mass fingerprinting was used to characterize proteins in floating material. Bands with molecular weights of approximately 40 and 55 kDa, derived from disrupted floating material, were cut from the gel for trypsin digestion according to Ratanapariyanuch (2009) and subsequent mass spectral analysis (National Research Council of Canada, Saskatoon, SK, Canada). Proteins were identified by peptide mass fingerprinting using m/z ratios of tryptic peptides. A scan range from *m/z* 700 to 3,200 was utilized to perform the MS survey. Data were processed using Protein Lynx Global Server 2.4 (Waters, Milford, MA, USA). The MS fragment data were searched against National Center for Biotechnology Information (NCBI) database using MASCOT (Matrix Science, London, UK) database search engine (http://www.matrixscience.com).

3.3.10 Amino Acid Profile

Amino acid profile analyses of floating material from 148 h fermentation replicate 1 and 92 h fermentation replicate 2 were conducted according to the Pico-Tag™ method (Waters, Milford,

MA, USA) at POS Bio-Sciences (Saskatoon, SK, Canada).

3.3.11 Statistical Analysis

Duplicate analyses were conducted for each sample. Means comparisons were made by analysis of variance (ANOVA) and Duncan's multiple-range test using SPSS statistical software (SPSS 21.0, IBM Corp., Armonk, NY, USA).

3.4 Results and Discussion

3.4.1 W-TS, W-B, and W-DS Composition

Protein and moisture contents of W-B, W-DS, and W-TS samples varied with batch (Table 3.1). It should be noted that not all nitrogen present in W-B, W-TS, or W-DS is protein nitrogen as these materials contain betaine and GPC. The nitrogen content contributed by these compounds should be subtracted from total nitrogen before calculating true protein content. The protein content of W-DS was higher than W-B. Moisture content of W-TS was higher than that of W-B and W-DS. After yeast fermentation ceases and ethanol is distilled from beer, whole stillage is typically filtered to produce TS and DWG. In most ethanol plants, water in TS is then evaporated to produce DS (Ingledew *et al*., 2009). Consequently, W-DS has lower moisture content and higher protein content than either W-B or W-TS. In addition, W-B had lower moisture content than W-TS due to the presence of wet grains. Organic solutes identified in W-B, W-TS, and W-DS were 1,3-PD, acetic acid, betaine, ethanol, glycerol, GPC, isopropanol, lactic acid, phenethyl alcohol, and succinic acid (Table 3.2). These compounds are products of grain metabolism by yeast and bacteria and results are consistent with those reported by Ratanapariyanuch (2009). As W-B has not been distilled, the ethanol concentration was higher than that of W-DS and W-TS. As would be expected, organic solute concentrations in W-TS were considerably lower than in W-DS. It should be noted that according to the concentration of ethanol and glycerol in W-DS1 sample, it suggested that W-DS1 could have been partially fermented prior to utilize as a raw material.

3.4.2 Effect of Clarifying Agents on W-DS Turbidity

Gelatin and silica gel are positively charged clarifying agents; however, bentonite, cellulose,

Table 3.1 Protein and moisture contents of W-B, W-DS, and W-TS samples

Characteristic (%, w/w)	W-B	W-TS1	W-TS2	W-TS3	W-DS1	W-DS2
Total nitrogen (wb)	0.59 ± 0.01	0.35 ± 0.00	0.62 ± 0.00	0.58 ± 0.00	1.05 ± 0.01	1.71 ± 0.02
Betaine nitrogen[1]	0.012 ± 0.001	0.010 ± 0.000	0.010 ± 0.000	0.008 ± 0.000	0.018 ± 0.000	0.045 ± 0.000
GPC nitrogen[1]	0.005 ± 0.000	0.005 ± 0.000	0.005 ± 0.000	0.004 ± 0.000	0.008 ± 0.000	0.020 ± 0.000
Moisture content	89.6 ± 0.2	93.40 ± 0.00	92.92 ± 0.02	93.40 ± 0.00	88.0 ± 0.1	75.94 ± 0.01
Protein[2] (wb)	3.35 ± 0.06	1.91 ± 0.04	3.45 ± 0.01	3.24 ± 0.00	5.83 ± 0.04	9.38 ± 0.03
Protein[2] (db)	32 ± 1	29.0 ± 0.6	48.70 ± 0.04	49.07 ± 0.09	48.5 ± 0.8	39.0 ± 0.1

Each value is presented as the mean ± standard deviation (SD, $n = 2$).
[1]Nitrogen contributed by GPC and betaine was determined by DPFGSE-NMR.
[2]Nitrogen contributed by these materials to total nitrogen was subtracted prior to calculation of protein content.

Table 3.2 Concentration (g/L) of organic solutes in W-B, W-DS, and W-TS samples

Compounds	W-B	W-TS1	W-TS2	W-TS3	W-DS1	W-DS2
1,3-PD	0.5 ± 0.1	0.16 ± 0.01	0.52 ± 0.01	0.38 ± 0.00	0.00 ± 0.00	2.06 ± 0.01
Acetic acid	0.70 ± 0.02	0.65 ± 0.03	1.10 ± 0.05	1.07 ± 0.02	0.67 ± 0.00	2.35 ± 0.00
Betaine	1.03 ± 0.07	0.83 ± 0.01	0.83 ± 0.01	0.67 ± 0.00	1.53 ± 0.01	3.72 ± 0.00
Ethanol	97.0 ± 1.0	0.04 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	10.74 ± 0.02	0.21 ± 0.01
Glycerol[1]	13.02 ± 0.01	8.7 ± 0.2	8.6 ± 0.2	7.8 ± 0.1	13.02 ± 0.06	28.10 ± 0.30
GPC	1.00 ± 0.00	0.87 ± 0.02	0.98 ± 0.02	0.81 ± 0.01	1.50 ± 0.02	3.79 ± 0.04
Isopropanol	0.3 ± 0.1	0.22 ± 0.00	0.29 ± 0.00	0.26 ± 0.00	0.89 ± 0.01	1.67 ± 0.00
Lactic acid	4.4 ± 0.3	3.12 ± 0.08	3.61 ± 0.04	3.42 ± 0.05	1.46 ± 0.05	5.07 ± 0.08
Phenethyl alcohol	0.36 ± 0.01	0.32 ± 0.01	0.36 ± 0.01	0.30 ± 0.01	0.30 ± 0.10	0.58 ± 0.01
Succinic acid	0.65 ± 0.01	0.63 ± 0.00	0.69 ± 0.01	0.62 ± 0.00	0.50 ± 0.01	1.80 ± 0.01

Each value is presented as the mean ± SD ($n = 2$). [1]The concentration of glycerol in W-B, W-DS, and W-TS samples may be affected by the presence of interfering resonances from carbohydrate and protein.

pectin, and tannic acid are negatively charged (Castellari *et al*., 2001). It was found that clarifying agents significantly affected centrifuged cotton-filtered diluted W-DS1 solution turbidity (Table 3.3, $P < 0.05$). Mixtures of diluted W-DS1 solution with bentonite had lower turbidity compared to control solutions without additives and diluted W-DS1 solutions after adding other clarifying agents.

In addition, 0.4% (w/v) bentonite produced solutions with the lowest turbidity achieved with a clarifying agent. Bentonite is a negatively charged layered aluminum silicate (Castellari *et al*., 2001). It has been utilized as clarifying agent due to its high specific area and high ion-exchange capacity (Kim, 2003). Bentonite particles are small and readily form colloidal solutions. When bentonite was suspended in water, its volume increased due to hydration (Olin and Peterson, 1937). Bentonite is easily suspended and flocculates when in contact with salts and other ionized substances. At a critical concentration, bentonite entrains and absorbs sediments and suspended solids. However, when concentration was increased to 0.5% (w/v), turbidity increased. This might be due to formation of bentonite colloids. Particle electrostatic repulsion in solution can overcome flocculation when excess clarifying agent is added (Menkhaus et al., 2010). However, the complex stillage matrix that contains residual ethanol, oils, proteins, salts, etc. should be taken into consideration as this complex solution could influence clarifying agent efficacy and particle interactions. In addition, low stillage pH could lead to formation of large cluster in the presence of anionic clarifying agents (Menkhaus et al., 2010). Moreover, zeta potential measurements might assist in understanding interactions between clarifying agents and W-DS or W-TS particles.

The turbidity of diluted W-DS1 solution without centrifugation and filtration (cotton) was similar to centrifuged cotton-filtered diluted W-DS1 solution. This indicated that centrifugation and cotton filtration were not necessary prior to adding the clarifying agents and also indicative that centrifugation would not be suitable as a clarification treatment. Bentonite (0.4%, w/v) effects on diluted W-DS1 solution clarification (without centrifugation or filtration prior to mixing with bentonite) were retested when holding the mixture at 25 °C for 24 h. It was confirmed that bentonite reduced diluted W-DS1 solution turbidity (Table 3.4).

In addition, bentonite clarification did not require holding mixtures at low temperature. This may help to reduce processing costs and space for a bentonite based clarification process.

Table 3.3 Turbidity of diluted W-DS1 solution when mixing with clarifying agents at different concentrations after storing at 4 °C for 72 h

Clarifying agent	Clarifying agent concentration (%, w/v)	OD_{600}[1]
Diluted W-DS1 solution	0.0	0.528 ± 0.001^{x}
Centrifuged and cotton-filtered diluted W-DS1 solution	0.0	0.534 ± 0.004^{y}
Bentonite	0.1	0.471 ± 0.001^{r}
	0.2	0.469 ± 0.000^{qr}
	0.3	0.454 ± 0.001^{hij}
	0.4	0.341 ± 0.001^{a}
	0.5	0.385 ± 0.000^{b}
κ-Carrageenan	0.1	0.481 ± 0.001^{s}
	0.2	0.465 ± 0.003^{opq}
	0.3	0.464 ± 0.002^{nop}
	0.4	0.471 ± 0.001^{r}
	0.5	0.460 ± 0.001^{lmn}
Celite 545	0.1	0.478 ± 0.001^{s}
	0.2	0.462 ± 0.001^{lmno}
	0.3	0.459 ± 0.001^{klm}
	0.4	0.453 ± 0.000^{ghi}
	0.5	0.447 ± 0.001^{f}
Cellulose	0.1	0.486 ± 0.001^{t}
	0.2	0.488 ± 0.001^{tu}
	0.3	0.489 ± 0.002^{tu}
	0.4	0.491 ± 0.003^{u}
	0.5	0.496 ± 0.003^{v}
Gelatin from bovine skin type B	0.1	0.478 ± 0.002^{s}
	0.2	0.469 ± 0.001^{qr}
	0.3	0.460 ± 0.001^{lmn}
	0.4	0.460 ± 0.002^{lmn}
	0.5	0.458 ± 0.001^{jkl}
Gelatin from porcine skin type A	0.1	0.459 ± 0.004^{kl}
	0.2	0.452 ± 0.002^{fghi}
	0.3	0.451 ± 0.002^{efgh}
	0.4	0.453 ± 0.001^{ghi}
	0.5	0.448 ± 0.002^{ef}
Pectin from citrus fruit esterified ≥ 85%	0.1	0.463 ± 0.001^{mnop}
	0.2	0.479 ± 0.000^{s}
	0.3	0.490 ± 0.001^{tu}
	0.4	0.506 ± 0.001^{w}
	0.5	0.528 ± 0.001^{x}

[1] OD_{600} was determined in duplicate and the values are presented as the mean ± SD (n = 2). Values followed by different letters are significantly different ($P < 0.05$).

Table 3.3 Turbidity of diluted W-DS1 solution when mixing with clarifying agents at different concentrations after storing at 4 °C for 72 h (cont'd)

Clarifying agent	Clarifying agent concentration (%, w/v)	OD_{600}[1]
Pectin from citrus fruit esterified ≤ 26%	0.1	0.413 ± 0.000^{c}
	0.2	0.469 ± 0.000^{qr}
	0.3	0.538 ± 0.000^{z}
	0.4	0.563 ± 0.001^{C}
	0.5	0.556 ± 0.003^{B}
GENU® pectin type D slow set z	0.1	0.443 ± 0.001^{d}
	0.2	0.499 ± 0.001^{v}
	0.3	0.545 ± 0.000^{A}
	0.4	0.599 ± 0.001^{D}
	0.5	0.639 ± 0.004^{E}
Silica gel 60	0.1	0.466 ± 0.003^{pq}
	0.2	0.455 ± 0.001^{ijk}
	0.3	0.453 ± 0.001^{ghi}
	0.4	0.449 ± 0.001^{efg}
	0.5	0.449 ± 0.001^{efg}
Tannic acid	0.1	2.203 ± 0.002^{F}
	0.2	2.271 ± 0.003^{H}
	0.3	2.324 ± 0.006^{I}
	0.4	2.356 ± 0.001^{J}
	0.5	2.267 ± 0.004^{G}

[1]OD_{600} was determined in duplicate and the values are presented as the mean ± SD (n = 2). Values followed by different letters are significantly different ($P < 0.05$).

Table 3.4 Turbidity of diluted W-DS1 solution when mixing with 0.4% (w/v) bentonite after storing at 25 °C for 24 h

Concentration of bentonite (%, w/v)	OD_{600}[1]
0.0	1.135 ± 0.001^{a}
0.4	0.140 ± 0.001^{b}

[1]OD_{600} was determined in duplicate and the values are presented as the mean ± SD (n = 2). Values followed by different letters are significantly different ($P < 0.05$).

3.4.3 Effect of W-DWG on W-TS Clarification

A piston screen press was used to press a mixture of W-DWG and W-TS at a pressure of 0.7 kPa and free solution released was removed with a syringe. Pressure was then increased to 1.0 kPa and free solution was again removed. The turbidity of free solution released by pressing at 1.0 kPa was lower than that of free solution released at 0.7 kPa (Table 3.5). Therefore, W-DWG acted as an elastic size-exclusion medium. When W-TS was in contact with W-DWG, particles present in W-TS, which had smaller sizes than W-DWG pore size and solutes likely entered W-DWG pores while larger particles were rejected (Fallah *et al*., 2012). Free solution released at 0.7 kPa was released from outside W-DWG and was enriched with larger particles compared to pressure 1.0 kPa. However, solution released at pressures 0.7 to 1.0 kPa came from pores that excluded larger W-TS particles and, therefore, had lower turbidity compared to W-TS.

This novel approach confirmed that W-DWG was substantially elastic and could be repeatedly pressed and imbibed with fresh W-TS (Table 3.6). Industrial scale equipment, e.g. belt presses, are available for continuous pressing and recycling of W-DWG to clarify W-TS or TSF products. Although W-DWG was effective in W-TS clarification, pore size and pore structure during and after pressing and after reuse should be investigated.

Transmission electron microscopy might be used to explore pore size and pore structure. Increased reuse cycles should also be tested as W-DWG might fatigue and decompose with more than 3 cycles in a clarification process. It should be noted that in a single cycle 4.4 g of free solution was recovered when pressing between 0.7 and 1.0 kPa (Table 3.5). Relatively small amounts of free solution were also recovered (2.5 to 10.5 g) in pressing 200 g of mixture (W-DWG and W-TS) between 0.7 and 1.0 kPa where the W-DWG was recycled. The recovery of solution might be greater if an aspiration system was used in conjunction with a belt press but without aspiration the yield is between 1.3 and 5.3 % of mixture (W-DWG and W-TS) mass. Therefore, it would be necessary to cycle the W-DWG many times to clarify all W-TS available.

3.4.4 Effect of Fermentative Coagulation on W-TS Clarification

Anoxic gas flotation (AGF) processes occur where anoxic gas produced by anaerobic digestion coalesce and float particles in solution during fermentation. Anoxic gases can be used to concentrate, float, and return bacteria, enzymes, organic acids, proteins, and undigested substrates to an anaerobic digester to increase its efficiency (Burke, 2000b).

Table 3.5 The turbidity of W-TS after pressing when washed W-DWG was used as a plastic size-exclusion medium to clarify W-TS

Rep	Mass blank piston screen press (g)	Mass of wet grain (g)	Force of pressing (kPa)	Mass of solution after pressing (g)	OD_{600}[1]
1	338.3	ND[2]	ND	ND	1.578 ± 0.001
		ND	0.7	12.1	0.796 ± 0.004
		177.76	1.0	8.4	0.360 ± 0.001
2	338.3	ND	ND	ND	1.583 ± 0.004
		ND	0.7	12.9	0.76 ± 0.02
		181.11	1.0	4.4	0.381 ± 0.001

[1]OD_{600} was determined in duplicate and the values are presented as the mean ± SD (n = 2).
[2]ND = not determined.

Table 3.6 The turbidity of W-TS after pressing when washed W-DWG was reused three times as a plastic size-exclusion medium to clarify W-TS[1]

Rep	Pressing	Mass of wet grain (g)	Force of pressing (kPa)	Mass of solution after pressing (g)	OD_{600}[2]
1	1st	ND[3]	0.7	13.9	0.565 ± 0.001
		179.3	1.0	4.8	0.380 ± 0.003
	2nd	ND	0.7	11.0	0.758 ± 0.004
		178.8	1.0	9. 7	0.214 ± 0.006
	3rd	ND	0.7	8.3	0.783 ± 0.002
		180.7	1.0	10.5	0.207 ± 0.006
2	1st	ND	0.7	13.7	0.733 ± 0.000
		182.0	1.0	2.5	0.452 ± 0.009
	2nd	ND	0.7	13.8	0.757 ± 0.002
		180.3	1.0	5.2	0.306 ± 0.001
	3rd	ND	0.7	11.1	0.818 ± 0.004
		181.0	1.0	7.6	0.329 ± 0.002

[1]W-TS sample had an OD_{600} of 1.581 ± 0.002 at 600 nm.
[2]OD_{600} was determined in duplicate and the values are presented as the mean ± SD (n = 2).
[3]ND = not determined.

L. panis PM1B glucose utilization pathways produced CO_2, an anoxic gas, during fermentation (Kang *et al*., 2014b). In our investigation we observed that particles floated to the fermentation medium surface during TSF. Furthermore, floating material increased as fermentation progressed and the amount of floating material decreased when fermentation ceased (Table 3.7 and Appendix B Table B.1). Therefore, TSF might be used as a novel approach for clarifying TSF solution.

The mechanism of fermentative coagulation during TSF of W-TS has not been reported previously but anoxic gas formation might aid solid aggregation. Furthermore, exopolysaccharides (EPSs) might also affect colloid stability and help to clarify W-TS media as TSF progresses. *L. casei* CG11 grown in basal minimum medium (Cerning *et al*., 1994) produces EPS. EPS is also formed in sour dough by lactic acid-producing bacteria including *L. sanfranciscensis* (Hammes and Gänzle, 1997; De Vuyst and Degeest, 1999) while *L. plantarum* EP56 also produces EPS (Tallon *et al*., 2003). Therefore, it is possible that *Lactobacillus* species in W-TS produce EPS during TSF. It is not known whether EPS and AGF act synergistically in W-TS clarification. The genome of *L. panis* PM1B was sequenced (Tanaka, T., personal communication, September 24, 2015) and genes encoding EPS pathways are present. The sequence data of *L. panis* PM1B and other *Lactobacillus* in stillage could assist in determining the nature of EPS. According to annotated genome sequences in the Rapid Annotation using Subsystem Technology database (Reaney *et al*., 2013), the *L. panis* PM1B genome contains sequences encoding pathway activators and enzymes involved in EPS production including; EPS biosynthesis transcriptional activator EpsA, glycosyl transferase, group 2 family protein, manganese-dependent protein-tyrosine phosphatase, tyrosine-protein kinase EpsD, and tyrosine-protein kinase transmembrane modulator EpsC, undecaprenyl-phosphate galactosephosphotransferase. These sequences are likely linked to EPS synthesis. However, EPS produced from *L. panis* PM1B have not been characterized. Gene sequences with substantial homology were identified in *L. acidophilus* NCFM, *L. casei* BL23, *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, *L. fermentum* IFO 3956, *L. gasseri* ATCC 33323, *L. helveticus* DPC 4571, *L. johnsonii* NCC 533, *L. plantarum* WCFS1, *L. reuteri* F275, and *L. reuteri* JCM 1112.

Importantly, floating material dry matter and protein contents were higher than the dry matter and protein contents of W-TS. As fermentation progressed, floating material dry matter and protein contents increased until fermentation ceased, meanwhile, dry matter and protein

Table 3.7 Protein and moisture contents of W-TS fermentation fractions

Fermentation time (h)	Sample	Mass of material (kg)	Dry matter (%, w/w)	Moisture (%, w/w, wb)	Protein[1] (%, w/w, wb)	Protein[1] (%, w/w, db)	OD_{600}
0	W-TS	ND	7.38 ± 0.02	92.62 ± 0.02	2.59 ± 0.07	35.1 ± 0.9	1.56 ± 0.03
23	F	0.04	10.46 ± 0.07	89.54 ± 0.07	4.3 ± 0.2	41 ± 2	ND
	M	ND	7.03 ± 0.01	92.97 ± 0.01	2.53 ± 0.01	36.1 ± 0.1	1.48 ± 0.02
47	F	0.22	7.21 ± 0.01	92.79 ± 0.01	3.0 ± 0.1	41 ± 2	ND
	M	ND	6.64 ± 0.00	93.36 ± 0.00	2.55 ± 0.00	38.40 ± 0.00	1.46 ± 0.01
73	F	1.19	13.68 ± 0.04	86.32 ± 0.04	6.40 ± 0.04	46.8 ± 0.1	ND
	M	ND	5.68 ± 0.02	94.32 ± 0.02	1.89 ± 0.02	33.4 ± 0.2	1.26 ± 0.01
92	F	1.08	13.73 ± 0.08	86.27 ± 0.08	6.2 ± 0.2	45 ± 2	ND
	M	ND	5.51 ± 0.02	94.49 ± 0.02	1.59 ± 0.02	28.8 ± 0.3	1.06 ± 0.04
119	F	0.92	12.6 ± 0.2	87.4 ± 0.2	5.49 ± 0.09	44 ± 1	ND
	M	ND	5.23 ± 0.09	94.77 ± 0.09	1.44 ± 0.01	27.5 ± 0.4	0.974 ± 0.004
148	F	0.77	10.72 ± 0.03	89.28 ± 0.03	4.7 ± 0.3	44 ± 2	ND
	M	ND	5.11 ± 0.05	94.89 ± 0.05	1.28 ± 0.09	25 ± 2	0.85 ± 0.04
167	F	0.43	8.94 ± 0.00	91.06 ± 0.00	3.52 ± 0.06	39.4 ± 0.6	ND
	M	ND	5.04 ± 0.01	94.96 ± 0.01	1.17 ± 0.00	23.12 ± 0.03	0.777 ± 0.008
191	F	0.08	9.14 ± 0.07	90.86 ± 0.07	3.51 ± 0.09	38 ± 1	ND
	M	13.18	4.11 ± 0.01	95.89 ± 0.01	0.53 ± 0.02	12.9 ± 0.6	0.140 ± 0.001
	B	6.00	7.22 ± 0.00	92.78 ± 0.00	2.85 ± 0.02	39.5 ± 0.2	1.52 ± 0.02

Each value except weight of material is presented as the mean ± SD (n = 2).
Abbreviation: F = Floating material; M = middle layer; B = bottom layer; ND = not determined.
[1]Protein content of floating material and bottom layer was not corrected for the nitrogen content of GPC and betaine as no data was available for this material.

contents of largely clarified medium decreased (Table 3.7 and Appendix Table B.1). The mass of floating material increased until fermentation stopped. Interestingly, skimming floating material from the fermentation medium daily led to decreased turbidity of the remaining fermentation medium (Table 3.7 and Appendix B Table B.1).

Anoxic gas (CO_2) was evolved before nutrients, like added glucose, were exhausted. As fermentation progressed, CO_2 evolution stopped, and floating material precipitated. In addition, floating material removal from the fermentation medium led to decreased turbidity. The major amino acid present in floating material proteins was glutamic acid (Table 3.8), which is also the major amino acid found in wheat gluten (Woychik *et al*., 1961). However, the concentrations of lysine, tryptophan, and sulfur containing amino acids (cysteine and methionine) were low in the floating material proteins.

The protein contents of floating material, measured by dye binding, before and after hydraulic disruption, were 0.503 and 0.750 μg/μL, respectively. Apparent increased protein suggested that the hydraulic press disrupted bacterial or yeast cell walls and grain material resulting in better access of dye to proteins. Supernatant samples of floating material were loaded onto SDS-PAGE gel at 20 μg protein per lane (Figures 3.3A and 3.3B). SDS-PAGE gel patterns were similar in samples with or without hydraulic press disruption. Protein bands observed by SDS-PAGE of disrupted samples provided better resolution than samples that were not disrupted. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight analysis of tryptic fragments from bands cut from the SDS-PAGE gels indicated that a band observed at 40 kDa was highly similar to glyceraldehyde 3-phosphate dehydrogenase from *L. plantarum* JDM1 while the band at 55 kDa was consistent with enolase from *L. helveticus* according to the NCBInr amino acid sequence database. This suggested that *L. panis* strain PM1B was not the source of floating material proteins.

The discovery that floating material in TSF was protein rich and readily recovered led us to consider using this material as a feed ingredient. The price retained by protein-rich sources for inclusion in animal feed is comparatively higher than for feed ingredients with lower protein content. Therefore, a process that concentrates wheat protein would be desirable for producing improved feed substances. Floating fraction protein is, at least, in part, from lactobacilli thus it might be a useful probiotic ingredient (Pedersen *et al*., 2004). In addition, solution clarified by floatation might be suitable for compound extraction/recovery; the original goal of this research,

Table 3.8 Amino acid composition (g/100 g of protein) of floating material

Amino acid	Floating material 148 h of fermentation replicate 1	Floating material 92 h of fermentation replicate 2
Aspartic acid	5.71	5.07
Glutamic acid	29.52	30.05
Serine	5.46	5.27
Glycine	4.22	3.90
Histidine	4.71	3.12
Arginine	7.69	5.27
Threonine	3.72	2.93
Alanine	4.71	4.29
Proline	11.41	10.93
Tyrosine	4.71	4.10
Valine	5.21	5.07
Methionine	1.98	1.95
Cystine	2.98	2.73
Isoleucine	4.47	4.29
Leucine	7.94	8.00
Phenylalanine	5.46	5.46
Lysine	3.97	4.10
Tryptophan	1.49	1.37

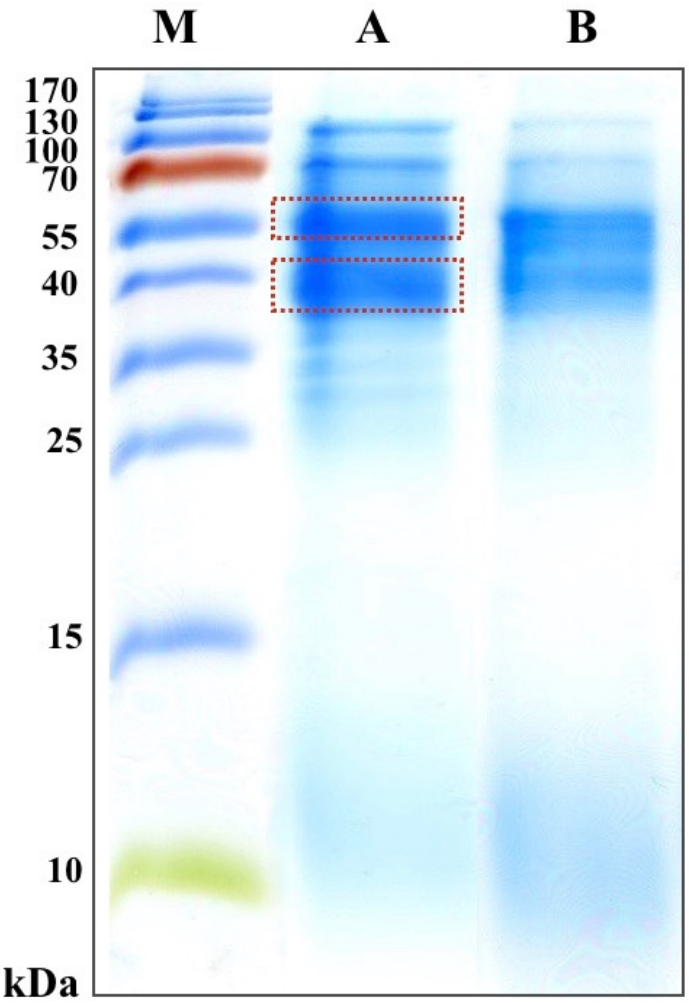

Figure 3.3 SDS-PAGE of floating material with (A) and without (B) disruption by pressing at 163 MPa

and a portion of the water might be recycled for use as backset in ethanol production.

3.4.5 Comparing Clarification Methods

Five clarification approaches were surveyed including the use of clarifying agents, the use of W-DWG as a size-exclusion medium, fermentative coagulation, centrifugation and membrane filtration. Centrifugation, clarifying agents, membrane filtration, and size exclusion medium are commonly utilize for industrial clarification. Fermentative coagulation and the use of W-DWG are novel methods for W-TS clarification. Centrifugation did not reduce relative water content markedly. Membrane filtration produced solutions with very low turbidity but transmembrane flux was slow and the amount of solution collected before flux became insignificant was not sufficient to warrant further investigation of membrane separations. The use of membranes in conjunction with TSF will be discussed in Chapter 5. Each approach achieved some clarification. Bentonite addition reduced diluted W-DS solution turbidity by 35% (Figure 3.4). Use of W-DWG as a size-exclusion medium reduced the turbidity of W-TS 74% and fermentation coagulation lowered turbidity 92% (Figure 3.5). Industrial implementation of the three methods varies in complexity. Using additives requires the ability to mix solution with the additive in a uniform fashion and then apply sufficient settling time for solid aggregation. The use of clarifying agents is inherently simple and widely practised. However, additives were unimpressive in their ability to produce clarified diluted W-DS solution. The use of W-DWG as a size-exclusion medium is an interesting possibility and this would require the use of continuous belt presses or similar devices to apply pressure to release solution held in pores. Aspiration would be required to recover free solution that was released. Such an approach is more mechanically complex than alternative approaches but large-scale belt pressing devices are available that can aspirate free solution released by pressing. The amount of solution recovered from stillage as a portion of W-DWG was small and clarification of large amounts of stillage would require many passes of DWG to clarify a substantial portion of the stillage. It is uncertain that W-DWG would tolerate repeated compression cycles or if greater pressure could be used to recover more solution for each extraction. Centrifugation and ultrafiltration were not promising and implementation of these approaches using industrial equipment is complex and requires expensive equipment. Fermentative coagulation is the most interesting approach for clarification of W-TS as the slurry remaining from clarification is a concentrated protein. Also separation

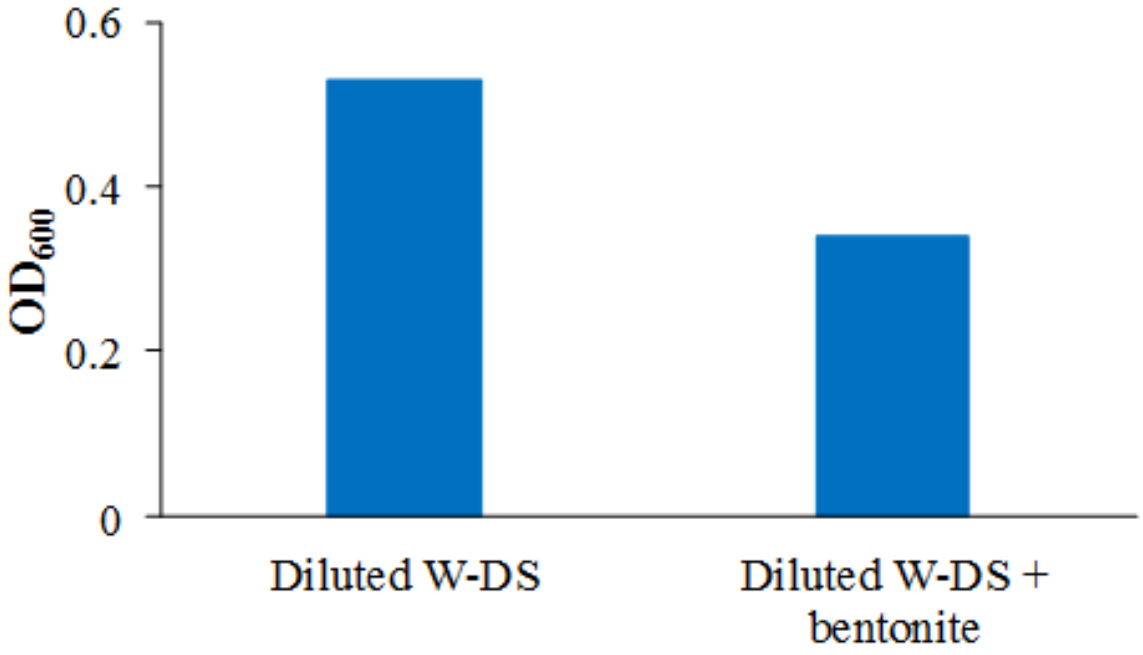

Figure 3.4 Clarification efficiency using additives (bentonite)

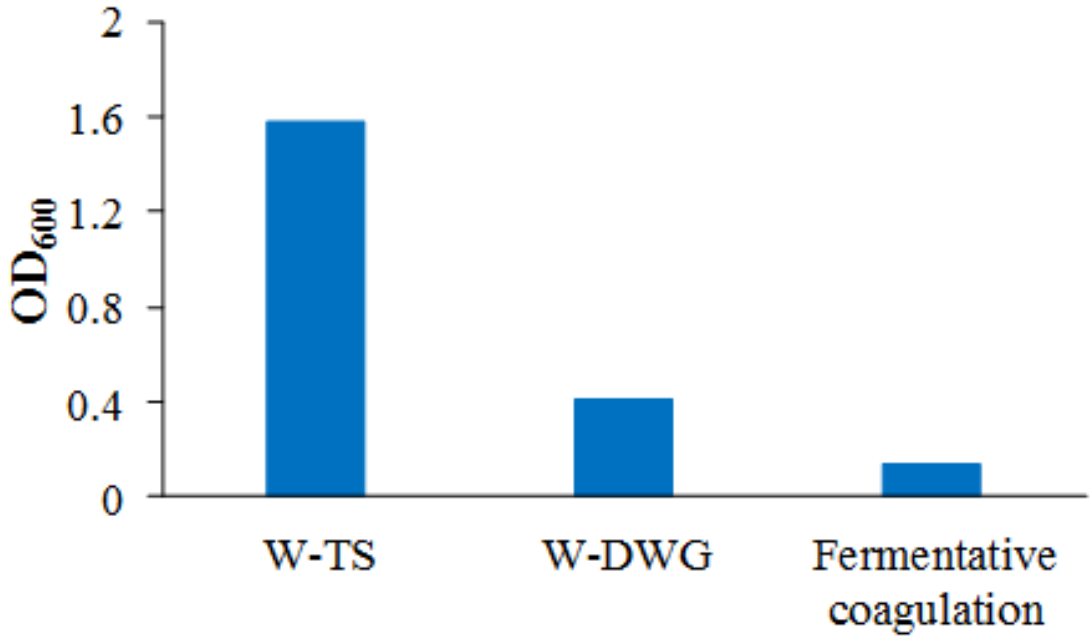

Figure 3.5 Comparison of clarification efficiency using size-exclusion with W-DWG and fermentative coagulation

devices for concentration of slurries are readily available. Recovery of this fraction presented in Chapter 6.

3.5 Conclusion

W-DS and W-TS are complex suspensions with organic solutes that contain organic solutes suited for use as chemicals and intermediates, e.g., 1,3-PD, acetic acid, and GPC. However, before extracting and recovering solutes, particles present in TSF products should be removed to mitigate equipment fouling. Three clarification processes were studied: the use of clarifying agents, size-exclusion with W-DWG, and fermentative coagulation. Fermentative coagulation was the most efficient method for clarifying W-TS. The turbidity of W-TS was decreased by 92%. Protein contents of solids floating at the fermenter surface during fermentative coagulation were 47–50% (w/w, db). This approach to clarification provides an opportunity to recover particles for use as protein rich animal feeds.

3.6 Brief Introduction to Chapter 4

As described in Chapter 3, particles present in W-TS should be removed prior to extraction and recovery of organic solutes in order to prevent equipment fouling that interferes with heat and mass transfer. Anoxic gas and EPS produced during TSF successfully coagulated the particulate matter in W-TS and clarified a large portion of the W-TS. TSF was the most effective process for particle removal. The first unit operation in separation of 1,3-PD, acetic acid, and GPC from TSF could be the clarification of W-TS using fermentation with *Lactobacillus*. A specific advantage of this process is the generation of a high protein of slurry I from TSF that might have utility as a protein source for animal feed. As TSF *Lactobacillus* naturally clarifies W-TS, the next goal is to improve the production of 1,3-PD and acetic acid. The cost of isolation of these compounds is related to their concentration in process streams. The greater the yield of 1,3-PD, acetic acid, GPC, and protein concentrate from TSF the more valuable the process.

CHAPTER 4
CONVERSION OF THIN STILLAGE COMPOUNDS USING ENDEMIC BACTERIA AUGMENTED WITH *LACTOBACILLUS PANIS* PM1B

4.1 Abstract

A consortium of organisms endemic in wheat-based thin stillage (W-TS) obtained from a commercial ethanol production facility converts glycerol to 1,3-propanediol (1,3-PD) and lactic acid to acetic acid. In this study, we sought to improve conditions for accumulation of 1,3-PD and acetic acid for later studies of industrial isolation of these compounds from two-stage fermentation (TSF). Occasionally stillage fermentation proceeded slowly but an inoculum of *Lactobacillus panis* PM1B augmented both fermentation rate and extent (data not shown). Effects of adding glycerol metabolites (1,3-PD and 3-hydroxypropionaldehyde: 3-HPA), and micro-nutrients were investigated. Additionally, culture conditions including agitation, pH adjustment, and substrate substitution were also studied. Fermentation rate and product yield were enhanced by adjusting media pH to 5 daily, adding glucose and glycerol (molar ratio 0.1:1), adding freeze-dried W-TS, and adding vitamins (B2, B3, and B12). 1,3-PD and 3-HPA did not inhibit 1,3-PD accumulation during fermentation. Moreover, agitation did not improve fermentation rate or extent. Corn sugar, a minimally refined glucose rich substrate, was a suitable substitute for glucose. A process requiring an inexpensive carbon source (corn sugar) and minimal agitation is ideal for low-cost industrial production of 1,3-PD. Fermentation was performed at both 20 and 150 L, with 1,3-PD production of 2% (w/v) (i.e., 20 g/L) being routinely achieved or exceeded.

4.2 Introduction

Thin stillage (TS) is a liquid coproduct of the bioethanol industry. Wheat-based thin stillage (W-TS) contains organic solutes from bacteria, plant, and yeast metabolism as well as protein, and salts. Glycerol is a major W-TS organic solute (Ratanapariyanuch, 2009; Ratanapariyanuch *et al*., 2011) and W-TS is a potential commercial source. However, crude glycerol is inexpensive (Tan *et al*., 2013) and purification of glycerol from W-TS may not be economically feasible as it is relatively dilute. Recently, Reaney *et al.* (2013) reported that a bacterial strain, *Lactobacillus panis* PM1B, selected from W-TS, converted glycerol to 1,3-propanediol (1,3-PD) and lactic acid to acetic acid. 1,3-PD is potentially more valuable than glycerol and, thus, it might prove more economically viable to recover this compound from W-TS than glycerol. Similarly, lactic acid is converted to acetic acid by the metabolic action of lactobacilli in stillage. The acetic acid boiling point is lower than that of lactic acid while the 1,3-PD boiling point is lower than that of glycerol. Therefore, distillation could potentially be used to recover 1,3-PD and acetic acid from W-TS residues.

It is recognized that W-TS likely contains many strains of bacteria other than *L. panis* PM1B that might also produce 1,3-PD. Other bacteria have metabolic pathways for conversion of glycerol to 1,3-PD including *L. diolivorans*, *L. reuteri*, *Clostridium butyricum* DSM 5431, *C. butyricum* CNCM 1211, *C. butyricum* VPI 3266, *Klebsiella oxytoca* M5al, *K. pneumoniae* ATCC 15380, *K. pneumoniae* M5al (Pflügl *et al*., 2012; Tobajas *et al*., 2009; Biebl *et al*., 1992; Himmi *et al*., 1999; Saint-Amans *et al*., 1994; Yang *et al*., 2007; Tag, 1990; Cheng *et al*., 2007). While some bacterial cultures can accumulate 1,3-PD ranging from 13.0 to 84.5 g/L a portion of these organisms are potentially pathogenic and might also produce toxins. Moreover, other bacteria are fastidious or strict anaerobes, requiring rigorously controlled fermentation conditions and/or costly nutrients.

Previous studies of *L. panis* PM1B mediated conversion of glycerol and lactic acid to 1,3-PD and acetic acid, respectively, were conducted primarily in de Man, Rogosa and Sharpe (MRS) medium and variants of MRS (Grahame *et al*., 2013; Kang *et al*., 2013b; Kang *et al*., 2014a; Khan *et al*., 2013). Controlled fermentation conditions utilized by Kang *et al*. (2013b) were important in elucidating biochemical pathways but may not reflect fermentation in stillage media. Compositions of both inorganic and organic solutes in MRS media tested by Kang *et al*.

(Grahame *et al*., 2013; Kang *et al.,* 2014a) are unlike W-TS solutes. Many MRS organic solutes could significantly modify bacterial metabolism and metabolic responses. Media, like MRS, are supplemented with extracts, detergent, proteins, essential salts, and vitamins. Each of these supplements contributes to medium suitability for growth of a wide range of microorganisms while sodium acetate is added to increase medium specificity for lactobacilli. Growth of lactobacilli and enhancement of metabolic pathways that convert glycerol and lactic acid in W-TS based media may require similar supplementation but there has been no attempt to improve W-TS medium for 1,3-PD and acetic acid production.

Glucose is the typical MRS medium carbon source though this may be substituted (Grahame *et al*., 2013) and MRS has other potential carbon sources (De Man *et al*., 1960). Stillage also has an array of potential carbon sources (Kim *et al*., 2008) with glucose levels being low due to prior metabolism by yeast. Carbon sources used in *Lactobacillus* fermentations can have a large effect on metabolism. It is not certain that growth and metabolism studies of organisms in MRS can reveal useful information regarding organism metabolism and growth in W-TS. In addition, other W-TS solutes might influence glycerol metabolism. Active bacterial metabolic pathways expressed when growing on MRS might differ greatly from pathways of the same organisms grown on W-TS. Additionally, stillage contains colloids and particulate solids that could have multiple effects on fermentation that are not comparable in MRS media. Colloids and particles can impede mass transfer and slow metabolic conversion of substrates. Conversely, stillage solids may form bacterial habitats, enabling biofilm formation that might improve metabolic efficiency.

The impact of solids present in W-TS on *Lactobacillus* growth and metabolism during fermentation is not known. Fermentation conducted in this research used corn sugar, largely α-D-glucose (U.S. Food and Drug Administration, 2016) instead of glucose, as had been used by Grahame *et al*. (2013). The price of refined glucose is too high to be economical for an industrial carbon source while corn sugar is more economical for industrial fermentation. Ethanol production facilities are large and in these commercial fermentation environments, it may be neither practical nor economical to impose sterile conditions on millions of liters of solution each day. Therefore, we chose to study fermentation in the presence of endemic cultures. Fermentation with endemic cultures is widely used for food production. Kim chi, sauerkraut, sourdough breads, and yoghurt are all examples (Nout, 1992). Our research describes

improvement of fermentation conditions for efficient production of 1,3-PD and acetic acid including glucose concentration, freeze-dried W-TS, vitamins, pH adjustment, and substrate substitution. In addition, fermentation scale was increased in stages from less than 100 mL to 150 L.

4.3 Materials and Methods

W-DS sample was obtained from Terra Grain Fuels (Regina, SK, Canada) on May 26, 2012 and stored at 4 °C). W-TS samples were obtained from Pound-Maker Agventures Ltd. (Lanigan, SK, Canada) and stored at 4 °C. W-TS samples collected on October 22, 2012, March 28, 2013, August 13, 2013, and November 13, 2013 were called W-TS1, W-TS2, W-TS3, and W-TS4, respectively. TS is normally concentrated to distillers' solubles (DS) by evaporation (Monceaux and Kuehner, 2009). W-DS was more stable than W-TS and, therefore, was used as a concentrate that could be diluted for specific studies where the same medium was needed over a long period of time. While W-DS is similar in composition to W-TS, heating during evaporation may degrade labile nutrients and remove volatile substances. *L. panis* PM1B was isolated previously by Khan *et al*. (2013) and Reaney *et al*. (2013) and available through the International Depository Authority of Canada; accession number 180310-01.

4.3.1 Analytical Materials

4.3.1.1 Protein Content

The Kjeldahl method (AOAC 981.10) was used to determine sample nitrogen content (Section 3.3.6). Protein content was calculated from nitrogen content after subtracting nitrogen contributed from non-protein material, betaine and glycerophosphorylcholine (GPC) (Ratanapariyanuch *et al*., 2011) then multiplying by a conversion factor of 5.7 (Sosulski and Imafidon, 1990).

4.3.1.2 Moisture Content

Moisture content was determined as previously described in the Section 3.3.7.

4.3.1.3 DPFGSE-NMR Spectrometry

DPFGSE-NMR was conducted to follow fermentation progress according to Ratanapariyanuch *et al.* (2011) (Section 3.3.8).

4.3.1.4 *Lactobacillus* Enumeration

Colony forming units (CFU) of *Lactobacillus* present in fermentation media were determined using the drop plate technique (Kirk *et al*., 2005) with *Lactobacillus* Heteroferm screening broth containing 2% agar. The agar plates were incubated for 72 h in a candle jar at 37 °C. Colonies that appeared after incubation were counted to determine CFU/mL (Kirk *et al*., 2005).

4.3.1.5 Glucose Content

Samples were centrifuged at 9,200 × *g* for 10 min and supernatant samples were filtered through 0.45 µm syringe filters prior to analysis. Filtrates were diluted with 0.5 M phosphate buffer (pH 8) to prepare a range of glucose concentrations. Glucose concentrations of diluted filtrates (10 µL) were determined using a glucose hexokinase assay kit following the manufacturer's guidelines.

4.3.2 Experimental Procedures

4.3.2.1 Fermentation Improvement

L. panis was a predominant endemic microorganism present in W-TS in Chapter 5. It is expected that the addition of *L. panis* PM1B would accelerate establishment 1,3-PD producing organisms. Therefore, all cultures were inoculated with *L. panis* PM1B under non-sterile conditions as described below. *L. panis* PM1B grown in double strength skim milk medium was regrown on W-DS agar (500 mL W-DS, 500 mL water, 8 g yeast extract, 4 g glucose, pH 5.1 ± 0.1 adjusted with 1 M NaOH, 15 g agar, autoclaved at 121 °C for 15 min). Agar plates were incubated at 37 °C in a candle jar for 72 h. *L. panis* PM1B colonies were chosen to inoculate starter cultures to supplement endemic W-TS organisms for 50 mL fermentation scale studies of the effects of pH, glucose concentration, freeze-dried W-TS, 1,3-PD, vitamins, and agitation on growth and media solutes. In subsequent studies, *L. panis* PM1B in double strength skim milk (25 µL) was inoculated in filtered sterilized (0.22 µm) 25% W-DS containing 1.1 M of glycerol (25 mL) in a 25 mL sterilized centrifuge tube. The tube was incubated at 37 °C for 72 h. After incubation, tube contents were divided into sterile 2 mL micro-centrifuge tubes and stored at −80 °C as culture stock. *L. panis* PM1B stock was grown on W-DS agar. Agar plates were incubated at 37 °C in a candle jar for 72 h. *L. panis* PM1B colonies were selected to inoculate starter

cultures for studies of the effects of corn sugar, 3-HPA, and pH and for 20 L fermentations. *L. panis* PM1B was used as an inoculum to supplement endemic W-TS organisms.

For 50 mL fermentations (the smallest scale non-sterile culture), W-DS was diluted to 25% W-DS and centrifuged at 7,000 × *g* for 15 min at 4 °C. A dilution 1:3 with water was selected as this essentially reconstitutes the W-DS to a similar concentration of compounds found in W-TS. After centrifugation, supernatant samples were filtered through cotton. Hereafter, these samples are called "clarified 25% W-DS". Clarified 25% W-DS was mixed with glycerol to a final concentration of 0.9 M and the solution (50 mL) was added to 50 mL sterile centrifuge tubes after which media were pasteurized by holding for 15 sec at 72 °C. The tube and contents were cooled prior to inoculation. Media were inoculated with 2−3 colonies of *L. panis* PM1B from the W-DS agar plates and incubated at 37 °C for 48 h (Figure 4.1). Inoculant for 20 L scale fermentations was prepared in six bottles (500 mL) nearly filled with pasteurized clarified 25% W-DS and 0.9 M glycerol. After cooling, pasteurized media were inoculated with *L. panis* PM1B and incubated at 37 °C for 72 h (Figure 4.1).

4.3.2.2 Fermentation in a 50 mL Fermenter

W-TS1 medium was mixed with 50 mL of starting culture to a final volume of 350 mL and divided into 50 mL sterilized centrifuged tubes (50 mL/tube).

4.3.2.2.1 Effect of pH

The tubes were incubated at 37 °C (Figure 4.1). The pH of media (excluding controls) was adjusted to their respective pH from 5.0−10.0 daily after approximately 46 h using 1 M NaOH and 1 M HCl. *Lactobacillus* populations were enumerated at 0 h. After approximately 46 h of incubation, fermentations were sampled daily and subjected to NMR analysis.

4.3.2.2.2 Effect of Glucose Concentration

Glucose was added to achieve final concentrations of 0.1, 0.2, 0.3, and 0.4 M and glycerol was added to final concentration 1 M. Cultures were incubated at 37 °C. After approximately 46 h, medium pH was adjusted to 5.0 and pH was readjusted to 5.0 every 24 h thereafter. When fermentation ceased, 1 g of glucose was added into 0.1 and 0.2 M of glucose treatments at 139 h of fermentation in an attempt to restart fermentation. Lactobacilli were enumerated as described above at 0 h. Fermentation media were sampled daily from each tube for analyses of organic solutes by NMR.

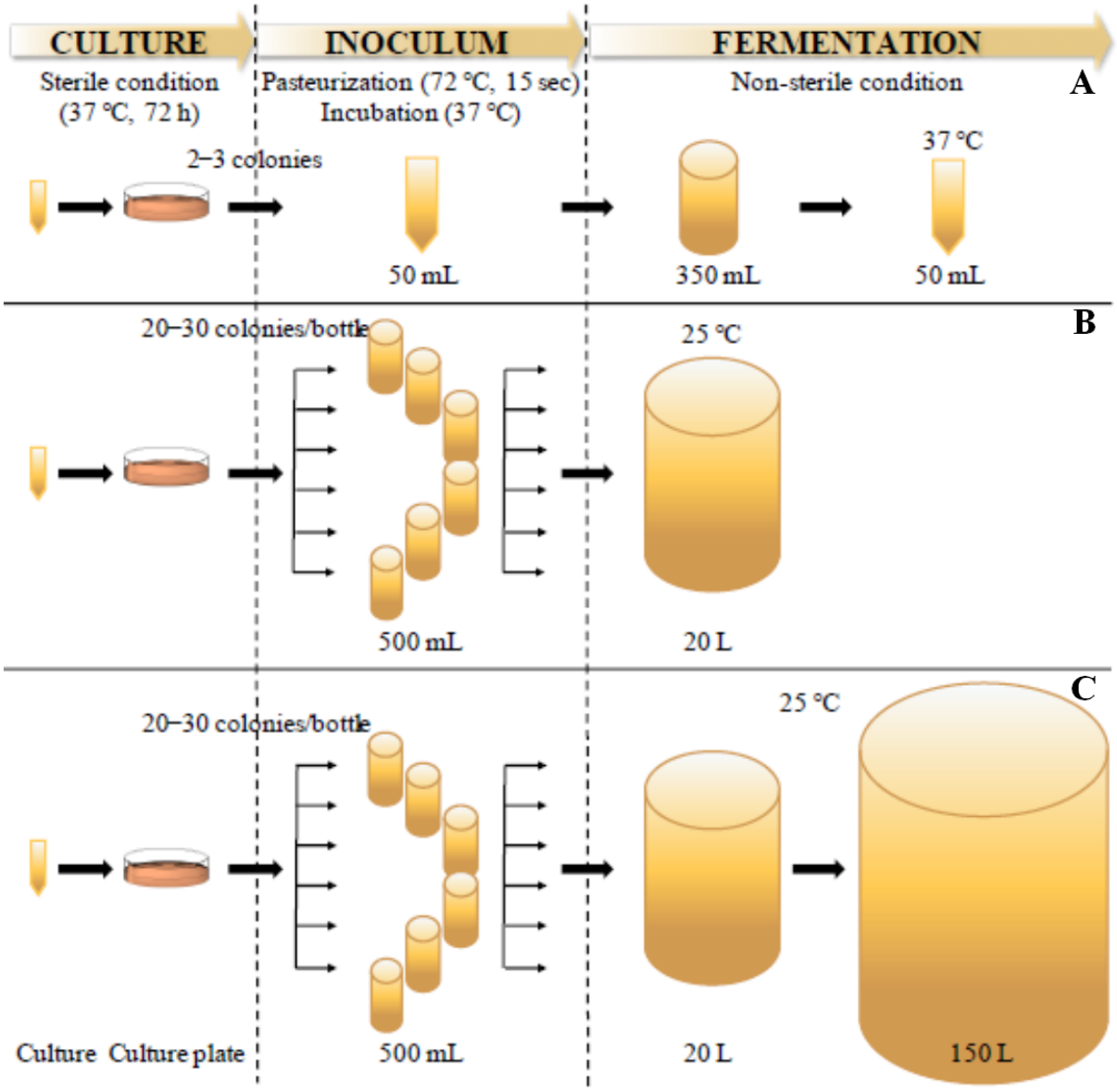

Figure 4.1 Flow chart for inocula preparation

4.3.2.2.3 Effect of Freeze-dried W-TS, 1,3-PD, and Vitamins

In prior work, pH 5, glycerol (1 M), and glucose (0.1 M) aided fermentation. Therefore, media were adjusted to these improved conditions for further work. It was hypothesized that organic solutes present in W-TS might act as micro-nutrients and aid metabolism and microbial growth. Furthermore, organic solutes might be heat labile. A concentrate, prepared to ensure retention of potentially heat labile organic solutes of W-TS, was made by freeze-drying W-TS (Stoppering Tray Dryer, 7948040, Labconco Inc., Kansas City, MO, USA). W-TS media were modified by addition of additives and a range of compounds then accumulation of 1,3-PD and acetic acid was assessed and compared with a W-TS control medium [glucose (0.1 M) and glycerol (1 M)] with added 1,3-PD (1.0 g). Media were also modified by adding freeze-dried W-TS1 (0.5 g) or vitamins (B2, B3, and B12, 0.5 mg of each). After inoculation (approximately 46 h), medium pH was adjusted daily to 5.0 using either 1 M NaOH or 1 M HCl. Cultures were incubated at 37 °C. Lactobacilli were enumerated as described above at 0 h while glucose content and NMR analyses were conducted daily. After fermentation ceased (190 h), glycerol and lactic acid conversion in control media was no longer observed. In an attempt to restart fermentation, freeze-dried W-TS1 (0.5 g) was added to media supplemented with freeze dried W-TS and control conditions. In addition, vitamins (0.5 mg each) were added to produce vitamin enriched culture media. The fermentation was continued until 233 h to determine effects of adding freeze-dried W-TS and vitamins.

4.3.2.2.4 Effect of Agitation and Freeze-dried W-TS

A temperature-controlled benchtop shaker (Excella E24, New Brunswick Scientific Co., Inc., Edison, NJ, USA) was used for temperature controlled agitation and incubation. Freeze-dried W-TS1 (0.5 g) was introduced to agitated (200 rpm) and static cultures (0.1 M glucose: 1.0 M glycerol) at 0 h. Medium pH was adjusted to 5.0 daily after approximately 46 h of fermentation except in condition 1. In that culture, medium pH was adjusted to 5.0 at 0 h. This culture was agitated and freeze-dried W-TS was included. The tubes were incubated at 37 °C. Lactobacilli were enumerated and organic solutes were determined from daily samples.

4.3.2.2.5 Effect of 3-hydroxypropionaldehyde (3-HPA), Corn Sugar, and pH

Four media were prepared to determine effects of 3-HPA, corn sugar, and pH on 1,3-PD accumulation. A control medium (0.1 M glucose: 1 M glycerol + 0.5 g freeze-dried W-TS1), a

similar medium with 3-HPA (0.3 g) added, a third medium similar to the control but substituting glucose with corn sugar (to produce 0.1 M glucose), and a control adjusted to pH 5.0 daily starting at inoculation. For other media, pH was adjusted to 5.0 daily after incubation for approximately 46 h. Cultures were incubated at 37 °C. W-TS solution was sampled daily for enumeration of lactobacilli and NMR analyses.

4.3.2.3 Fermentation in a 25 L Vessel

Freeze-dried W-TS (2.48 kg) was prepared by drying W-TS2 at temperatures below 80 °C from 30.84 kg to 7.58 kg in a rotary evaporator (R-220) equipped with a vacuum controller (V-800), all from Büchi Labortechnik AG (Flawil, Switzerland) then drying the concentrate in a freeze dryer as described previously. Freeze-dried W-TS was used in media preparation as described below. A 25 L polyethylene pail and lid equipped with a gas trap (Wine Kitz, Saskatoon, SK, Canada) was used as a fermenter (Figure 4.2). W-TS2 was mixed with freeze-dried W-TS2 (200 g) and starting culture (total volume 3 L; Figure 4.1). Corn sugar (approximately 0.1 M glucose) and glycerol (final concentration 1 M) were also added. The fermentation medium volume was adjusted to 20 L and incubated at 25 ± 2 °C. Medium pH was adjusted to pH 5.0 daily. Media were stirred daily using a Polytron homogenizer (45TE, Bronwill Scientific, Rochester, NY, USA) during pH adjustment and sampled afterwards. Glucose content, *Lactobacillus* enumeration, and NMR analyses of organic solutes were conducted daily using these samples.

4.3.2.4 Fermentation in a 210 L Vessel

W-TS2 was mixed with corn sugar and glycerol to final concentration 0.1 and 1.0 M, respectively. In addition, freeze-dried W-TS2 (200 g), and starting culture (3 L, Figure 4.1) were added. The medium volume was adjusted to 20 L in 25 L fermenters, which were then incubated at 25 ± 2 °C. Fermentation media were stirred using a Polytron homogenizer (described above) while adjusting pH to 5.0. After adjusting pH, fermentation medium were sampled for glucose content, *Lactobacillus* enumeration and organic solute analysis. Fermentation continued until the 1,3-PD concentration reached approximately 6 and 15 g/L for replicate 1 and 2, respectively. After, this culture was used to inoculate a 210 L fermenter equipped with a gas trap (Figure 4.3). W-TS3 was utilized as fermentation medium and the experiment was repeated with W-TS4 for two 150 L fermentations. W-TS was mixed with corn sugar (2.7 kg), freeze-dried W-TS2 (1.5 kg), and

Figure 4.2 25 L wine pail equipped with a gas trap

Figure 4.3 210 L polyethylene drum equipped with a gas trap

glycerol (12 kg). Fermentation medium volume was adjusted with W-TS to 150 L. The fermentation medium was mixed daily using a hand pump (SP-280P-V, Standard Pump Inc., Duluth, GA, USA) at a setting of 5 for 10 min while adjusting pH to 5.0 during mixing. Fermentation medium was sampled daily after adjusting pH to enumerate bacteria, and determine organic solute content.

4.3.3 Statistical Analysis

Effects of agitation, glucose concentration, inhibitors, micro-nutrients, pH, and substrate substitution, were determined. The simultaneous study of all of these treatments was impractical with available facilities. The process was iterative, with each successive round of fermentations building upon prior findings. Conditions in iterations that produced the highest concentration of 1,3-PD were used as the starting point for subsequent iterations. Nonetheless, additional response surface studies will be required to determine optimum conditions for large-scale fermentation. The largest fermentation scale possible using available equipment was 150 L. The nearest ethanol plant is 150 km from the research facility and it was impractical to transport more than 300 kg of stillage to the laboratory in a rental vehicle. Due to constraints of working with large samples, 20 and 150 L fermentations were done in duplicate only.

4.4 Results and Discussion

4.4.1 Characterization of W-TS

The protein and moisture contents and organic solutes varied among W-TS batches (Tables 4.1 and 4.2). Not all nitrogen present in W-DS and W-TS arises from protein. W-DS and W-TS contain betaine and GPC, which contribute to total nitrogen. Therefore, nitrogen content was corrected before calculating protein content. As expected, W-DS protein content was higher than that of W-TS while moisture content was lower. DS had solid contents from 25 to 28% (Meredith, 2003) while Liu and Barrows (2013) noted that corn-based TS and DS had moisture contents of 90−95 and 50−75%, respectively. 1,3-PD, acetic acid, betaine, ethanol, glycerol, GPC, isopropanol, lactic acid, phenethyl alcohol, and succinic acid were present in both W-DS and W-TS samples (Table 4.2) (Ratanapariyanuch, 2009; Ratanapariyanuch *et al*., 2011). In addition, non-volatile compounds (e.g. glycerol) present in W-DS were more concentrated than

Table 4.1 Protein and moisture contents of W-DS and W-TS samples

Characteristic (%, w/w)	W-DS	W-TS1	W-TS2	W-TS3	W-TS4
Total nitrogen	1.71 ± 0.01	0.62 ± 0.00	0.58 ± 0.00	0.64 ± 0.04	0.52 ± 0.00
Betaine nitrogen[1]	0.045 ± 0.000	0.010 ± 0.000	0.008 ± 0.000	0.011 ± 0.000	0.010 ± 0.000
GPC nitrogen[1]	0.020 ± 0.000	0.005 ± 0.000	0.004 ± 0.000	0.006 ± 0.000	0.005 ± 0.000
Moisture	75.94 ± 0.00	92.92 ± 0.02	93.40 ± 0.00	91.64 ± 0.00	92.3 ± 0.2
Protein[2] (wb)	9.38 ± 0.03	3.45 ± 0.01	3.24 ± 0.00	3.6 ± 0.2	2.89 ± 0.01
Protein[2] (db)	39.0 ± 0.1	48.70 ± 0.04	49.07 ± 0.09	43 ± 2.0	37.8 ± 0.9

Each value is presented as the mean ± standard deviation (SD, $n = 2$).
[1]Nitrogen contributed by GPC and betaine was determined by DPFGSE-NMR.
[2]Nitrogen contributed by these materials to total nitrogen was subtracted prior to calculation of protein content.
The pH of W-TS was pHs 3.4–3.9.

Table 4.2 Concentration (g/L) of organic solutes of W-DS and W-TS samples

Component	W-DS	W-TS1	W-TS2	W-TS3	W-TS4
1,3-PD	2.06 ± 0.01	0.52 ± 0.01	0.38 ± 0.00	0.69 ± 0.02	0.72 ± 0.00
Acetic acid	2.35 ± 0.00	1.10 ± 0.05	1.07 ± 0.02	1.44 ± 0.05	1.53 ± 0.00
Betaine	3.72 ± 0.00	0.83 ± 0.01	0.67 ± 0.00	0.88 ± 0.02	0.83 ± 0.01
Ethanol	0.21 ± 0.00	0.13 ± 0.00	0.13 ± 0.00	0.26 ± 0.01	0.24 ± 0.02
Glycerol[1]	28.1 ± 0.3	8.6 ± 0.2	7.8 ± 0.1	10.2 ± 0.4	8.73 ± 0.02
GPC	3.79 ± 0.04	0.98 ± 0.02	0.81 ± 0.01	1.10 ± 0.03	0.90 ± 0.02
Isopropanol	1.67 ± 0.00	0.29 ± 0.00	0.26 ± 0.00	0.37 ± 0.01	0.35 ± 0.01
Lactic acid	5.07 ± 0.08	3.61 ± 0.04	3.42 ± 0.05	4.84 ± 0.04	4.93 ± 0.00
Phenethyl alcohol	0.58 ± 0.01	0.36 ± 0.01	0.30 ± 0.01	0.40 ± 0.01	0.36 ± 0.00
Succinic acid	1.80 ± 0.01	0.69 ± 0.01	0.62 ± 0.00	0.73 ± 0.03	0.77 ± 0.01

Each value is presented as the mean ± SD ($n = 2$).
[1]The concentration of glycerol in W-DS and W-TS samples may be affected by the presence of interfering resonances from carbohydrate and protein.

in W-TS.

4.4.2 Fermentation Improvement

Fermentation media pH was adjusted from 5.0−10.0. The growth of microorganisms present in W-TS supplemented with *L. panis* PM1B lowered the pH of W-TS media presumably due to production of lactic and acetic acids (Figures 4.4C and 4.4D) during normal metabolism of lactobacilli (data not shown). The highest accumulation of both 1,3-PD and acetic acid were achieved when culture pH was adjusted to 5 (Figures 4.4B and 4.4D). Moreover, glycerol conversion was nearly complete with little glycerol remaining in the media at the end of the fermentation period at pH 5, while residual glycerol was present in cultures adjusted to pH 9, 10, and the unadjusted control (pH 3.4–3.9; Figure 4.4A). The highest residual glycerol and lowest 1,3-PD accumulation were noted after fermentation at pH 10.0 (Figures 4.4A and 4.4B). These results contrast with observations of Grahame *et al.* (2013). They noted that when *L. panis* PM1B was grown in MRS medium at initial pHs 9.0 and 10.0, 1,3-PD accumulation was faster and that cell density increased more slowly compared to other pH conditions tested. These responses may have been related to differences between MRS media and W-TS used or the use of endemic bacteria and not a pure strain of *L. panis* PM1B. The observation of slow accumulation of 1,3-PD at higher pH is in agreement with Khan *et al*. (2013) who noted that the optimum pH for growth of *L. panis* PM1B was 4.5. The growth of *L. panis* PM1B was slower at higher pH in the present study and that of Khan *et al*. (2013). It should be noted however that Grahame *et al*. (2013) did not maintain the pH throughout their experiment, but only performed an initial pH adjustment, and pH likely decreased through fermentation. Additionally, they utilized modified versions of MRS medium as a base to prepare specific fermentation media rather than W-TS as was done in this present study. This choice may have led to differences observed with the findings reported herein. Based on results of our initial test of optimal pH for improved production accumulation of 1,3-PD (Figure 4.4), subsequent experiments in this study use a pH 5.0 and 1 M of glycerol were used to ensure an adequate supply of glycerol for conversion and higher accumulation of 1,3-PD during fermentation.

Grahame *et al*. (2013) found that glucose was the best carbon source tested for growth of *L. panis* PM1B in variations of MRS media that included alternate sugars. Here, we tested the effects of glucose added to W-TS on glycerol and lactic acid metabolism during fermentation in

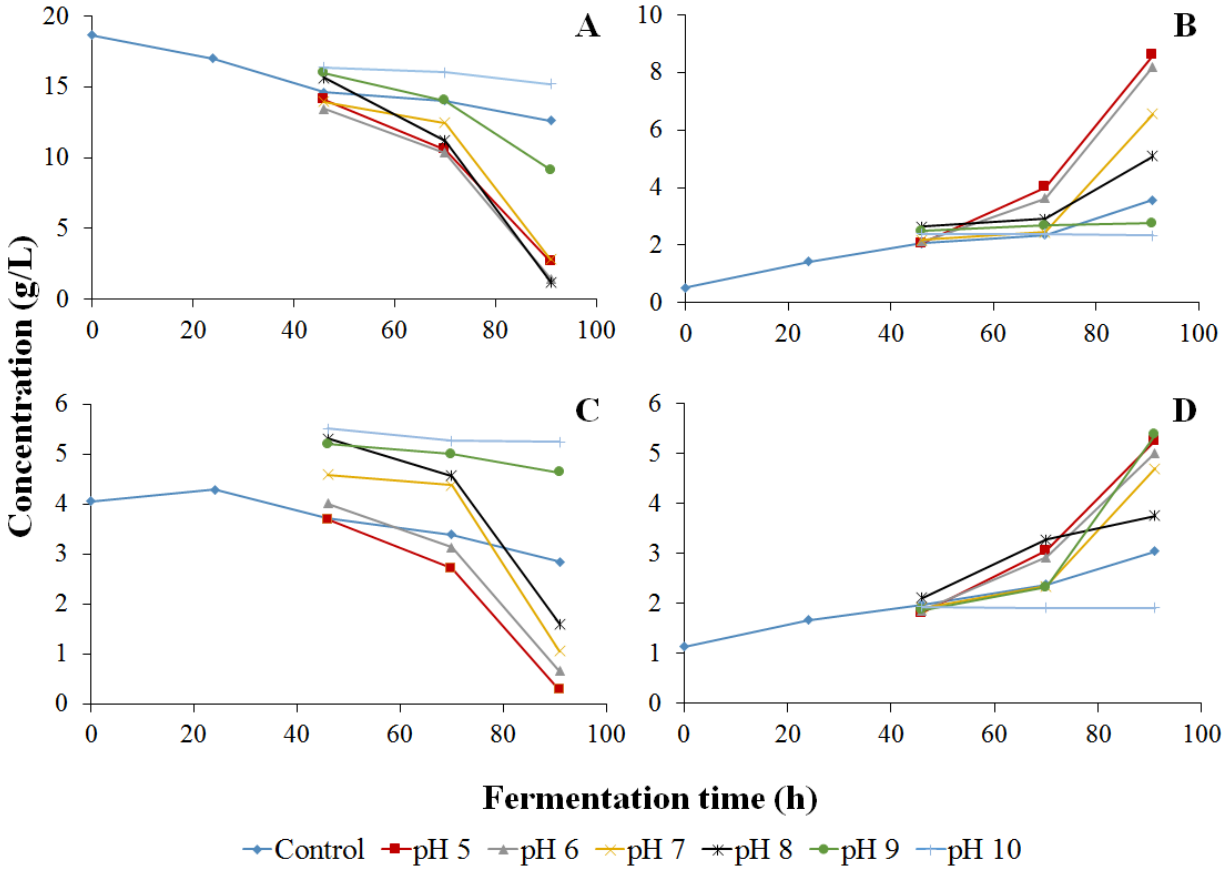

Figure 4.4 The effect of pH on concentration of A) glycerol, B) 1,3-PD, C) lactic acid, D) acetic acid, and E) glucose during fermentation with endemic bacterial populations augmented with *L. panis* PM1 inoculum

comparison to a control without added glucose. Concentrations up to 0.4 M glucose were tested in W-TS. The number of lactobacilli in the W-TS fermentation solution was 7×10^7 CFU/mL at 0 h. A glucose concentration of 0.1 M combined with 1 M of glycerol at pH 5 resulted in the greatest accumulation of 1,3-PD and acetic acid when compared with other glucose concentrations tested (Figures 4.5B and 4.5D). In addition, it was observed that accumulation of 1,3-PD began when glucose in the fermentation medium was nearly depleted (Figures 4.5B and 4.5E). This result was in agreement with Grahame *et al*. (2013) who reported that under anaerobic conditions 1,3-PD production did not occur until glucose was mostly depleted in late log to early stationary phase. According to Khan *et al*. (2013), 1,3-PD production utilized $NADH+H^+$ from glucose consumption to regenerate NAD^+ for continued glucose metabolisms under anaerobic condition. However, when glucose was depleted, 1,3-PD accumulation still occurred. $NADH+H^+$ for 1,3-PD accumulation after glucose depletion could arise from a pathway that converts lactic acid to acetic acid and, thereby, generates $NADH+H^+$ (Figure 2.3). Nevertheless, it should be noted that energy would be involved in converting lactic acid to acetic acid. Higher starting concentrations of glucose (0.2, 0.3, and 0.4 M) did not yield greater concentrations of 1,3-PD or more effective use of glycerol (Figure 4.5). We hypothesize that the glucose may have been in excess for the reaction, and may have increased osmotic pressure leading to cell stress and lower bacterial growth rates and 1,3-PD production (Figure 4.5B). In addition, it was observed that adding 1 g of glucose at 139 h of fermentation showed no improvement for 1,3-PD accumulation, though glucose concentration decreased and lactic acid was produced (Figure 4.5). These findings suggest that other essential nutrients e.g. amino acids and minerals for microorganisms might be depleted. Therefore, fermentation could not be restarted. Based on these findings, 0.1 M glucose was used for subsequent fermentations in this study.

W-DS is prepared by distillation of W-TS, which requires extensive exposure to elevated temperatures that could inactivate important labile nutrients. Freeze-dried W-TS was tested as a potential source of concentrated nutrients to replace W-DS to determine if heat labile nutrients were lost in preparing W-DS. According to Pflügl *et al*. (2012), vitamins (B2, B3, and B12) might enhance 1,3-PD accumulation in fermentation media because vitamin B2 and B3 are the compositions in yeast extract which involve in vitamin B12 biosynthesis pathway (Pflügl *et al*., 2012).

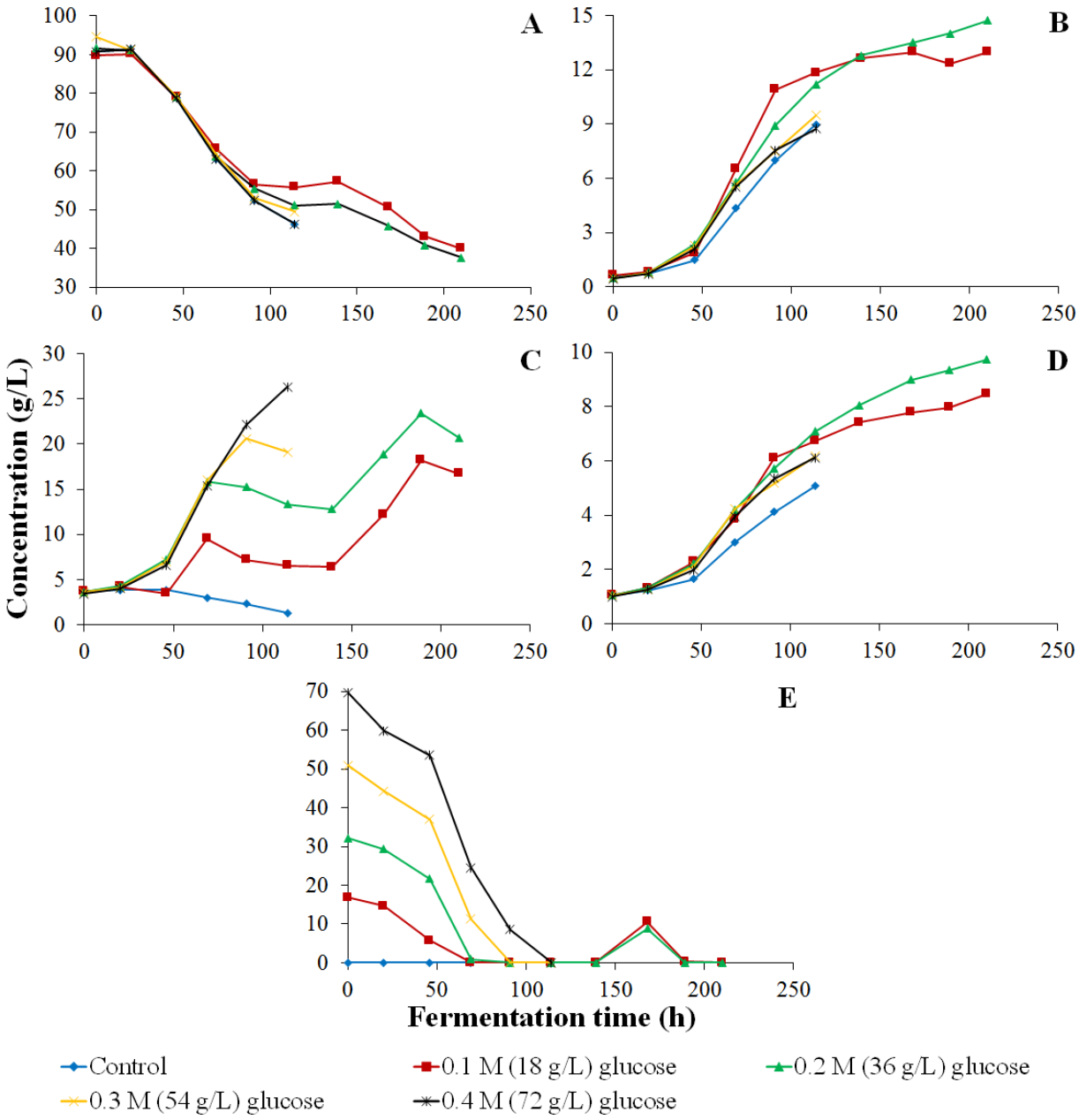

Figure 4.5 The effect of glucose addition to W-TS on concentration of A) glycerol, B) 1,3-PD, C) lactic acid, D) acetic acid, and E) glucose during fermentation with endemic bacterial populations augmented with *L. panis* PM1 inoculum. Glucose concentration was not determined when its concentration was lower than the assay detection limit. Glucose concentration 0.1 and 0.2 M conditions were analyzed after 139 h fermentation.

In addition, 1,3-PD could also potentially act as an end product inhibitor of its own accumulation. Consequently, we explored the effects of freeze-dried W-TS, vitamins, or 1,3-PD added to W-TS media. At the beginning of fermentation 7×10^7 CFU/mL were enumerated. Adding either freeze-dried W-TS or vitamins enhanced 1,3-PD and acetic acid accumulation compared to controls (Figure 4.6). Sluggish growth and fermentation rates at the end of fermentation might be due to exhaustion of essential nutrients and carbon source e.g. amino acids and minerals and glucose. Freeze-dried W-TS was tested because vitamins are relatively expensive and may be cost prohibitive for commercial use. W-TS dried under mild conditions might be preferred as a source of essential nutrients. Nevertheless, adding freeze-dried W-TS or vitamins after 190 h fermentation neither improved 1,3-PD production nor restarted fermentation (Figures 4.6A−4.6D). 1,3-PD addition did not inhibit accumulation of 1,3-PD in W-TS (Figure 4.6B). Therefore, it appears that end product inhibition by 1,3-PD does not block 1,3-PD accumulation. In addition, glycerol consumption still occurred after 1,3-PD accumulation reached its maximum. These phenomena could be the result of endemic microorganisms that utilize glycerol or other carbon sources.

Both agitated (0.5 g freeze-dried W-TS; pH adjusted to 5.0 at 0 h) and static (0.5 g freeze-dried W-TS) cultures produced similar amounts of 1,3-PD indicating that agitation had little effect on product accumulation (Figures 4.7A−4.7E). Agitation during fermentation can aid in gas-liquid mass transfer (Maier *et al*., 2004) and increase oxygen transfer from headspace to fermentation media. This might impede 1,3-PD and acetic acid accumulation according to Khan *et al*. (2013) who observed 1,3-PD production only under anaerobic and microaerobic conditions and Oude Elferink *et al*. (2001) who proposed a pathway for lactic acid degradation by *L. buchneri* under anaerobic condition. One of the highest concentrations of 1,3-PD and acetic acid was observed in cultures that were adjusted to pH 5.0 daily (Figures 4.7B and 4.7D). The greatest accumulation of 1,3-PD and acetic acid was noted when acid produced by microorganisms was neutralized with NaOH. In addition, the increase of *Lactobacillus* CFU was faster when pH was adjusted to 5 at 0 h along with agitation compared to other conditions (Figure 4.7F). Based on these investigations, addition of freeze-dried W-TS and static conditions were used in further studies.

Prior to increasing fermentation scale, factors that might affect fermentation were tested

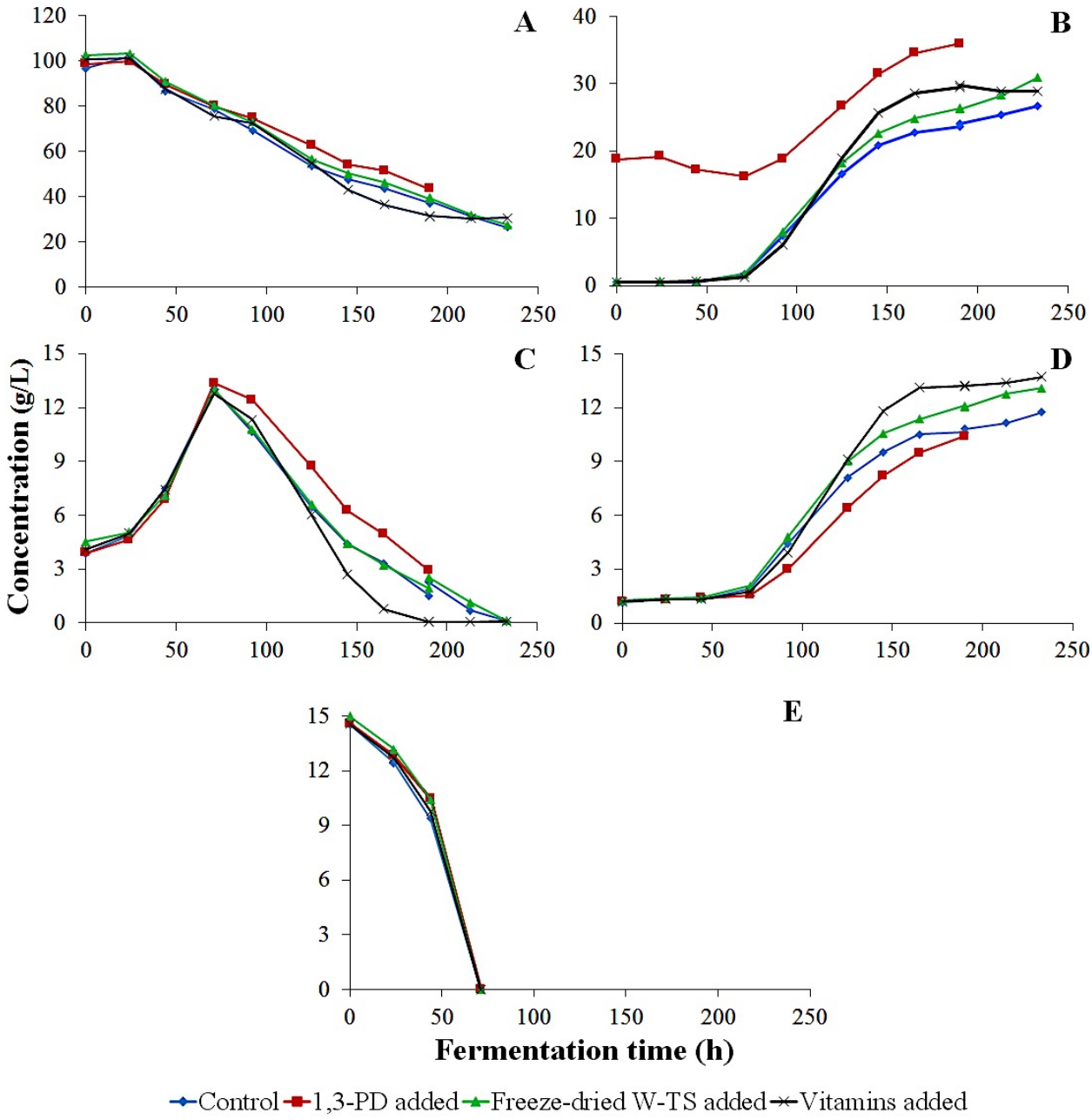

Figure 4.6 Effect of 1,3-PD, freeze-dried W-TS, and vitamins on concentration of A) glycerol, B) 1,3-PD, C) lactic acid, D) acetic acid, and E) glucose during fermentation with endemic bacterial populations augmented with *L. panis* PM1 inoculum. Glucose concentration was not determined when its concentration was lower than the assay detection limit. Control, freeze-dried W-TS added, and vitamin added conditions were analyzed after 190 h fermentation.

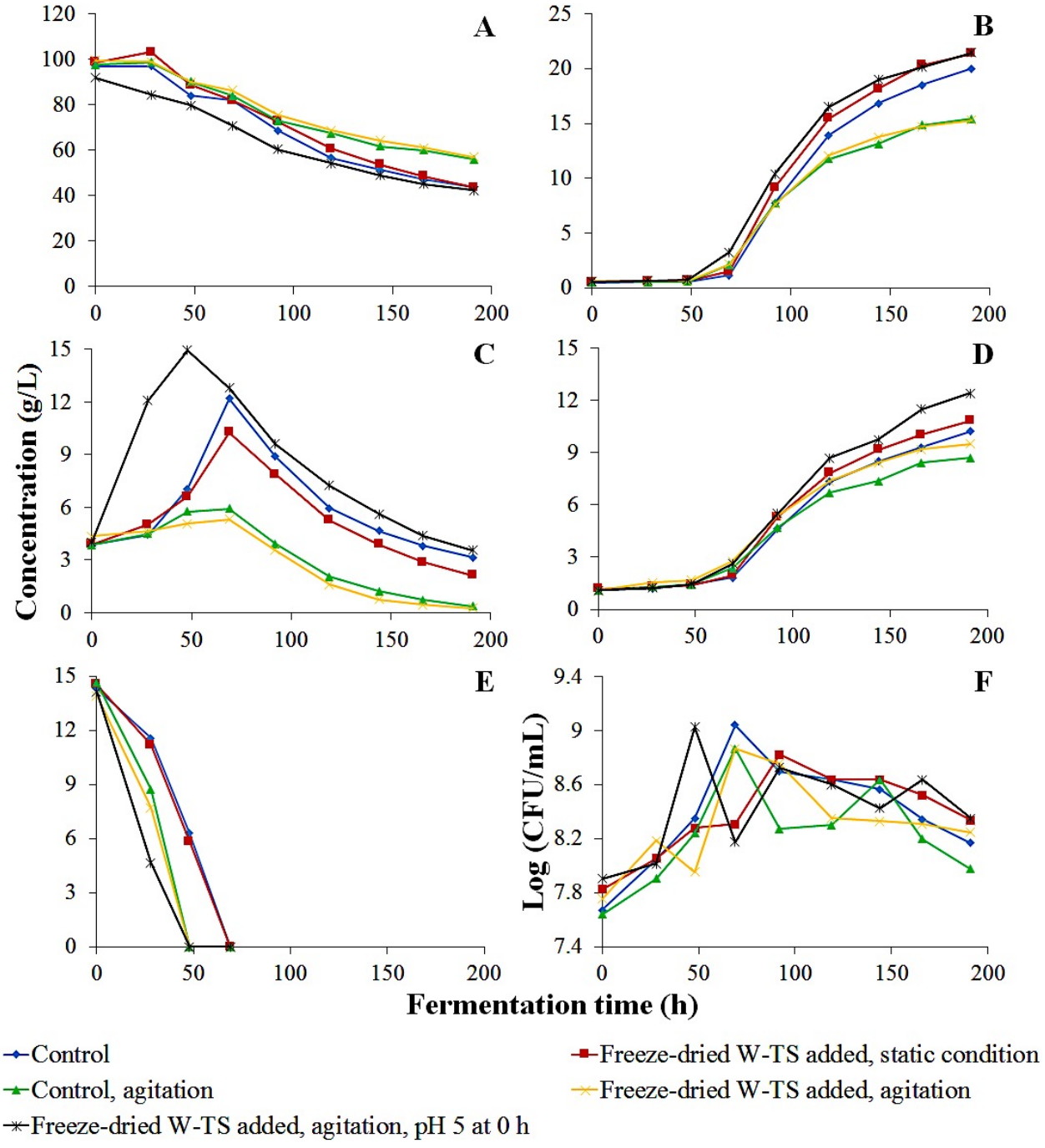

Figure 4.7 Effect of freeze-dried W-TS, agitation, and pH adjusting on the concentration of A) glycerol, B) 1,3-PD, C) lactic acid, D) acetic acid, and E) glucose, and F) number of bacteria during fermentation with endemic bacterial populations augmented with *L. panis* PM1 inoculum. Glucose concentration was not determined when its concentration was lower than the assay detection limit.

including; substitution of refined glucose with corn sugar, adjustment of pH at time 0, and the addition of 3-HPA, a potentially toxic intermediate (Saxena *et al*., 2009). Corn sugar is produced by enzymatic hydrolysis of cornstarch and is a crude product containing mostly glucose but also containing the disaccharide maltose, and higher polysaccharides. Glucose is often used in fermentation media for research purposes but corn sugar is a preferred carbon source for commercial fermentation. As was expected corn sugar could be substituted for laboratory glucose without affecting glycerol conversion to 1,3-PD or lactic to acetic acid (Figure 4.8). The CFU number was also unaffected. In addition, there is no apparent effect of either maltose or polysaccharides present in this carbon source. We noted above that pH 5 appeared to be a superior for substrate conversion. The effect of adjusting pH to 5.0 at 0 h was tested to compare with other treatments. It was found that CFU increased more rapidly in cultures when pH was adjusted to 5.0 than for cultures grown without this pH adjustment at 0 h of fermentation. Moreover, it was confirmed that metabolism was more efficient when pH was adjusted to 5.0 at 0 h and daily thereafter. Therefore, corn sugar and pH adjustment to 5.0 daily were employed for subsequent experiments. According to Saxena *et al*. (2009), 3-HPA is toxic to bacteria and its presence during fermentation might slow cell growth or inhibit substrate conversion. Adding 3-HPA to media affected neither 1,3-PD accumulation nor culture growth.

4.4.3 Fermentation in a 25 L Vessel

1,3-PD increased to 40 and 36 g/L with the conversion efficiency 47% and 43% in replicates 1 and 2, respectively (Figures 4.9A and 4.9B), which were the greatest amounts of 1,3-PD observed in any of the fermentations reported here. Concentrations of acetic acid in replicate 1 and 2 were 12 and 13 g/L, respectively. Fuchs *et al*. (1971) and Junker (2004) stated that surface aeration decreased when fermenter size increased. Larger fermenters often have lower headspace to fermenter volume ratios than smaller fermenters. The 25 L fermenter had a lower ratio of surface area and headspace to culture volume compared to 50 mL fermenter. The larger fermenter may have less oxygen that may lead to greater 1,3-PD accumulation. However, small amounts of oxygen introduced while stirring fermentation media using a homogenizer might enhance growth of endemic flora and *L. panis* PM1B. CFU in these fermenters showed no clear trend during incubation (Figures 4.9C and 4.9D). Interestingly, when the growth of microorganisms reached stationary phase, glycerol consumption and 1,3-PD accumulation still

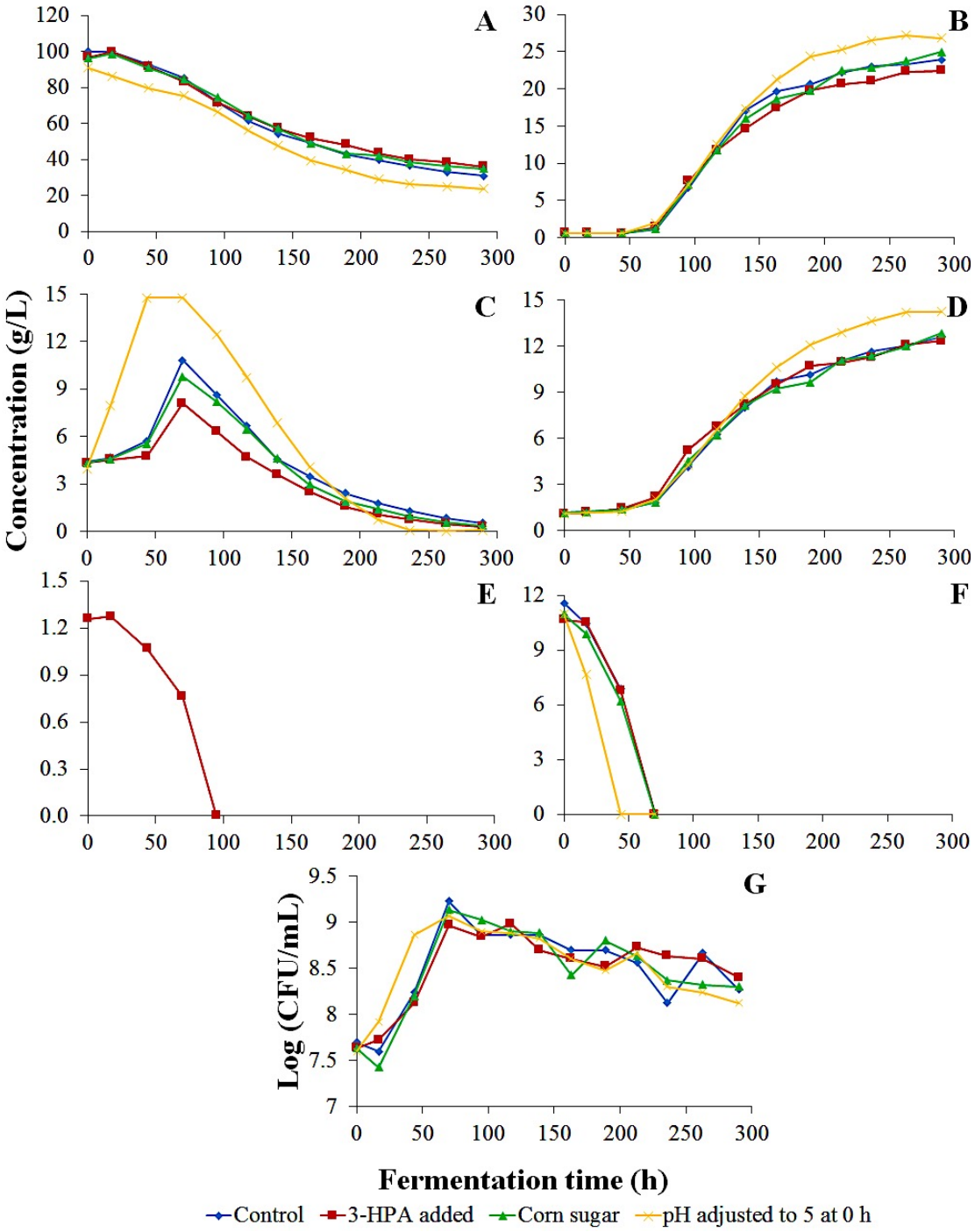

Figure 4.8 Effects of 3-HPA, corn sugar, and adjusting pH on the concentrations of A) glycerol, B) 1,3-PD, C) lactic acid, D) acetic acid, E) 3-HPA, and F) glucose, and G) bacteria populations during fermentation with endemic bacterial populations augmented with *L. panis* PM1 inoculum. Glucose and 3-HPA concentrations were not determined when their concentrations were lower than the assay detection limit.

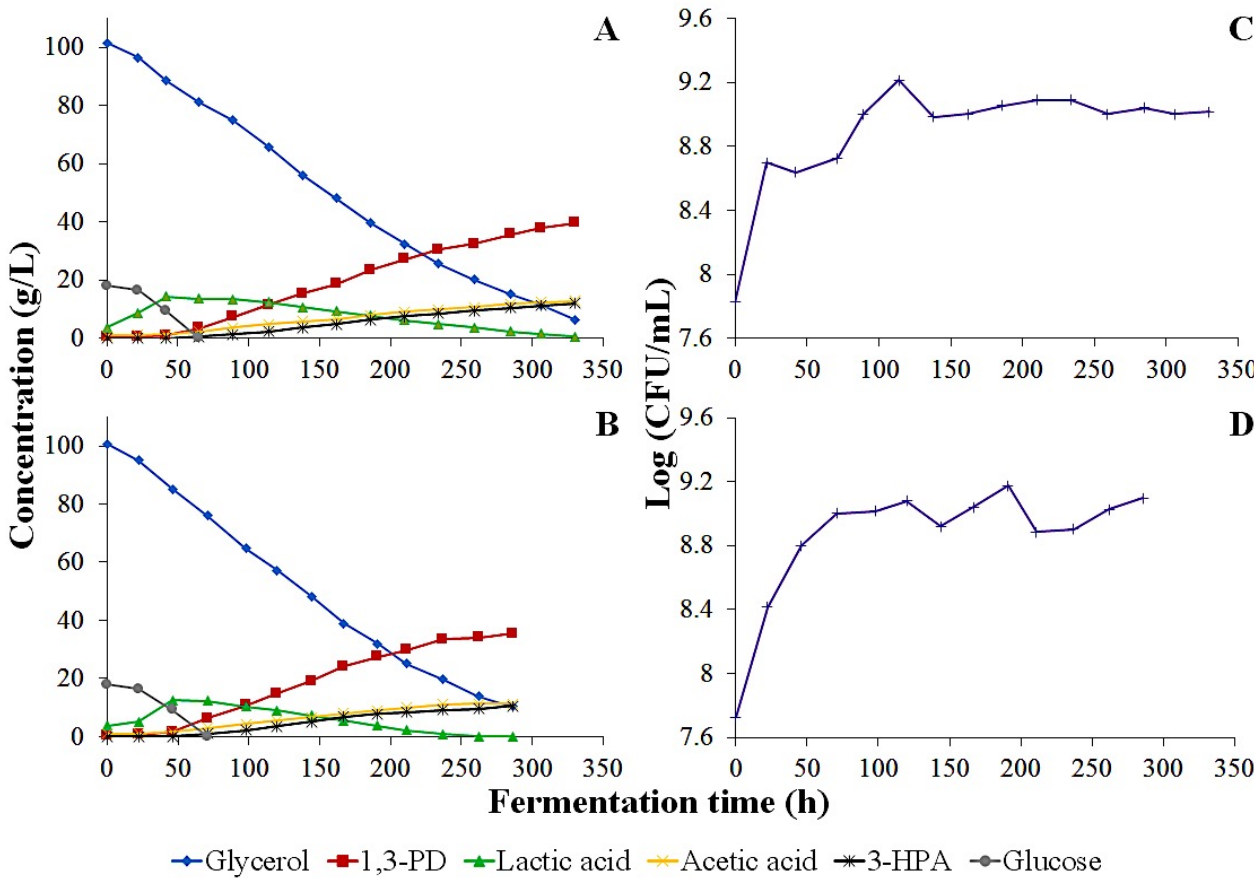

Figure 4.9 Media concentration of A) glycerol, lactic acid, 1,3-PD, acetic acid, 3-HPA, and glucose of duplicate 1, B) glycerol, lactic acid, 1,3-PD, acetic acid, 3-HPA, and glucose of duplicate 2, C) number of bacteria (CFU/mL) duplicate 1, and D) number of bacteria (CFU/mL) duplicate 2 during fermentation with endemic bacterial populations augmented with *L. panis* PM1 inoculum. Glucose concentration was not determined when its concentration was lower than the assay detection limit.

occurred suggesting that endemic microorganisms might utilized other forms of carbohydrate or protein as energy sources for their metabolism. Fermentation progressed at 25 °C, which might benefit commercial production, as energy input might not be required for heating these cultures.

4.4.4 Fermentation in a 210 L Fermenter

For replicate 1, starting culture (20 L) with a concentration of 6 g/L 1,3-PD was utilized as an inoculum (Figure 4.10A). The concentration of 1,3-PD and acetic acid in 150 L fermentation reached 23 (conversion efficiency 28%) and 11 g/L, in that order (Figure 4.10A). For repeated experiment, the concentration of 1,3-PD in starting culture was approximately 15 g/L (Figure 4.10B). The finishing concentration of 1,3-PD and acetic acid from repeated experiment of 150 L fermentation reached 22 (conversion efficiency 30%) and 11 g/L, respectively (Figure 4.10B). Accumulation of 1,3-PD (2%) and acetic acid was possible in a 150 L fermenter at 25 °C. This accumulation was lower than that of 50 mL and 20 L scale fermentations but greater than achieved for cultures grown in MRS medium by Grahame *et al*. (2013), Kang *et al*. (2013b, 2014a), Khan *et al*. (2013), and Reaney *et al*. (2013). The inoculation train for larger scale fermentation was greatly different than that of smaller fermentations and this could lead to effects that might impact conversion and culture stability (Okonkowski *et al*., 2005). With larger scale fermentations, longer mixing times were required to increase media homogeneity and larger stagnant regions could occur (Junker, 2004). This mixing likely introduces oxygen. Mixing efficiency is also reduced (Junker, 2004) and heat could be generated for longer mixing times (Schmidt, 2005). These factors can stress microorganism (Schmidt, 2005) leading to decreased ability to effect desired conversions. In addition, a larger fermentation reactor has lower surface area to volume ratio compared to a small reactor. This would reduce heat transfer surface to volume ratio fermentation (Junker, 2004). CFU of 150 L fermentations and starting cultures increased after inoculation (Figure 4.11A) and became constant at about 60 h. A similar pattern was observed in 150 L fermentations where CFU also increased then became constant after 60 h (Figure 4.11B). Nonetheless, longer incubation times did not improve fermentation yield. Therefore, shorter incubation times of the 20 L starting culture could be preferred to achieve more rapid conversion.

4.5 Conclusion

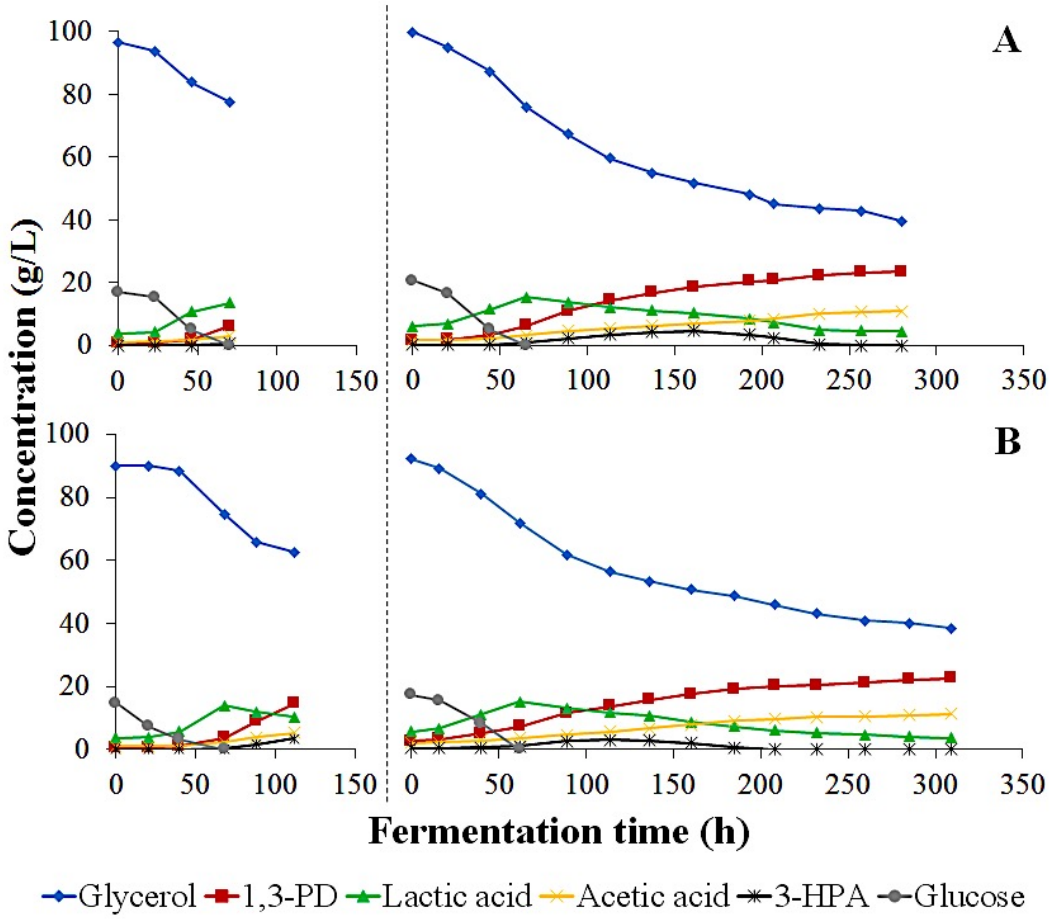

Figure 4.10 Media concentration of glycerol, lactic acid, glucose, 1,3-PD, acetic acid, and 3-HPA of A) starting culture and 150 L fermentation and B) starting culture and 150 L fermentation of repeated experiment during the fermentation with endemic bacteria populations augmented with *L. panis* PM1. Glucose concentration was not determined when its concentration was lower than the assay detection limit.

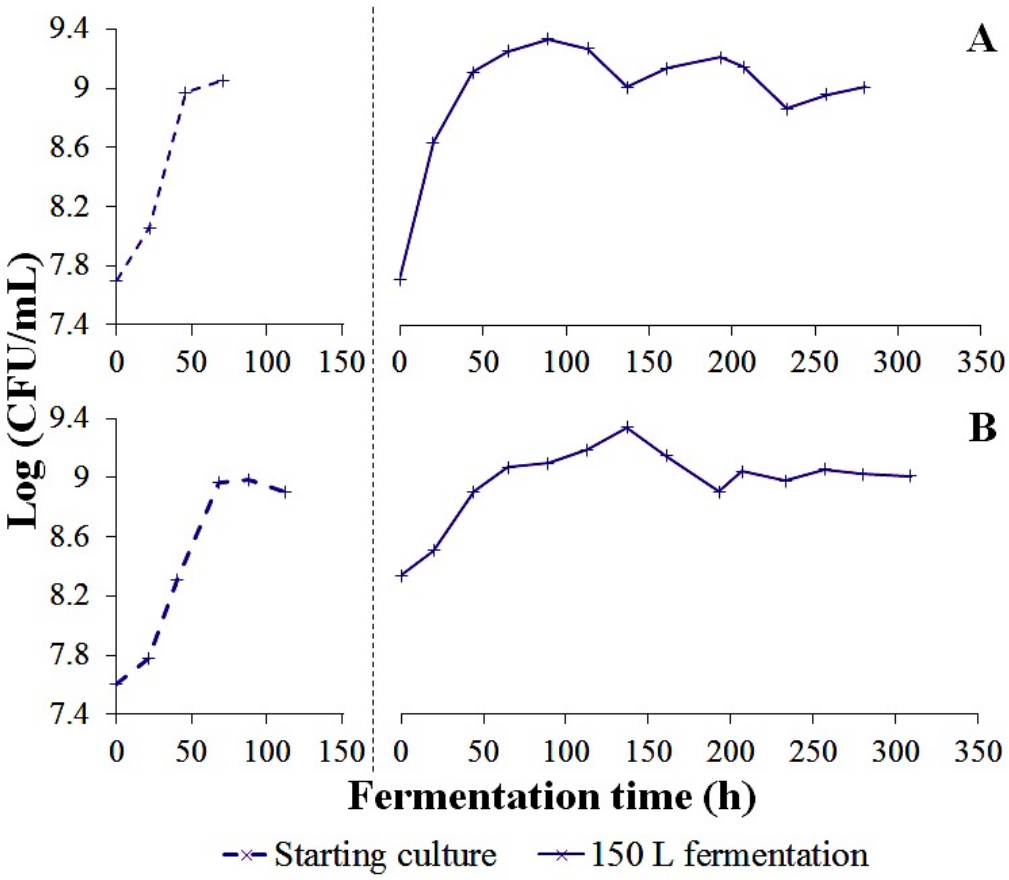

Figure 4.11 Number of bacteria for cultures with augmented inoculation with *L. panis* PM1 (CFU/mL) for starting culture and 150 L fermentation for two separate fermentations (A and B).

W-TS contained organic solutes, for instance 1,3-PD, acetic acid, glycerol, and lactic acid. Even though glycerol is a major compound present in W-TS it is inexpensive. In addition, a complex process for purification of glycerol would be required. Therefore, it is likely that it would not be profitable to recover and purify glycerol from W-TS. In addition, W-TS contained high boiling point and hygroscopic solutes (glycerol and lactic acid) leading to obstacles for valuable compound (1,3-PD, acetic acid, and GPC) extraction and recovery. Endemic flora especially *L. panis* PM1B could convert glycerol and lactic acid to 1,3-PD and acetic acid, respectively. 1,3-PD and acetic acid have lower boiling points than glycerol and lactic acid. Consequently, fermentation of W-TS followed by isolation of 1,3-PD and acetic acid could be a strategy for increasing the value of commercial ethanol production. The effect of fermentation parameters of W-TS in the presence of endemic microorganisms supplemented with *L. panis* PM1B were studied to improve compound conversion. We discovered that freeze-dried W-TS, pH 5 adjusted daily, ratio of glucose: glycerol 0.1: 1.0 (mol: mol), vitamins, and static conditions favoured conversion. It was also noted that product, 1,3-PD, and intermediate, 3-HPA, did not block conversion. Moreover, corn sugar could be utilized as a glucose substitute and agitation was not required. Furthermore, fermentation scale was increased to 20 L and 150 L at 25 °C. At least 2% (20 g/L) of 1,3-PD was produced in all scaled up fermentations. Conditions that lowered production costs such as culture at 25 °C, use of corn sugar as a carbon source, and minimal agitation could be beneficial in this regard.

4.6 Brief Introduction to Chapter 5

As described in Chapter 4, major compounds present in W-DS and W-TS included glycerol and lactic acid. These compounds are not expensive. In addition, their high boiling point and hygroscopic characteristics could impede extraction of other valuable compounds, for instance, 1,3-PD, acetic acid, and GPC. Therefore, it is not likely to be profitable to recover and purify these compounds from W-TS. Findings presented above show that using TSF with endemic flora supplemented with *L. panis* PM1B could offer a way to add value to W-TS. Conversion of glycerol and lactic acid to 1,3-PD and acetic acid, respectively was approximately 30% efficient. The boiling point of 1,3-PD and acetic acid are lower than those of glycerol and lactic acid. A lower boiling point could lead to lower cost compound extraction and recovery. Consequently, it is likely that TSF is a simple process for converting less-valuable compounds to more valuable compounds and for reducing high boiling point and hygroscopic solutes in W-TS. Unfortunately, W-TS contains colloidal and unfermented particles. These might cause equipment fouling utilized for compound extraction and recovery processes. Therefore, clarification of fermentation media prior to extraction is an essential step. The metabolic pathways of lactobacilli especially *L. panis* PM1B produce anoxic gas (CO_2). In addition, they produce exopolysaccharides (EPSs). It is likely that dissolved air filtration (DAF) using anoxic gas produced by endemic flora and EPS could be a potential clarification process.

CHAPTER 5
PRODUCTION OF PROTEIN CONCENTRATE AND 1,3-PROPANEDIOL BY WHEAT THIN STILLAGE FERMENTATION

5.1 Abstract

Fermentation with yeast produces ethanol and wheat-based thin stillage (W-TS). A second fermentation of W-TS, a two-stage fermentation (TSF) of wheat, with endemic bacteria at 25 °C decreased glycerol and lactic acid concentrations while 1,3-propanediol (1,3-PD) and acetic acid accumulated. Increasing fermentation temperature to 37 °C increased 1,3-PD and acetic acid accumulation markedly when compared with fermentation at 25 °C. During anaerobic TSF, W-TS colloids coagulated to produce largely clarified liquid and slurry phases. The slurry floated to the top of the fermentation medium. The endemic bacteria, present in W-TS, were largely members of *Lactobacillus panis*, *L. gallinarum*, and *L. helveticus* and this makeup did not change substantially as fermentation progressed. Furthermore, genome sequence data indicated that *L. panis* PM1B possessed sequences involved in exopolysaccharides (EPSs) production. The presence of carbon dioxide bubbles and EPSs may contribute to particle-liquid separation. As fermentation media were exhausted of nutrients, floating particles precipitated. Protein contents of slurry and liquid increased and decreased, respectively, as TSF progressed. The liquid phase was easily processed by ultrafiltration without membrane fouling. These results demonstrated that TSF is a novel method for W-TS clarification.

5.2 Introduction

The production of ethanol by yeast fermentation followed by distillation produces thin stillage (TS), which is composed of organic solutes, suspended particles and inorganic salts (Meredith, 2003; Ratanapariyanuch, 2009; Ratanapariyanuch *et al*., 2011). Typically, wheat-based thin stillage (W-TS) contains colloids, microorganisms, inorganic and organic solutes, particulate matter, polysaccharides, and proteins (Ratanapariyanuch, 2009). Organic solutes present in W-TS included 1,3-propanediol (1,3-PD), acetic acid, glycerol, glycerophosphorylcholine (GPC), and lactic acid (Ratanapariyanuch *et al*., 2011). These compounds are potentially valuable without modification or as precursors for additional processing. 1,3-PD may be used to replace ethylene glycol (Liu *et al*., 2010) or as an intermediate chemical for synthesis of polyamides, polyesters, and polyurethanes (Biebl *et al*., 1999; Saxena *et al*., 2009). Acetic acid might be utilized as a food ingredient, a precursor for production of polyvinyl acetate for synthetic fibres, or vinegar (Manning and Hutten, 1992; New World Encyclopedia, 2012). GPC is a cholinergic substance that releases choline when consumed. Cholinergic compounds can be used to mitigate the effects of Alzheimer disease (Parnetti *et al*., 1993) and transient ischemic attacks (Sangiorgi *et al.*, 1994). GPC can also be esterified with fatty acids for the synthesis of lecithin and lysolecithin.

Microorganisms present in W-TS include bacteria, fungi, and yeast (Ratanapariyanuch, 2009). Some of these microorganisms, specifically *Lactobacillus panis* PM1B, may be used to conduct a second fermentation of W-TS (Kang *et al*., 2014a; Reaney *et al*., 2013). The modified two-stage fermentation (TSF) is, potentially, a novel intermediate process for adding value to W-TS. Recovery of soluble organic compounds from TS is a challenging step (Chapter 3) due to the high concentration of particulates and high boiling point and hygroscopic solutes. The complexity of TS limits options for developing an inexpensive enrichment process.

Inorganic solutes, particles, and soluble protein and non-protein biopolymers remain in W-TS after ethanol fermentation. TSF occurred when endemic flora including *L. panis* PM1B, an organism discovered in W-TS, are allowed to proliferate in W-TS. *L. panis* PM1B converted glycerol to 1,3-PD and lactic acid to acetic acid (Chapter 4; Kang *et al*., 2014a; Khan *et al*., 2013; Reaney *et al*., 2013). W-TS solutes, therefore, are converted by TSF to less hygroscopic forms that have lower boiling points.

Conversion of these solutes might enable approaches for simplified processing to recover W-TS compounds.

TSF also produces CO_2, an anoxic gas, from *Lactobacillus* metabolism (Biebl *et al*., 1999; Kang *et al*., 2014b). Others have used anoxic gas generated by anaerobic fermentation to clarify dairy manure and sewage sludge in a process known as anoxic gas flotation (AGF). AGF can concentrate and return organic acids, protein, and partially digested substances to anaerobic digesters (Burke, 1997a, 1998, 2000b). Typically, gas bubbles in water have negative surface charges that repel adjacent bubbles due to electrostatic forces. Positively charged particles bind to bubble surfaces and reduce the net charge. In addition, van der Waals, hydrodynamic retardation, and hydrophobic forces are also associated with bubble and particle interactions (Edzwald, 2010). These phenomena enhance coagulation of colloids (Rubio *et al*., 2002). Colloids or particles present in W-TS could adhere to anoxic gas bubbles produced by lactobacilli. Particles adhering to bubble surfaces are aggregated and float in fermentation media as TSF progresses. Furthermore, lactobacilli also produce exopolysaccharides (EPSs) that might affect colloid stability and clear solutions as TSF progresses. EPSs are produced by *L. casei* CG11 grown in a basal medium (Cerning *et al*., 1994), *L. plantarum* EP56 (Tallon *et al*., 2003), *L. sanfranciscensis* in sour dough (Hammes and Gänzle, 1997), and lactic acid-producing bacteria present in sour dough (De Vuyst and Degeest, 1999). Therefore, it is possible that endemic flora present in W-TS specifically *Lactobacillus* species produce EPS during TSF. It is not known if AGF and EPS production might act synergistically to aggregate, coagulate, and separate W-TS particles from colloids during fermentation. The genome of *L. panis* PM1B has been sequenced and genes encoding the EPS pathway can be identified. The sequence data of *L. panis* PM1B and other stillage lactobacilli could be used to determine the nature of EPS. The objective of this study was to utilize CO_2 released by TSF to clarify W-TS. Once the stillage is clarified, numerous solution processes might be devised to separate useful compounds from solution and enrich protein particles.

5.3 Materials and Methods

5.3.1 Materials

W-TS samples were collected from Pound-Maker Agventures Ltd. (Lanigan, SK, Canada)

on May 12, August 28, September 25, December 3, 2014, and February 23, 2015 called W-TS1, W-TS2, W-TS3, W-TS4, and W-TS5, respectively. W-TS samples were stored in 10 L containers at 4 °C until utilized.

5.3.2 Methods

5.3.2.1 Protein and Moisture Contents

Protein content of sample was determined using the Kjeldahl method in the Section 3.3.6. Moisture content was determined by heating a weighed sample as previously described in the Section 3.3.7.

5.3.2.2 DPFGSE-NMR Spectrometry

DPFGSE-NMR was utilized to follow the progress of TSF in the Section 3.3.8.

5.3.2.3 16S Ribosome Sequencing

Slurry I (Figure 5.1) from Section 5.3.3.1 replicate 2 at 0, 46, and 94 h of fermentation and from Section 5.3.3.2 replicate 1 at 0, 47, and 101 h of fermentation were frozen and sent to Contango Strategies Ltd. (Saskatoon, SK, Canada) to enumerate microorganisms present in slurry I using 16S ribosome sequencing (Klindworth *et al*., 2012). Slurry I (500 μL) was centrifuged and decanted to obtain a pellet (between 0.33 to 0.37 g). Pellet DNA was extracted using a MoBio Powerlyzer Powersoil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) following the manufacturers protocol. The 16S metagenomic sequencing library was prepared utilizing the MiSeq V3 paired-end 300 base pairs according to the Illumina MiSeq system. Mock communities and negative control were included in sequencing runs as controls. Raw data sequences from sequencing runs were filtered. Sequences with an average quality of less than Q30, shorter than 350 base pairs, not having an exact match with the base-call of N, forward 16S primer, and forward and reverse primer were discarded. Several internal scripts as well as selected QIIME Scripts were utilized to analyze filtered sequences. The *de novo* option with QIIME, with a 97% identity threshold was utilized to generate operational taxonomic units. Greengenes Database (version 13-8) was used to perform taxonomic classifications of operational taxonomic units.

5.3.3 Experimental Procedures

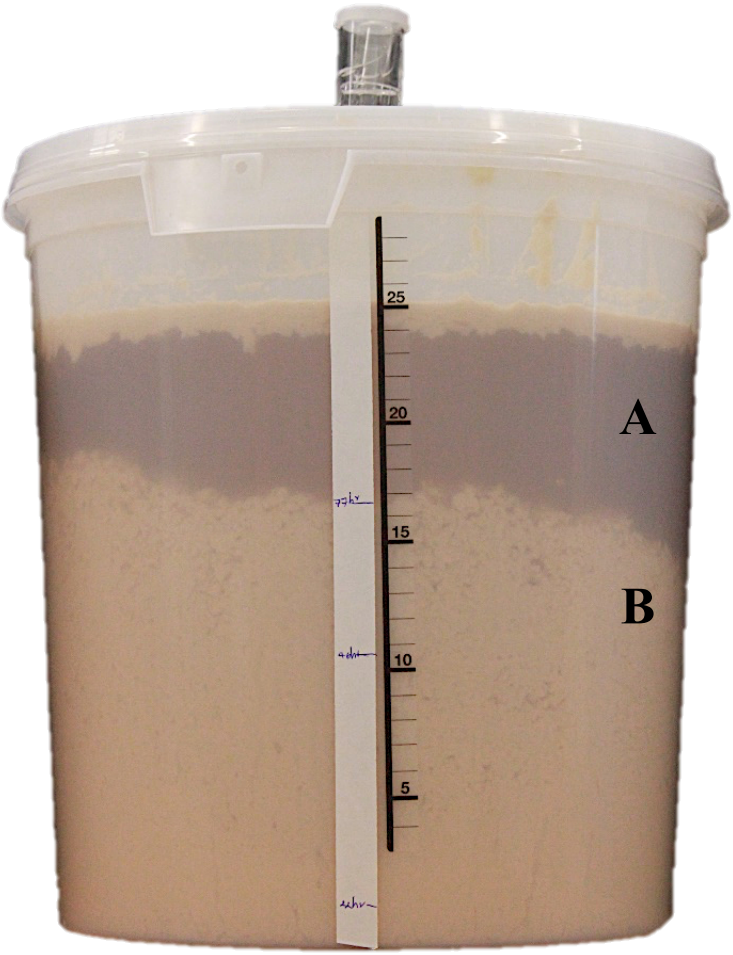

Figure 5.1 Liquid I (A) and slurry I (B) from fermentation.

5.3.3.1 Small-scale Fermentation

W-TS1 and W-TS2 were utilized as fermentation medium for replicate 1 and 2, respectively. Two 30 L semi-transparent polypropylene plastic pails (described as fermenter 1 and fermenter 2) with lids equipped with fermentation gas traps were utilized as fermenters. Twenty-five liters of W-TS was added to each fermenter. Fermentation was allowed to progress at 25 °C until gas evolution ceased and particles precipitated. Slurry I and liquid I were sampled daily for analysis of protein and moisture contents and organic solutes by NMR. The volume of liquid I was recorded daily. Slurry I samples from replicate 2 at 0, 46, and 94 h were collected and stored at –80 °C for 16S ribosome sequencing (Section 5.3.2.3). Liquid I samples from small-scale TSF at 25 °C were filtered with regenerated cellulose membranes (10 kDa molecular weight cut-off (MWCO) PL type; Millipore Corp., Bedford, MA, USA) at 380 kPa in a stirred ultrafiltration cell (8010, Millipore Corp., Bedford, MA, USA) and effective membrane area 4.1 cm^2. The membrane was prepared according to manufacturer's guidelines to remove preservative deposited on the membrane. The elastomer tubing from stirred cell was inserted into a 10 mL graduate cylinder sealed with flexible film (Parafilm M®, Bemis Company Inc., Neenah, WI, USA) to limit liquid I filtrate evaporation. Liquid I (10 mL) was added to the stirred cell. Agitation speed was held at 600 rpm. The volume of filtrate was recorded every 10 min and filtration proceeded until the solution volume was reduced from 10 mL to 1.0 mL and filtrate volume reached approximately 9.0 mL. Flux and solution volume concentration were calculated using Equations 5.1 and 5.2, respectively.

Solution flux from step 2 = $L/M^2/h$ (5.1)

Where, L = filtrate volume, M^2 = membrane surface area, h = time of filtration in hours

$$\text{Volume concentration} = \frac{\text{volume of sample}}{\text{volume of retentate}} \quad (5.2)$$

Filtrate protein contents were estimated using the modified Bradford protein assay (Bradford, 1976) with bovine serum albumin as a standard.

5.3.3.2 Temperature Effects on Fermentation

Fermentation was compared at two incubation temperatures (25 and 37 °C). W-TS4 and W-TS5 (25 L) were utilized as fermentation media for replicate 1 and 2, respectively. Fermentation was conducted in 30 L semi-transparent polypropylene plastic pails equipped with lids and gas traps at 25 and 37 °C until fermentation ceased. Slurry I and liquid I from fermentation media

were sampled daily as described in Section 5.3.3.1. Protein content, moisture content, and the concentration of organic solutes of liquid and slurry were determined as previously described. The volume of liquid I was recorded daily. Microbial populations of slurry I at 0, 47, and 101 h of fermentation replicate 1 were characterized using 16S ribosome sequencing as described above.

5.3.3.3 Replications of Small-Scale Fermentation

W-TS2 and W-TS3 were employed as fermentation media for replicate 1 and replicate 2. Twelve 30 L transparent polypropylene plastic pails with lids equipped with gas traps were utilized as fermenters. Media (25 L) was fermented in each fermenter at 25 °C until gas evolution ceased and slurry I precipitated. Liquid I and slurry I from replications of small-scale TSF were sampled each day from fermenter 1 and fermenter 2 as representative of the twelve fermenters as described in Section 5.3.3.1. Protein content, moisture content, and organic solute content were conducted on samples. The volume of liquid I was recorded daily.

5.3.4 Statistical Analysis

Data was obtained from analysis of duplicate samples. Means comparisons were made by analysis of variance (ANOVA).

5.4 Results and Discussion

5.4.1 Protein and Moisture Contents and Organic Solute in W-TS

W-TS protein (38–43% w/w, db) and moisture contents (91–94%, w/w) varied with sample collection date (Table 5.1). This result agreed with other studies of W-TS, which indicated W-TS protein at 36.6 and 45.7% (w/w, db) (Mustafa *et al*., 1999; Mustafa *et al*., 2000). TS (corn) moisture content was approximately 90–95% (w/w) in another report (Liu and Barrows, 2013). Wheat endosperm and endemic TS microorganisms might contribute to the observed protein.

W-TS contained 1,3-PD, acetic acid, betaine, ethanol, glycerol, GPC, isopropanol, lactic acid, phenethyl alcohol, and succinic acid. W-TS composition and organic compound concentrations differed on each sample collection date (Table 5.2). In addition, glycerol and lactic acid were major W-TS organic solutes. These organic solutes are products of microorganisms and wheat and are similar to compounds reported previously (Ratanapariyanuch,

Table 5.1 Protein and moisture contents of W-TS samples

Characteristic (%, w/w)	W-TS1	W-TS2	W-TS3	W-TS4	W-TS5
Total nitrogen	0.58 ± 0.02	0.68 ± 0.00	0.66 ± 0.00	0.46 ± 0.01	0.56 ± 0.01
Betaine nitrogen[1]	0.011 ± 0.001	0.009 ± 0.000	0.013 ± 0.000	0.009 ± 0.000	0.010 ± 0.000
GPC nitrogen[1]	0.006 ± 0.000	0.006 ± 0.000	0.008 ± 0.000	0.005 ± 0.000	0.006 ± 0.000
Moisture	91.74 ± 0.02	91.05 ± 0.00	91.15 ± 0.00	93.64 ± 0.01	92.01 ± 0.00
Protein[2] (wb)	3.2 ± 0.1	3.77 ± 0.01	3.65 ± 0.06	2.5 ± 0.1	3.10 ± 0.04
Protein[2] (db)	39 ± 1	42.1 ± 0.2	41.3 ± 0.7	40 ± 2.0	38.9 ± 0.3

Each value is presented as the mean ± standard deviation (SD, $n = 2$).
[1]Nitrogen contributed by GPC and betaine was determined by DPFGSE-NMR.
[2]Nitrogen contributed by these materials to total nitrogen was subtracted prior to calculation of protein content.

Table 5.2 Concentration (g/L) of organic solutes of W-TS samples

Compounds	W-TS1	W-TS2	W-TS3	W-TS4	W-TS5
1,3-PD	0.63 ± 0.01	2.91 ± 0.01	0.61 ± 0.01	0.31 ± 0.01	0.80 ± 0.01
Acetic acid	1.85 ± 0.01	3.92 ± 0.06	1.99 ± 0.04	0.89 ± 0.02	2.34 ± 0.06
Betaine	0.94 ± 0.03	0.77 ± 0.05	1.12 ± 0.02	0.74 ± 0.01	0.87 ± 0.05
Ethanol	0.35 ± 0.00	0.40 ± 0.01	0.47 ± 0.01	0.12 ± 0.01	0.29 ± 0.02
Glycerol[1]	10.56 ± 0.08	7.6 ± 0.1	11.2 ± 0.2	9.2 ± 0.2	8.3 ± 0.2
GPC	1.06 ± 0.01	1.16 ± 0.01	1.40 ± 0.07	0.89 ± 0.04	1.05 ± 0.04
Isopropanol	0.34 ± 0.00	0.38 ± 0.00	0.40 ± 0.01	0.32 ± 0.01	0.42 ± 0.02
Lactic acid	6.04 ± 0.07	6.76 ± 0.02	6.0 ± 0.2	3.28 ± 0.02	5.8 ± 0.3
Phenethyl alcohol	0.38 ± 0.00	0.43 ± 0.03	0.47 ± 0.01	0.34 ± 0.01	0.34 ± 0.02

Each value is presented as the mean ± SD ($n = 2$).
[1]The concentration of glycerol in W-TS samples may be affected by the presence of interfering resonances from carbohydrate and protein.

2009; Ratanapariyanuch *et al*., 2011).

5.4.2 Small-Scale TSF at 25 °C

Proton NMR analysis showed that as fermentation progressed glycerol and lactic acid concentrations decreased with simultaneous increases in 1,3-PD and acetic acid concentrations (Figure 5.2 and Appendix C Figure C.1). These changes were associated with metabolism of flora endemic in W-TS especially lactobacilli like *L. panis* PM1B that consumes glycerol and lactic acid to produce 1,3-PD and acetic acid, respectively (Kang *et al*., 2014a; Khan *et al*., 2013; Reaney *et al*., 2013). Moreover, TSF can proceed at 25 °C enabling fermentation to be conducted in unheated fermenters. The concentrations of glycerol and lactic acid in slurry I decreased more rapidly than in liquid I while 1,3-PD and acetic acid concentrations increased more rapidly than for liquid I suggesting that metabolic activity in the slurry was likely greater than in the liquid. Slurry formation might have been aided by EPS formed by fermentation organisms (Cerning *et al*., 1994; De Vuyst and Degeest, 1999; Hammes and Gänzle, 1997; Tallon *et al*., 2003). Though not investigated here, EPS production may stabilize bacteria adhesion to particle surfaces and allow biofilm formation (Lebeer *et al*., 2007; Yildiz and Schoolnik, 1999). Biofilms are conducive to improved metabolism. EPS produced by bacteria plays numerous roles including: acting as adhesives for interactions with other bacteria or surfaces or substrata, hiding bacteria surfaces, protective agents against environment, signalling molecules, structure stabilizer in biofilms, and substances for bacteria aggregation in rhizosphere communities (Badel *et al*., 2011). EPS produced from lactobacilli in slurry I might serve many roles. The discovery that metabolism of slurry I was more rapid than that of liquid I might be utilized to improve conversion of glycerol and lactic acid to 1,3-PD and acetic acid, respectively.

Ribosome sequencing (16S) revealed the species of microorganisms present in slurry I, e.g. lactobacilli, *Bifidobacteriacae*, etc. Lactobacilli constituted approximately 99% of slurry I organisms. Other bacteria, contributed approximately 1% of sequences, included members of *Acetobacteraceae*, *Bifidobacteriacae*, and unidentified bacteria (Figure 5.3). Two genera of lactobacilli present in slurry I accounted for approximately 91% of all sequences included *L. panis* (approximately 51%) and *L. helveticus* (approximately 41%). *L. panis* and *L. helveticus* have been identified and used in production of sour dough breadsand Swiss cheese, respectively (Arendt *et al*., 2007; Corsetti and Settanni, 2007; De Vuyst and Vancanneyt, 2007; Plessas *et al*.,

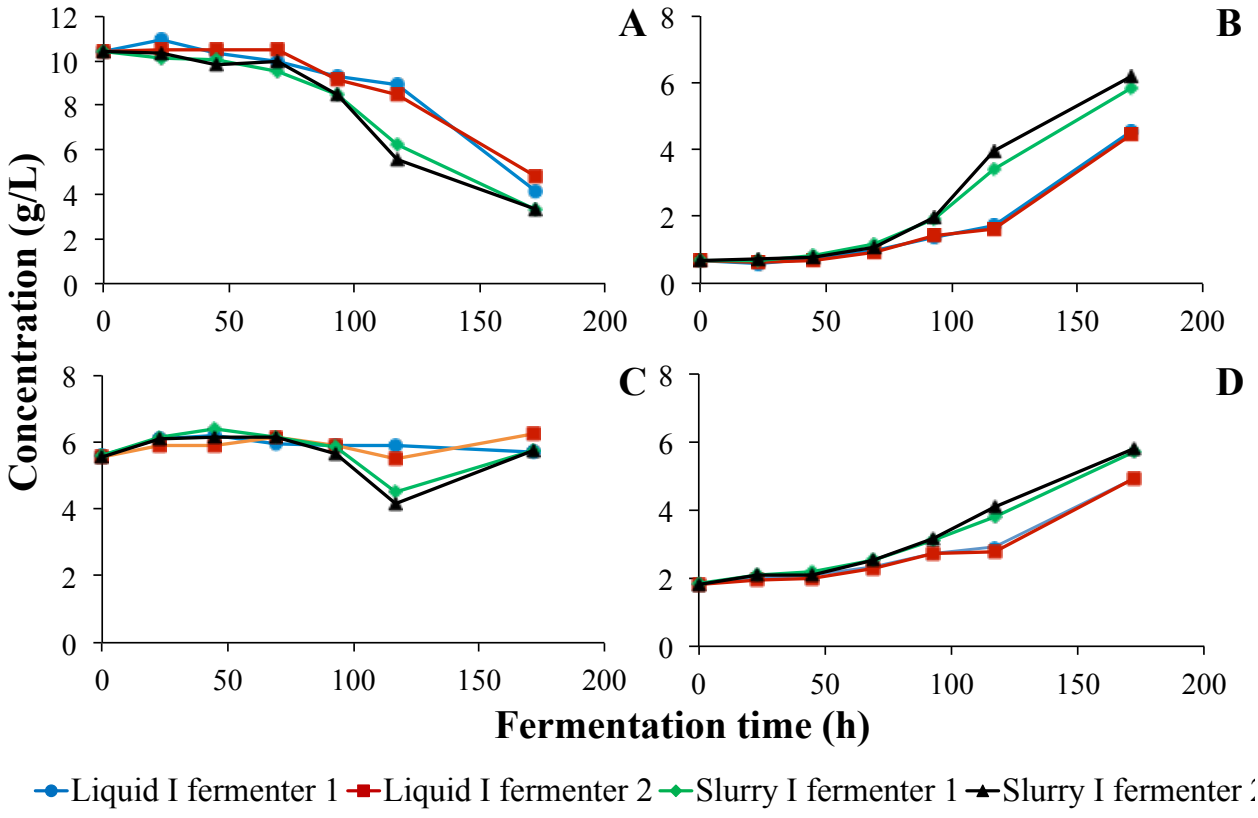

Figure 5.2 Metabolisms of (A) glycerol, (B) 1,3-PD, (C) lactic acid, and (D) acetic acid from small-scale TSF

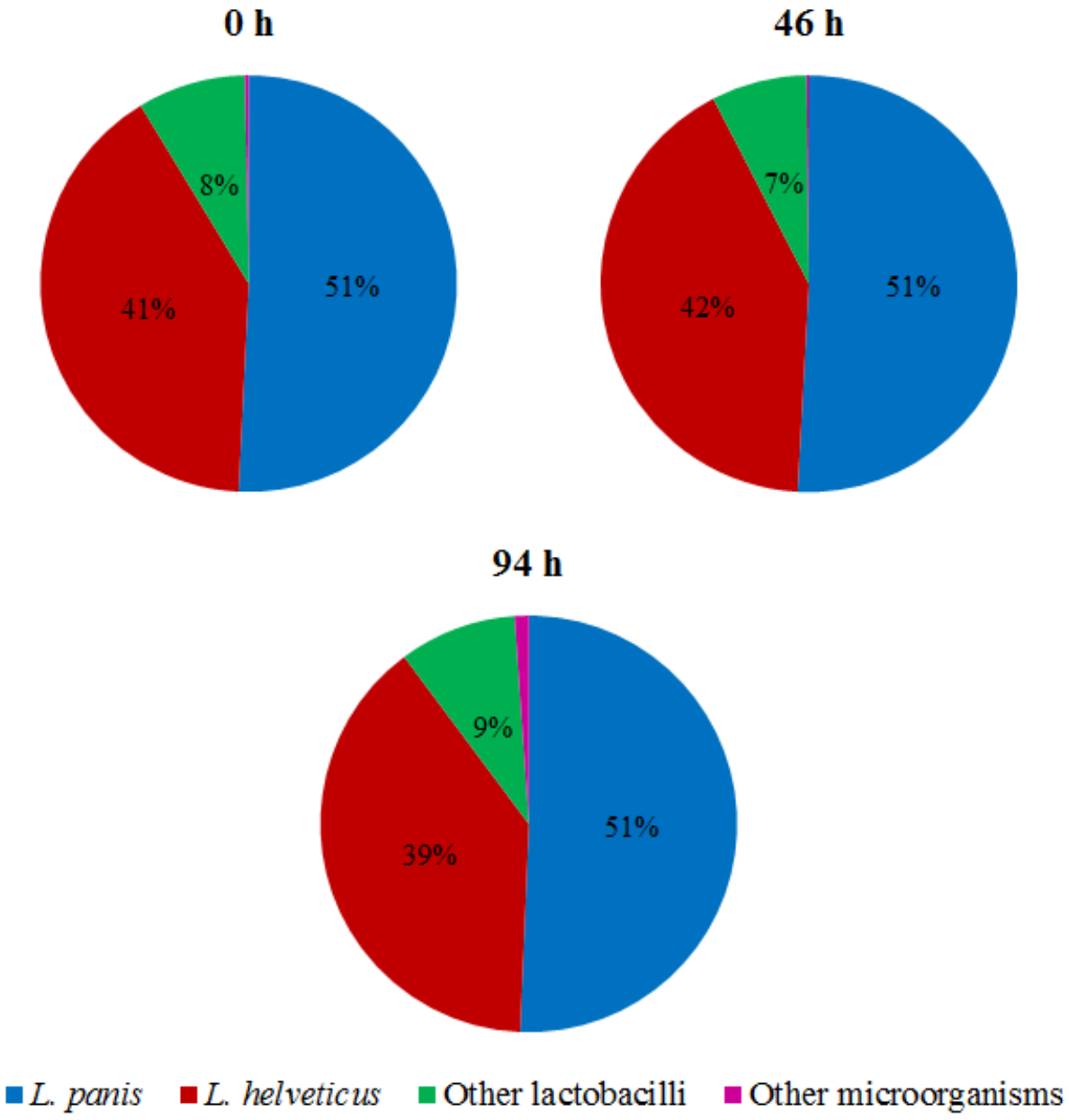

Figure 5.3 Graphical representation of main bacteria group present in slurry I in small-TSF. Other microorganisms indicated *Acetobacteraceae*, *Bifidobacteriacae*, and unidentified bacteria. Percentage was not indicated if less than 3%.

2008; Valence *et al*., 2000). Bacterial taxonomic classification indicates that slurry I might be considered potential as a probiotic material would require further investigations (Pedersen *et al*., 2004; Wine *et al*., 2009). In addition, genes encoding glycerol dehydratase and 1,3-PD oxidoreductase should be investigated in other lactobacilli present in slurry I to determine the ability of those lactobacilli to facilitate 1,3-PD production. Conversion of glycerol and lactic acid observed in the replications of small-scale fermentation indicated similar metabolic action (Figures C.2 and C.3).

5.4.3 Fermentation at 25 and 37 °C

The effect fermentation temperature was determined at 25 and 37 °C. At both temperatures, glycerol and lactic acid decreased and 1,3-PD and acetic acid increased and these conversions occurred more rapidly in slurry I than in liquid I (Figure 5.4 and Appendix C Figure C.4). Glycerol conversion to 1,3-PD at 37 °C was much faster than at 25 °C with full conversion occurring in half the time (50–60 h earlier). Khan *et al*. (2013) reported that the optimum temperature for *L. panis* PM1B growth and metabolism in de Man, Rogosa and Sharpe (MRS) media is 32–37 °C. In addition, 3-hydroxypropionaldehyde (3-HPA), an intermediate product conversion of glycerol to 1,3-PD was mostly observed at 25 °C (Figure 5.4 and Appendix C Figure C.4). There was no indication that 3-HPA impeded fermentation. This finding is in agreement with our observation that adding 3-HPA did not inhibit either 1,3-PD or acetic acid accumulation in Chapter 4. Rapid fermentation could benefit industrial scale fermentation by increasing 1,3-PD and acetic acid yield while decreasing fermentation time. Commercial fermenter volumes could be decreased by half and associated capital costs could be decreased. It is unlikely that heating the stillage would be a major cost as stillage exits the ethanol distillation unit at temperatures in excess of 37 °C. Taxonomic classification based on 16S ribosome sequences indicated that major microbial populations in slurry I from fermentation at 25 and 37 °C were comprised of lactobacilli which accounted for more than 93% of microorganisms (Figures 5.5 and 5.6). In addition, *L. panis* (33–38%) and *L. gallinarum* (33–37%) were major slurry I *Lactobacillus* species. *L. gallinarum* is a microorganism found in dairy products (Giraffa *et al*., 2010) and potentially could survive in the gastrointestinal tract and impact intestinal microbial metabolism (Fujiwara *et al*., 2001). Therefore, *L. gallinarum* could have utility as a probiotic *Lactobacillus* (Roy *et al*., 2000; Saito, 2004).

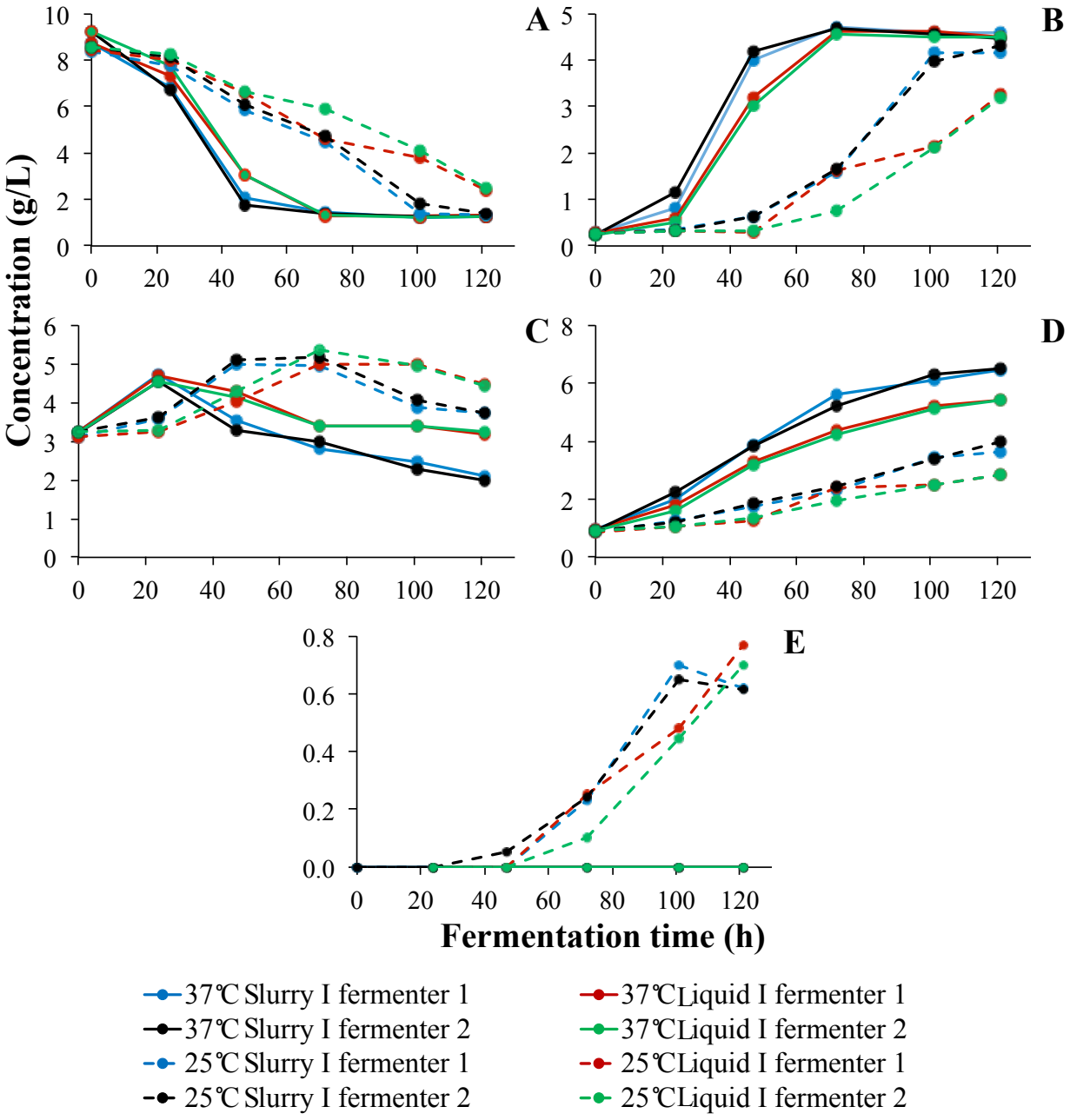

Figure 5.4 Concentration of small solutes in duplicate 25 L fermentation (A) glycerol, (B) 1,3-PD, (C) lactic acid, (D) acetic acid, and (E) 3-HPA when fermenting at 25 and 37 °C. The concentration of 3-HPA was under detection limit when fermenting W-TS at 37 °C.

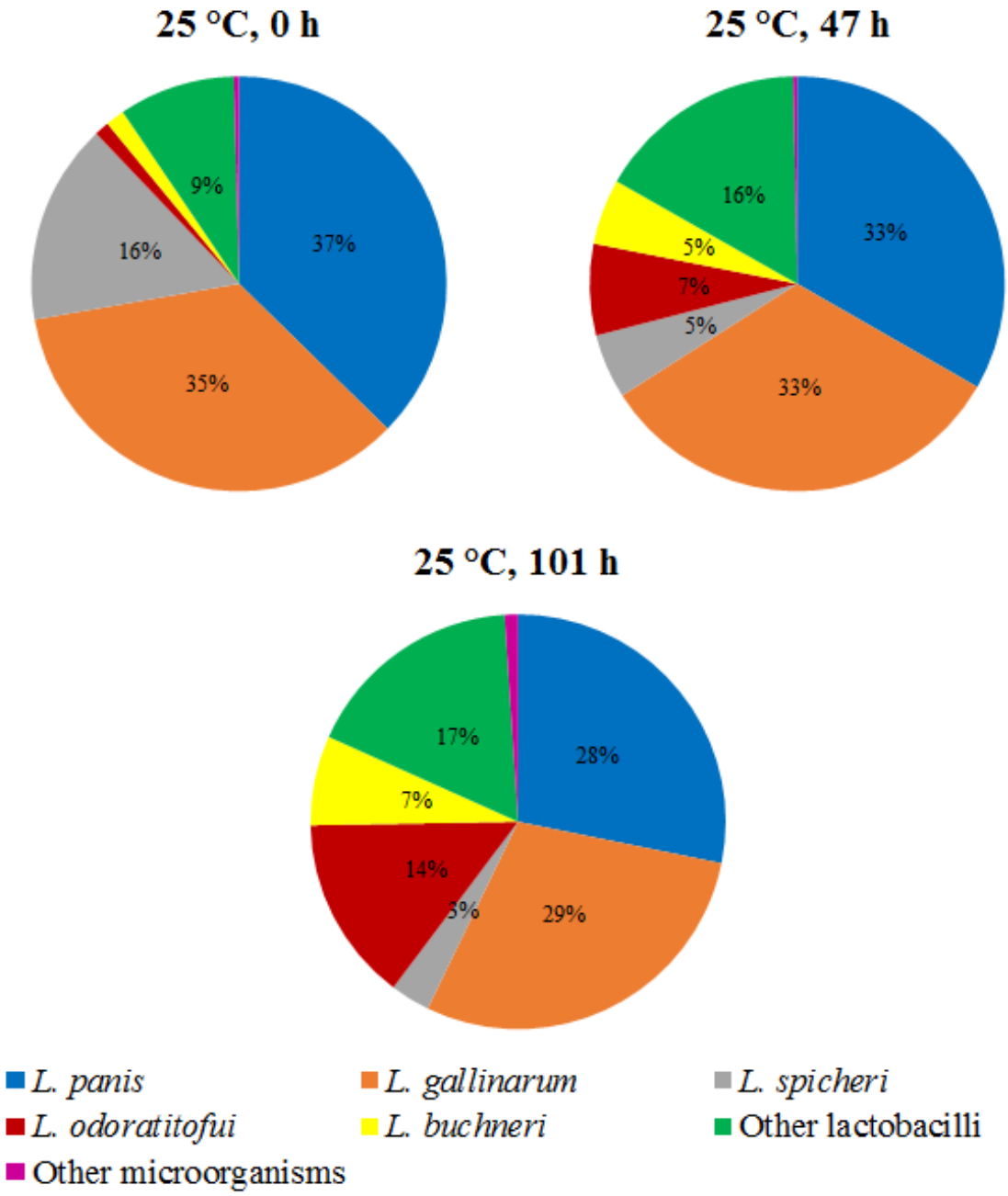

Figure 5.5 Graphical representation of main bacteria group present in slurry I from 25 °C fermentation. Other microorganisms indicated *Acetobacteraceae*, *Acinetobacter*, *Alicyclobacillus aciducaldarius*, *Bacillus*, *B. coagulans*, *Bifidobacteriacae*, *Chryseobacterium*, *Clostridiacae*, *Clostridium*, *Comamodaceae*, *Delftia*, *Enterococcus*, *Flectobacillus*, *Gluconobacter*, *Janthinobacterium lividum*, *Mogibacteriaceae*, *Pediococcus*, *P. acidilactici*, *Pedobacter*, *Prevotella*, *Rhodocyclaceae*, *Thermoanaerobacterium saccharolyticum*, *Xanthomonadaceae*, and unidentified bacteria. Percentage was not indicated if less than 3%.

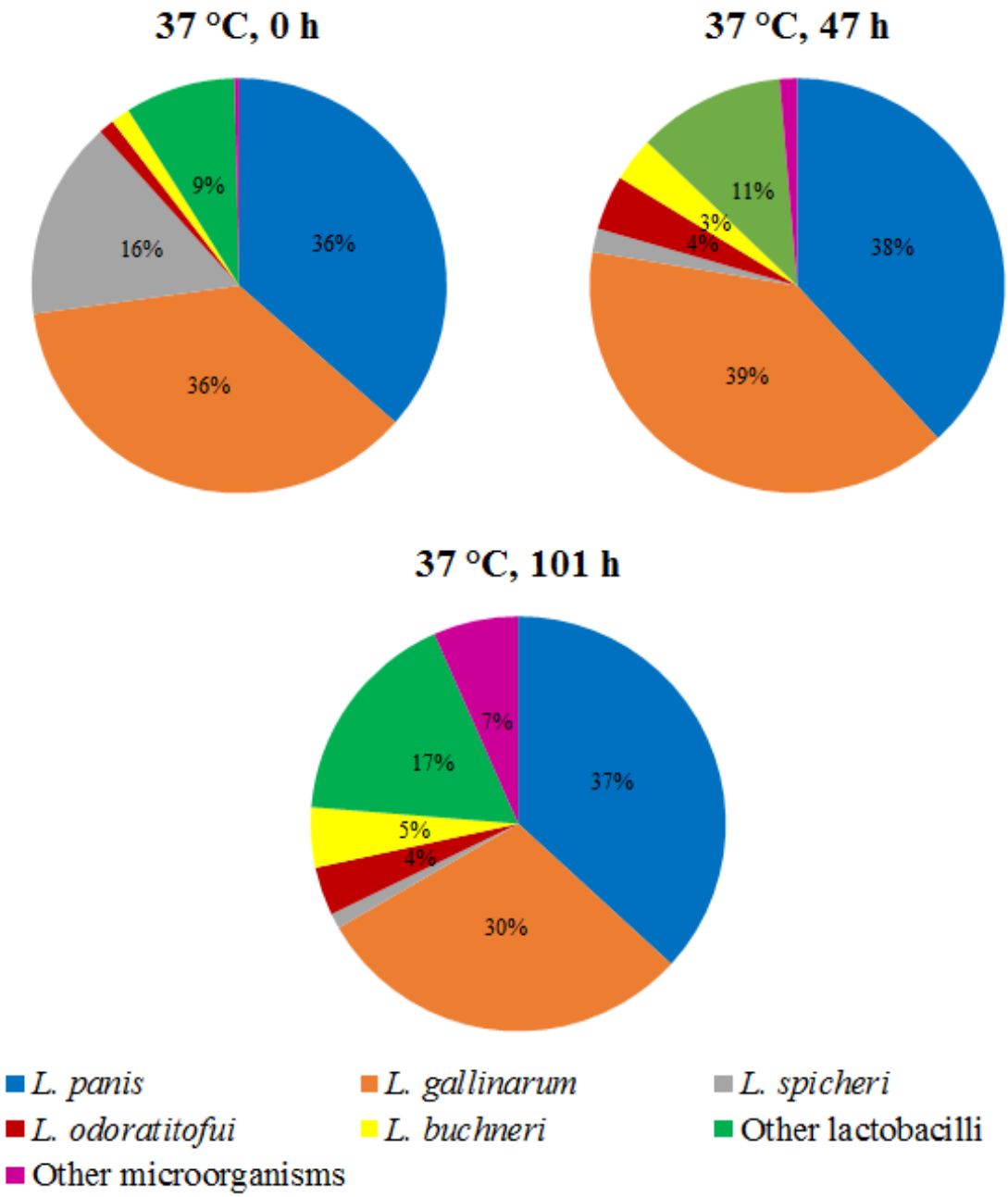

Figure 5.6 Graphical representation of main bacteria group present in slurry I from 37 °C fermentation. Other microorganism indicated *Acetobacteraceae*, *Acinetobacter*, *Alicyclobacillus aciducaldarius*, *Bacillus*, *B. coagulans*, *Bifidobacteriacae*, *Chryseobacterium*, *Clostridiacae*, *Clostridium*, *Comamodaceae*, *Delftia*, *Enterococcus*, *Flectobacillus*, *Gluconobacter*, *Janthinobacterium lividum*, *Mogibacteriaceae*, *Pediococcus*, *P. acidilactici*, *Pedobacter*, *Prevotella*, *Rhodocyclaceae*, *Thermoanaerobacterium saccharolyticum*, *Xanthomonadaceae*, and unidentified bacteria. Percentage was not indicated if less than 3%.

5.4.4 Progress of Small-Scale Fermentation

Early in TSF, slurry I settled and liquid I separated due to the greater slurry density (Figure 5.7A). As fermentation proceeded, slurry I floated and separated producing a more clear solution (Figure 5.7B). Finally, when fermentation ceased, slurry I sank to the bottom of the fermenter (Figure 5.7C).

Based on the predominance of lactobacilli in slurry I it would be expected that these organisms would produce CO_2 (anoxic gas) (Kang *et al*., 2013b, Kang *et al*., 2014b; Oude Elferink *et al*., 2001, Biebl *et al*., 1999) in Figure 5.8. At the beginning of fermentation, CO_2 production was insufficient to separate slurry from W-TS. Therefore, liquid I and slurry I separated partially due to gravity. However, as fermentation progressed lactobacilli produced sufficient CO_2 to assist coagulation, which was evident as slurry I layer floated in the fermentation medium. Burke (2000b) stated that anoxic gas produced from AGF process under anaerobic condition concentrated, floated, and returned bacteria, enzyme, organic acids, protein, and undigested substrates. Once floated, these materials could be skimmed and returned to the anaerobic digester (Burke, 2000b). As the duration of fermentation increased, nutrients consumed by lactobacilli were reduced until CO_2 production and fermentation ceased. The AGF ceased and slurry I precipitated in the fermenter. Solids floated after 45 and 22 h of fermentation in replicate 1 and 2, respectively. Fermentation ceased at 172 and 94 h in replicate 1 and 2, in that order.

For slurry I, as fermentation progressed moisture content decreased while protein content increased (Table 5.3 and Appendix C Table C.1). The opposite trend occurred with liquid I. At the end of TSF the protein content of slurry I was approximately 50% (w/w, db) compared to approximately 40% (w/w, db) for W-TS at 0 h. The higher protein concentration observed in slurry I indicated that a greater portion of protein present in fermentation medium at 0 h aggregated into slurry I. Aggregation might involve simple coalescence of protein rich particles or might involve microbial growth during fermentation and protein metabolism in solution and on particles. If slurry I protein content reflects high bacterial uptake and incorporation of protein by endemic *L. panis* and *L. helveticus* (Figure 5.3), it is possible that feed arising from slurry I produced from TSF could be considered to be a protein concentrate and it might have probiotic organisms when included in animal feed (Pedersen *et al*., 2004). Lactobacilli present in wet wheat distillers' grain have potential use as probiotics and *L. helveticus* in distillers' grain

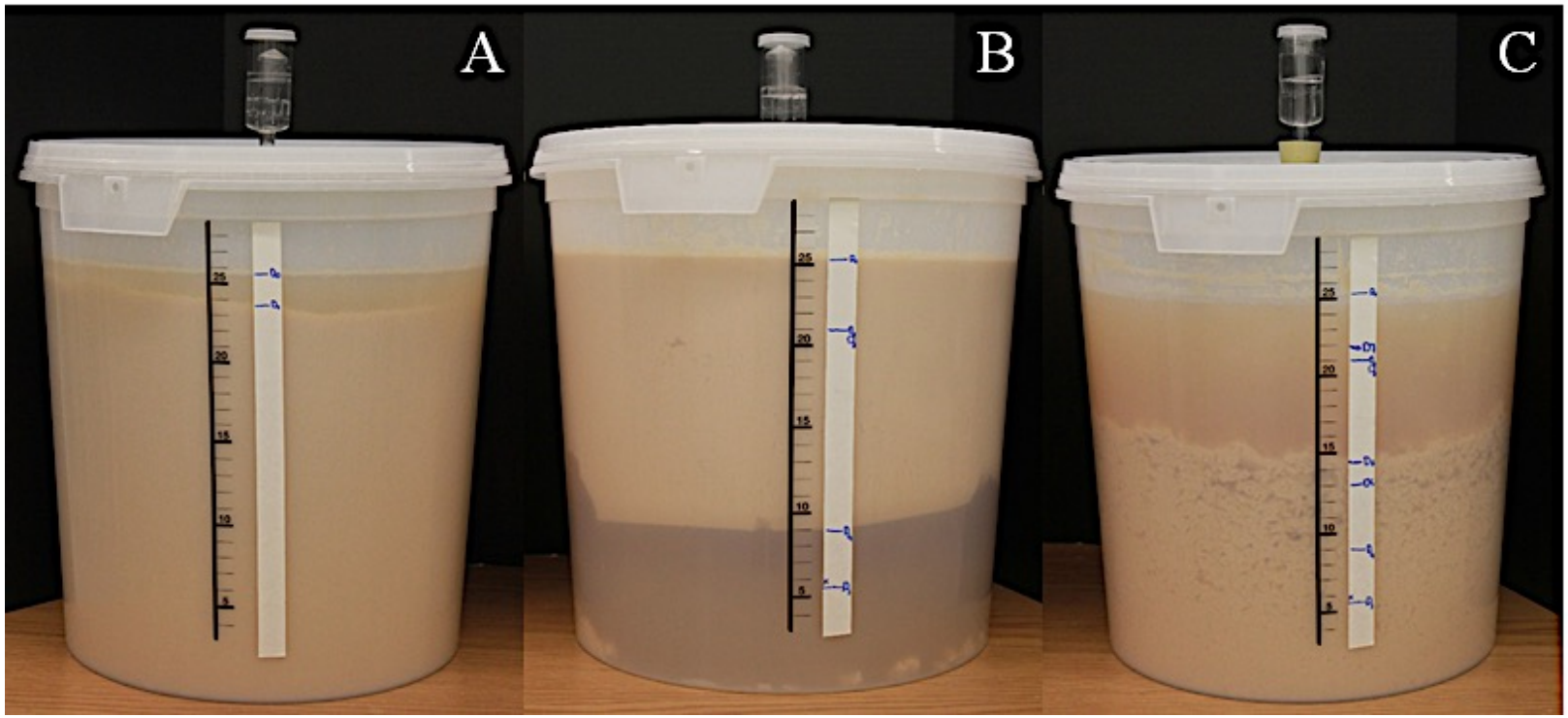

Figure 5.7 Fermentation stages in (A) 23 h, (B) 93 h, and (C) 172 h in small-scale TSF

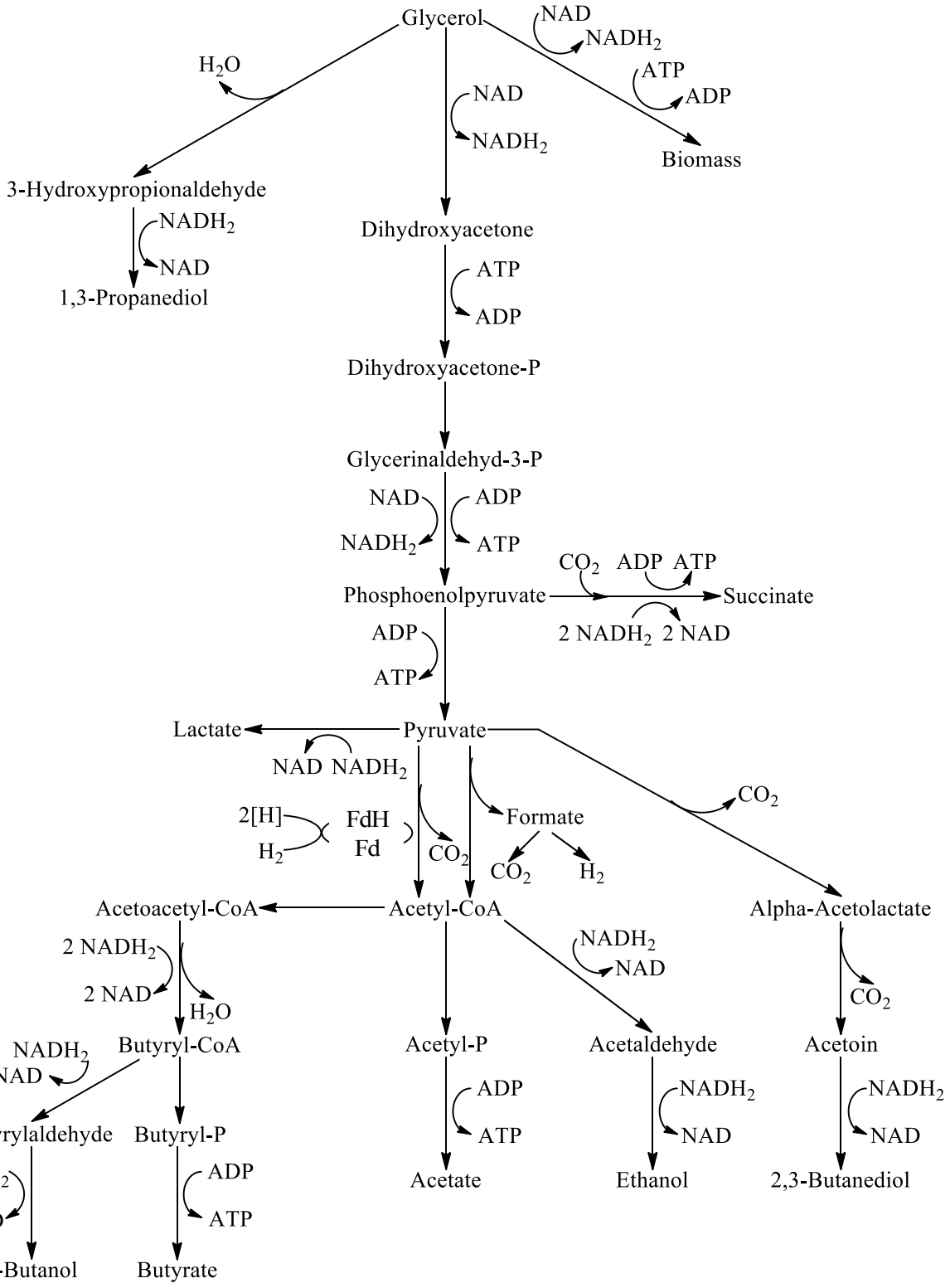

Figure 5.8 Biochemical pathways of glycerol fermentation. Source: Adapted with permission by Biebl *et al*. (1999) from Springer.

Table 5.3 Protein and moisture contents of TSF products from fermentation at 25 °C of small-scale fermentation

Sample	Hour of fermentation	Fermenter 1				Fermenter 2			
		Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)	Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Fermentation medium (W-TS)	0	0	91.89 ± 0.02	3.14 ± 0.04	38.66 ± 0.41	0	91.87 ± 0.03	3.14 ± 0.06	38.6 ± 0.9
Slurry I	23	2	91.68 ± 0.02	3.28 ± 0.07	39.5 ± 0.9	5	91.24 ± 0.00	3.45 ± 0.05	39.3 ± 0.5
Liquid I			96.87 ± 0.02	0.58 ± 0.01	18.4 ± 0.4		96.80 ± 0.02	0.54 ± 0.01	17.0 ± 0.2
Slurry I	45	5	90.97 ± 0.02	3.55 ± 0.07	39.3 ± 0.7	5	92.47 ± 0.02	2.84 ± 0.04	37.7 ± 0.6
Liquid I			96.31 ± 0.03	0.90 ± 0.01	24.3 ± 0.2		96.87 ± 0.02	0.58 ± 0.02	18.5 ± 0.4
Slurry I	69	7	89.73 ± 0.00	4.3 ± 0.1	42.0 ± 1.0	6.5	90.39 ± 0.04	4.17 ± 0.03	43.4 ± 0.1
Liquid I			96.60 ± 0.03	0.78 ± 0.01	23.0 ± 0.5		96.55 ± 0.07	0.83 ± 0.01	24.0 ± 0.1
Slurry I	93	10	88.82 ± 0.05	5.27 ± 0.01	47.12 ± 0.07	9	89.21 ± 0.05	5.00 ± 0.00	46.3 ± 0.2
Liquid I			96.74 ± 0.05	0.83 ± 0.04	26.0 ± 2.0		96.83 ± 0.01	0.78 ± 0.01	24.5 ± 0.3
Slurry I	117	14	87.47 ± 0.02	6.08 ± 0.04	48.5 ± 0.4	13	87.2 ± 0.1	6.30 ± 0.01	49.3 ± 0.6
Liquid I			96.94 ± 0.00	0.73 ± 0.01	23.8 ± 0.3		97.02 ± 0.05	0.65 ± 0.00	21.8 ± 0.5
Slurry I	172	10	89.98 ± 0.03	4.6 ± 0.2	46.0 ± 2.0	10	89.79 ± 0.01	4.75 ± 0.02	46.5 ± 0.3
Liquid I			97.23 ± 0.02	0.49 ± 0.00	17.7 ± 0.2		97.25 ± 0.07	0.50 ± 0.02	18.0 ± 1.0

Each value is presented as the mean ± SD (n = 2).

There was no sampling at 141 h of fermentation as the solution was settling at this time. Therefore, the fermentation medium was left for 24 h to let the slurry I precipitate.

inhibited *Campylobacter jejuni* invasion of human intestinal epithelial cell cultures (Wine *et al*., 2009). The volume of liquid I increased as fermentation time increased. In total, approximately 28−40% of clarified liquid or 7–10 L of liquid I were obtained from 25 L of fermentation medium. The volume of low turbidity solution released was substantially greater than produced by additives, centrifugation, filtration, and size exclusion in earlier studies (Chapter 3). This confirmed the potential for using TSF for W-TS clarification without using additives.

TSF could lower processing costs for recovering materials from W-TS. Two actions of bacterial growth may aid in this separation and density increase. First, lactobacilli produce bubbles of anoxic CO_2 gas when metabolizing glucose and other carbohydrates, bubbles attach to suspended particles to form aggregates that float to the fermenter surface (Figure 5.9). Bubbles not only induce particle aggregation but can also be used with coagulants to collapse solution colloids (Edzwald, 1995). Second, many *Lactobacillus* sp. also produce EPS (Badel *et al*., 2011). *L. panis* strains have been identified that produce levansucrose, which changes rheological properties and might act as a stabilizer or stimulate preferential microbial growth (Waldherr *et al*., 2008). Genome sequences of *L. panis* PM1B, retrieved from the Rapid Annotation using Subsystem Technology database shows that genes for EPS biosynthesis are present including; EPS biosynthesis transcriptional activator EpsA, glycosyl transferase, group 2 family protein, manganese-dependent protein-tyrosine phosphatase, tyrosine-protein kinase EpsD, and tyrosine-protein kinase transmembrane modulator EpsC, undecaprenyl-phosphate galactosephosphotransferase. Similar sequences were also identified in *L. acidophilus* NCFM, *L. casei* BL23, *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365, *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, *L. fermentum* IFO 3956, *L. gasseri* ATCC 33323, *L. helveticus* DPC 4571, *L. johnsonii* NCC 533, *L. plantarum* WCFS1, *L. reuteri* F275, and *L. reuteri* JCM 1112. However, the EPS produced by *L. panis* PM1B has not been characterized or quantified.

5.4.5 Ultrafiltration of W-TS and Liquid I

The flux and volume concentration through ultrafiltration membranes were determined for unfermented W-TS (0 h) and samples of liquid I collected as fermentation progressed (Figure 5.10) and Appendix C Figure C.5). The transmembrane flux unfermented W-TS (31 $L/M^2/h$) was initially high but decreased rapidly as filtration progressed. Colloids present in W-TS prevented effective volume concentration. This finding was in good agreement with Porter (1972) who

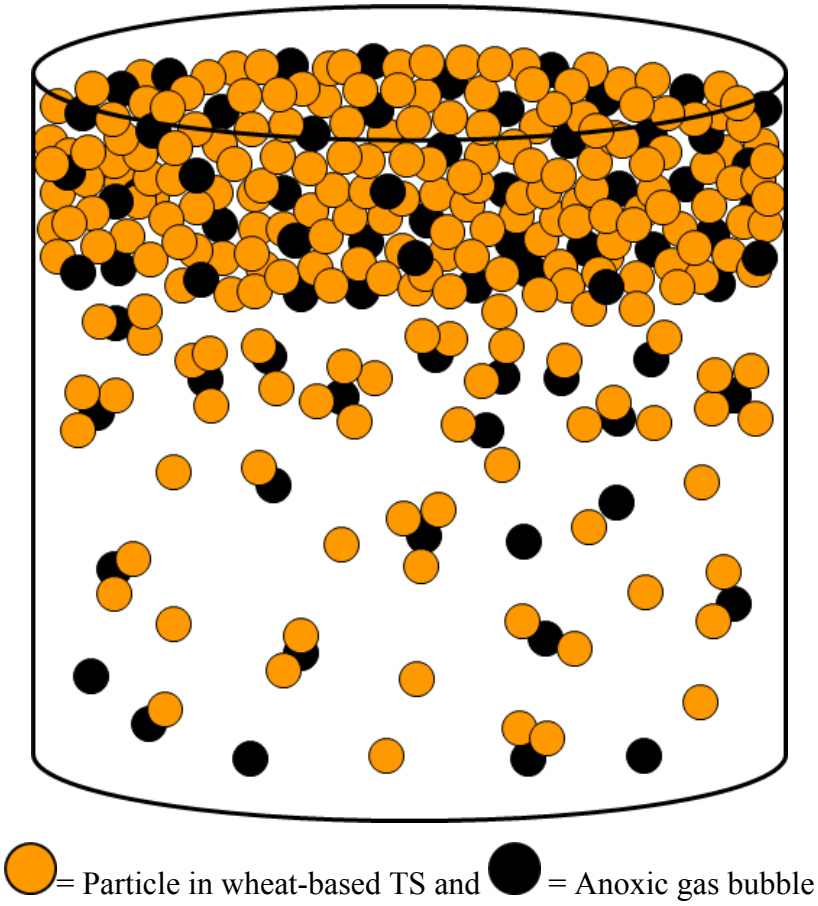

Figure 5.9 Diagram of AGF process.

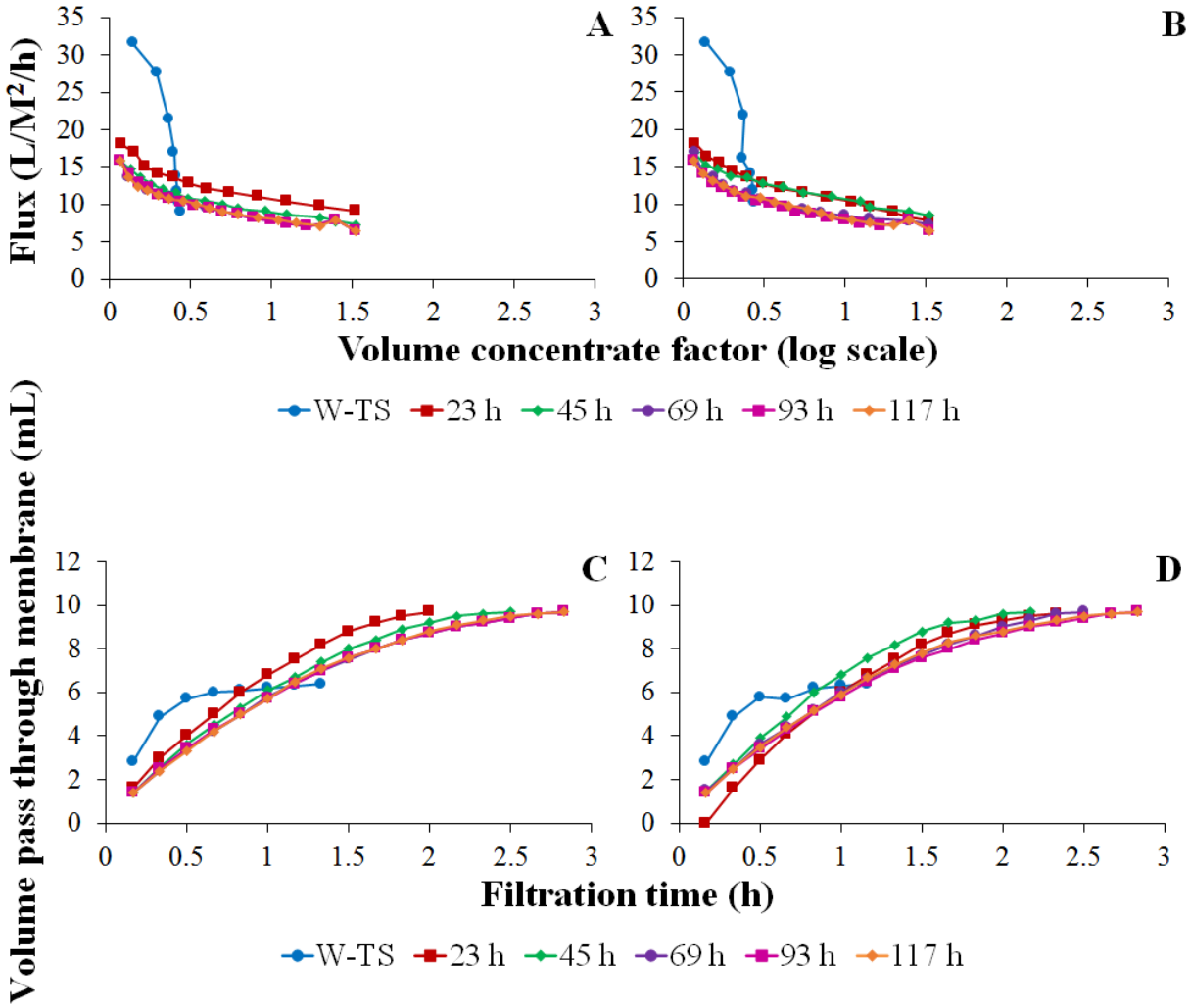

Figure 5.10 Transmembrane (10 kDa MWCO) flux of liquid I from (A) fermenter 1 and (B) fermenter 2. Filtered volume of liquid I passed through a 10 kDa MWCO membrane of liquid I from (C) fermenter 1, and (D) fermenter 2. The time in the legend presents the fermentation time.

noted that colloids or macromolecules that deposited on membranes led to reversible flux losses. The transmembrane flux of liquid I was substantially lower than W-TS flux, but flux remained constant during liquid I volume concentration. As most of liquid I passed through the membrane, liquid I is mostly a solution and not a strong colloid. Liquid I was approximately 3–5% (w/w) solid (Table 5.3 and Appendix C Table C.1) and contained small solutes and may also contain macromolecules. Liquid I filtrate protein content (Bradford assay) was less than 0.5 g/L (Table 5.4 and Appendix C Table C.2). It is possible that protein concentration was as low as indicated or that proteins present did not bind Coomassie Blue dye. Protein content, determined by dye binding, cannot be directly compared with protein content determined by nitrogen (Kjeldahl protein assay). The dye binds weakly with histidine, lysine, phenylalanine, tryptophan, and tyrosine residues. In addition, the Bradford assay is insensitive to peptides with masses below 3–5 kDa. Amino acids and smaller peptide, therefore, would not bind with the dye (Lucarini and Kilikian, 1999).

5.4.6 Protein and Moisture Content of TSF Products

The separation of slurry I and liquid I from fermentation at 25 and 37 °C occurred due to gravity and anoxic gas produced from lactobacilli as previous described. Protein and moisture contents of liquid I and slurry I indicate that as fermentation progressed, protein content increased and moisture content decreased. However, the results were opposite with liquid I (Tables 5.5 and 5.6 and Appendix C Tables C.3 and C.4). Aggregation was likely induced by anoxic gas and EPSs as previously described.

In addition, the quantity of liquid I from fermentation at 25 and 37 °C was approximately 8–14 L accounting for 32–56% liquid-solid separation. Moreover, protein content at the end of TSF increased to approximately 50% (w/w, db) with fermentation at 25 and 37 °C. These results are similar to those for moisture content and quantity of liquid I from small-scale fermentation at 25 °C (Table 5.3 and Appendix C Table C.1).

5.4.7 Replications of Small-Scale TSF

During fermentation, particle floatation produced low turbidity solutions in all twelve fermenters (Table 5.7 and Appendix Table C.5). AGF using autogenic gas likely aided in breaking colloids and clearing solution.

Table 5.4 Protein content (g/L) of filtrate from ultrafiltration of liquid I from fermentation at 25 °C of small-scale fermentation at different fermentation time

Container	0 h	23 h	45 h	69 h	93 h	117 h
Fermenter 1	0.39 ± 0.01	0.21 ± 0.00	0.20 ± 0.02	0.22 ± 0.00	0.19 ± 0.00	0.19 ± 0.00
Fermenter 2	0.41 ± 0.01	0.21 ± 0.00	0.20 ± 0.01	0.21 ± 0.00	0.19 ± 0.01	0.19 ± 0.00

Each value is presented as the mean ± SD (n = 2).

Table 5.5 Protein and moisture contents of TSF products from fermentation at 25 °C

Sample	Hour of fermentation	Fermenter 1				Fermenter 2			
		Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)	Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Fermentation medium (W-TS)	0	0	93.80 ± 0.01	2.46 ± 0.02	39.6 ± 0.3	0	93.60 ± 0.00	2.51 ± 0.01	39.2 ± 0.2
Slurry I	24	5.5	92.93 ± 0.00	3.01 ± 0.09	43 ± 1	5	92.73 ± 0.01	3.17 ± 0.05	43.6 ± 0.6
Liquid I			97.15 ± 0.00	0.50 ± 0.02	17.6 ± 0.6		97.12 ± 0.01	0.57 ± 0.02	19.7 ± 0.7
Slurry I	47	8	92.13 ± 0.00	3.52 ± 0.02	44.7 ± 0.3	7	92.04 ± 0.01	3.8 ± 0.1	47 ± 1
Liquid I			97.32 ± 0.00	0.51 ± 0.02	19.2 ± 0.7		97.29 ± 0.00	0.53 ± 0.03	20 ± 1
Slurry I	72	10	92.56 ± 0.00	3.8 ± 0.1	51 ± 2	11.5	91.77 ± 0.00	3.83 ± 0.06	46.5 ± 0.7
Liquid I			97.43 ± 0.00	0.51 ± 0.01	20.0 ± 0.5		97.36 ± 0.01	0.54 ± 0.01	20.6 ± 0.4
Slurry I	101	10.5	91.20 ± 0.04	4.33 ± 0.03	49.2 ± 0.5	12.5	91.65 ± 0.01	3.97 ± 0.02	47.5 ± 0.3
Liquid I			97.56 ± 0.02	0.51 ± 0.02	21.0 ± 0.6		97.49 ± 0.00	0.51 ± 0.03	20 ± 1
Slurry I	121	11	91.64 ± 0.00	4.07 ± 0.02	48.7 ± 0.3	12.5	90.71 ± 0.01	4.57 ± 0.02	49.3 ± 0.3
Liquid I			97.57 ± 0.00	0.48 ± 0.02	19.8 ± 0.7		97.48 ± 0.01	0.54 ± 0.01	21.4 ± 0.5

Each value is presented as the mean ± SD (n = 2).

Table 5.6 Protein and moisture contents of TSF products from fermentation at 37 °C

Sample	Hour of fermentation	Fermenter 1				Fermenter 2			
		Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)	Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Fermentation medium (W-TS)	0	0	93.61 ± 0.00	2.53 ± 0.01	39.7 ± 0.1	0	93.80 ± 0.00	2.46 ± 0.04	39.6 ± 0.6
Slurry I	24	9.5	92.65 ± 0.01	3.34 ± 0.01	45.4 ± 0.2	7	92.60 ± 0.00	3.26 ± 0.06	44.0 ± 0.8
Liquid I			97.36 ± 0.00	0.56 ± 0.00	21.1 ± 0.1		97.37 ± 0.02	0.50 ± 0.01	19.0 ± 0.3
Slurry I	47	12	93.16 ± 0.00	3.24 ± 0.02	47.4 ± 0.3	12	93.07 ± 0.00	3.26 ± 0.04	47.0 ± 0.6
Liquid I			97.50 ± 0.00	0.50 ± 0.02	20.2 ± 0.8		97.55 ± 0.01	0.47 ± 0.02	19.2 ± 0.7
Slurry I	72	13.5	91.33 ± 0.04	4.33 ± 0.07	50.0 ± 0.5	14	92.14 ± 0.01	3.85 ± 0.02	49.0 ± 0.2
Liquid I			97.62 ± 0.01	0.53 ± 0.03	23 ± 1		97.66 ± 0.01	0.53 ± 0.03	23 ± 1
Slurry I	101	13.5	92.24 ± 0.00	3.98 ± 0.01	51.3 ± 0.2	15	90.30 ± 0.00	5.03 ± 0.03	51.9 ± 0.3
Liquid I			97.77 ± 0.01	0.52 ± 0.01	23.4 ± 0.2		97.84 ± 0.01	0.49 ± 0.03	23 ± 1
Slurry I	121	13.5	92.45 ± 0.02	3.84 ± 0.02	50.9 ± 0.4	14	90.45 ± 0.01	5.08 ± 0.09	53.2 ± 0.9
Liquid I			97.91 ± 0.01	0.50 ± 0.01	24.1 ± 0.4		97.92 ± 0.01	0.47 ± 0.01	22.5 ± 0.3

Each value is presented as the mean ± SD (n = 2).

Table 5.7 Protein and moisture contents of small-scale TSF of twelve fermenters

Sample	Hour of fermentation	Fermenter 1				Fermenter 2			
		Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)	Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Fermentation medium (W-TS)	0	0	90.95 ± 0.00	3.74 ± 0.06	41.3 ± 0.6	0	90.74 ± 0.01	3.82 ± 0.09	41 ± 1
Slurry I	22	4	90.40 ± 0.01	4.0 ± 0.1	42 ± 1	2	90.19 ± 0.02	4.37 ± 0.09	45 ± 1
Liquid I			95.86 ± 0.00	1.09 ± 0.04	26.4 ± 0.9		95.91 ± 0.00	1.12 ± 0.03	27.3 ± 0.7
Slurry I	46	9	86.59 ± 0.02	6.10 ± 0.09	45.5 ± 0.7	7	87.40 ± 0.04	5.74 ± 0.03	45.6 ± 0.1
Liquid I			95.79 ± 0.00	1.08 ± 0.01	25.6 ± 0.2		95.98 ± 0.02	1.05 ± 0.04	26.1 ± 0.8
Slurry I	94	7	88.47 ± 0.00	5.35 ± 0.00	46.40 ± 0.02	7	88.64 ± 0.01	5.1 ± 0.1	45 ± 1
Liquid I			96.35 ± 0.01	0.84 ± 0.00	23.15 ± 0.09		96.32 ± 0.00	0.84 ± 0.02	22.8 ± 0.7

Each value is presented as the mean ± SD (n = 2).

There was no sampling at 77 h of fermentation as the solution was settling at this time. Therefore, the fermentation medium was left for 17 h to let the slurry I precipitate.

While particle flotation occurred during early stages of fermentation processes, settling occurred after approximately 46 and 100 h for replicate 1 and 2, respectively. The settling completed after 94 and 167 h for replicates 1 and 2 (out of twelve fermenters), in that order, confirming that fermentation had ceased. At the end of TSF, there were 67 L (22%) and 83 L (28%) of liquid I from replicate 1 and replicate 2, respectively, from 300 L of fermentation medium. The protein content of slurry I was higher than that of liquid I (Table 5.7 and Appendix C Table C.5). In addition, liquid I had higher moisture content than slurry I. At the end of TSF, slurry I protein content increased (44–47%, w/w, db) compared to original medium before fermentation (40–42%, w/w, db). Thus, most protein from the fermentation medium was recovered from W-TS in slurry I.

5.5 Conclusion

W-TS is a colloidal solution with organic solutes. Valuable compounds present in W-TS were 1,3-PD, acetic acid, and GPC. Unfortunately, these valuable compounds cannot be extracted from W-TS easily due to colloids remaining from yeast fermentation and high boiling point and hygroscopic solutes. Colloidal materials were converted to slurries during TSF using endemic populations of lactobacilli. This fermentation converts glycerol and lactic acid (high boiling point and hygroscopic solutes) to 1,3-PD and acetic acid (lower boiling solutes), respectively while maintaining GPC content. W-TS was processed using TSF at both 25 and 37 °C. Therefore, these three potentially valuable compounds may be extracted and recovered. Fermentation time was substantially reduced when fermenting W-TS at 37 °C. Fermentation at higher temperatures could save processing space and, thereby, reduce the need for capital investment in large volume fermenters. Interestingly, 16S ribosome sequencing of slurry I revealed that *L. panis, L. helveticus*, and *L. gallinarum* were the major endemic microorganisms and lactobacilli accounted for more than 93% of microbial populations.

Fermentative coagulation was a novel and efficient method to clarify fermentation medium using anoxic gas produced from endemic flora present in W-TS. Approximately 22–56% separation of liquid I and slurry I from fermentation medium could be achieved using TSF. The liquid from TSF could be ultrafiltered through a 10 kDa MWCO membrane in a high shear cell without membrane fouling. Therefore, liquid from TSF is a weak colloid or not colloidal, contains small solutes, and is largely free of particulate matter. Consequently, it is suggested that

TSF could be utilized as a preparation step for removing particles prior to filtration. Slurry I from TSF product had a higher protein content (approximately 50%, w/w, db) than that of W-TS (38–43%, w/w, db). Microbial growth during fermentation concentrated the protein. In addition, approximately 93% of microbial population were lactobacilli. Due to its high protein content and microbial population, it might be possible to utilized slurry I as a protein source in animal feed. Further studies will explore the use of commercial separation tools for separation of protein rich slurries produced by TSF.

5.6 Brief Introduction to Chapter 6

Glycerol and lactic acid were major organic solutes present in W-TS but are readily converted to 1,3-PD and acetic acid for subsequent extraction and recovery. W-TS also contained colloids and particles, which could introduce problems such as equipment fouling that blocks membranes and limits heat transfer during compound extraction and recovery processes. TSF is a novel and efficient method for particle-solid separation. It utilizes anoxic gas produced from microorganisms present in W-TS during TSF to separate particles. Liquid I from TSF had substantially fewer particles compared with W-TS and could be filtered to obtain clear solutions for compound recovery without indication of membrane blocking or fouling. In addition, slurry I from TSF had a high protein content (approximately 50%), which was higher than that of W-TS. The protein concentration might be the result of microorganism growth. Furthermore, major microorganisms present in slurry I were *L. panis*, *L. gallinarum* and *L. helveticus* which are found in sour dough, dairy products, and Swiss cheese. The high protein content and presence of lactobacilli in slurry I indicate that it would have utility as a protein source for animal feed. Additionally it is possible that slurry I could have probiotic microorganisms when fed to animals. The protein content of slurry I likely can be improved and valuable compounds remaining in liquid I surrounding particles in slurry I could be recovered by washing the slurry. Therefore, washing may be an essential process to produce protein concentrate and recover valuable compounds remaining in slurry I.

CHAPTER 6
PROTEIN CONCENTRATE PRODUCTION AND COMPOUND RECOVERY

6.1 Abstract

Sequential fermentation of saccharified wheat first with *Saccharomyces cerevisiae* and subsequently with a consortium of endemic lactobacilli or two-stage fermentation (TSF) produced anoxic gas (CO_2) and induced colloid separation. High-moisture-protein-rich slurry (90% moisture and 50% protein on a dry basis, db) was isolated from wheat-based thin stillage (W-TS) by either decanting solution or skimming floating material. Slurries were obtained by decanting in a centrifuge to remove entrained spent TSF medium with lower protein content than suspended solids. Processes that involved centrifugation treatments or washing the slurry were tested to increase slurry protein content. Washed slurries, were rich in protein and hereafter, are called protein concentrate. Dry matter and protein of approximately 13–14% (w/w) and 60% (w/w, db) were typically achieved by slurry centrifugation in a decanter. In addition, soluble compounds were present in free solution removed from slurry by washing. Solution solute content decreased with each wash of the slurry. The centrifugation and washing processes improved slurry protein and could improve recovery of valuable entrained solutes including 1,3-propanediol (1,3-PD), acetic acid, and glycerophosphorylcholine (GPC). The protein concentrate might have utility as a protein source for animal feed. Liquid from TSF and slurry washing was readily filtered through a cross flow filtration unit with 0.2 μm membrane pore size. There was no indication of decreasing flux and membrane fouling during microfiltration. Moreover, microfiltration retentate 1,3-PD, acetic acid, glycerol, GPC, lactic acid, and protein contents were slightly higher than in the corresponding permeate. Therefore, microfiltration partially concentrates protein and soluble compounds. The recoveries of protein, glycerol, 1,3-PD, lactic acid, acetic acid, and GPC from TSF were 66, 76, 72, 77, 74, and 84%, respectively. Protein concentrate was dried with a spray drier or drum drier or tray drier to a water activity

of 0.23–0.30. The protein concentrate produced from tray drying had low lysine content but high lysine availability (95%), and low heavy metal content. Lysine supplements may be necessary prior to use as a protein source for animal feed.

6.2 Introduction

The production of ethanol by yeast fermentation followed by distillation produces a dilute aqueous stream (thin stillage, TS) of inorganic salts, organic solutes, and suspended particles (Meredith, 2003; Ratanapariyanuch, 2009; Ratanapariyanuch *et al*., 2011). Water in TS can be evaporated to concentrate TS to distillers' soluble (DS). DS could be sold or mixed with wet grain to produce dried distillers' grain with solubles (DDGS), which can be used as animal feed (Ingledew *et al*., 2009). However, DS is inexpensive. For example, the price of corn-based DS varied from CAD $ 7.4 − 51.8/tonne depending on ethanol manufacturer (Hoppe, 2010). In addition, the TS concentration by evaporation is not energy efficient as this process consumes approximately 40−45% of thermal energy and 30−40% of electrical energy utilized in dry grind ethanol production facilities (Wilkins *et al*., 2006). Therefore, research has been conducted to utilize corn-based TS and wheat-based thin stillage (W-TS) as an animal feed (Ham *et al*., 1994; Mustafa *et al*., 2000). Some ethanol industries utilize W-TS as a water source for animals (e.g. Pound-Maker Agventures, Lanigan, SK, Canada). The use of energy for water evaporation is greatly reduced with this practise when compared to more conventional production practises.

As previously stated, corn-based DS can be utilized as an animal feed ingredient. Corn-based DS contains essential amino acids and approximately 18−23% protein (db). Lysine varied from 0.8−1.3% of protein (Belyea *et al*., 1998). Corn-based DS lysine content is lower than in soy protein concentrates (4.23%) (ADM, 2015a, 2015b).

In Chapter 5, clarification of W-TS was described. Slurry obtained from TSF contained high protein (approximately 50%, db, Chapter 5) with lactobacilli contributing more than 93% of endemic flora present in the slurries. These observations indicate that slurry I had higher protein than W-TS and that TSF could be used to produce W-TS protein concentrates. The high protein fraction from TSF could be utilized as protein source for animal feed. Lactobacilli are considered non-toxic bacteria and could have probiotic effects, as described in Pedersen *et al*. (2004), when the slurry is utilized as an animal feed protein source. However, slurry moisture content could

increase shipping costs and lead to spoilage. Furthermore, valuable solutes dissolved in liquids surrounding the slurry could be recovered from the protein concentrate before drying.

Slurry washing involves centrifugation in a decanter then mixing slurry with water followed by centrifugation (mixture) and decanting again. Centrifugation increases the force on particles many fold, which accelerates sedimentation (Geankoplis, 2003c). Centrifugation can generate pressure that presses liquid surrounding particles and generates sediment with increased density. Water added to sediments could extract soluble compounds from liquid surrounding centrifuged sediments and enable their recovery. These methods were also utilized to recover bacteria for DNA extraction as employed by Steffan *et al*. (1988).

Liquid I and liquid fractions from washing could be combined for compound extraction and compound recovery. From Chapter 4 and 5, it was observed that 1,3-propanediol (1,3-PD) and acetic acid increased while glycerol and lactic acid decreased. If 1,3-PD and acetic acid were extracted from TSF liquid fractions and slurry wash water, the water after compound extraction would be less acidic (less acetic acid). Both acetic and lactic acids cause yeast death or stress (Ingledew, 2009a). Therefore, liquid recovered after TSF and compound extraction would be better than backset to recycle water to ethanol production as it has less acetic acid and lactic acid.

After washing, slurries from TSF contained approximately 86–87% moisture. Therefore, drying processes are necessary to prevent spoilage and reduce shipping costs. Three drying methods were studied: spray drying, drum drying, and tray drying. The spray drier creates droplets from solution by passing it through a nozzle at high pressures. Solute in the droplets migrates to droplet surfaces (Deegan, 2000; Deegan *et al*., 2000) producing dried products. Drum driers utilize steam applied to the inner surface of a drum that heats the drum surface. Moist material contacting the drum coats its surface and it reaches the boiling point and water evaporates quickly (Tang *et al*., 2003). Tray drying dries materials by diffusion that transports moisture to material surfaces to be evaporated (Doymaz, 2004).

Water activity (a_w) for growth of microorganisms in feed/food (Tables 6.1 and 6.2) could be utilized to predict feed/food spoilage. The spoilage of feed/food could be prevented when feed/food has low a_w. Therefore, it is expected that dried protein concentrate would have low a_w which could extend its shelf life.

After drying, dried protein concentrate will be investigated for its potential utility as a protein source for animal feed e.g. proximate analysis, amino acid profile, lysine availability,

Table 6.1 Minimal a_w for growth of food-borne bacterial pathogens (optimum pH and temperature)

Microorganism	a_w
Campylobacter jejuni	0.990
Aeromonas hydrophila	0.970
Clostridium botulinum E	0.965
C. botulinum G	0.965
Shigella spp.	0.960
Yersinia enterocolitica	0.960
C. perfringens	0.945
C. botulinum A and B	0.940
Salmonella spp.	0.940
Escherichia coli	0.935
Vibrio parahaemolyticus	0.936
Bacillus cereus	0.930
Listeria monocytogenes	0.920
Staphylococcus aureus (anaerobic)	0.910
S. aureus (aerobic)	0.860

Source: Adapted with permission by Chirife (1993) from Elsevier.

Table 6.2 Minimal a_w for growth of moulds of public health concern (Modified from Beuchat, 1981)

Mould	a_w
Aspergillus flavus	0.78–0.80
A. clavatus	0.85
A. parasiticus	0.82
A. orchraceus	0.77–0.83
Byssochlamys nivea	0.84
Penicillium cyclopium	0.81–0.85
P. viridicatu	0.83
P. cyclopium	0.82–0.87
P. martensii	0.79–0.83
P. islandicum	0.83
P. patulum	0.81–0.85
P. expansum	0.83–0.85
Stachybotrys atra	0.94
Trichothecium roseum	0.90

heavy metals etc. It should be noted that lysine is important for muscle growth and is reflected in body weight gain of animals (Tesseraud *et al*., 1992). Therefore, if lysine quantity is low, supplementation might be considered.

6.3 Materials and Methods

6.3.1 Materials

Slurry I samples from small-scale fermentation at 25 °C replicates 1 and 2 were utilized to study washing at 30 mL scale. Slurry I samples of replications of small-scale fermentation replicates 1 and 2 were employed to study washing at a pilot scale (approximately 230 kg) using continuous processing equipment.

A solid-bowl decanter (CA225-010, Westfalia, GEA Mechanical Equipment US, Inc., GEA Westfalia Separator Division, Northvale, NJ, USA) and a disc stack desludger centrifuge (SA7-06-576, Westfalia, GEA Mechanical Equipment US, Inc., GEA Westfalia Separator Division, Northvale, NJ, USA) were utilized for washing slurry I from replications of small-scale fermentation to produce protein concentrate. In addition, a digital scale (MS 5060S, Ishida Digital Scale, Ishida Co. Ltd, Sakyo-ku, Kyoto, Japan) was used to record the weight of wash fractions.

A crossflow filter (Sartocon®2 plus 1–10 cassette system, Sartorius AG, Göettingen, Germany), with 5 spaced cassettes (0.6 m^2, wide spacer), and microfiltration membranes (pore size 0.2 μm) and a standard industrial three phase motor and pump (CM3558T, Baldor electric Co., Fort Smith, AR, USA) were utilized to filter liquid from TSF and liquid fractions from washing process of replications of small-scale replicate 1.

Spray drying (B-290, BÜCHI Labortechnik AG, Flawil, Switzerland), drum drying (Buflovak, Buflovak equipment division of Blaw-knox Co., Buffalo, NY, USA), and forced-air drying (1390 FM, Sheldon Manufacturing Inc., Cornelius, OR, USA) were employed to produce dried protein concentrate.

6.3.2 Methods

6.3.2.1 Protein Content

Protein content of samples was estimated from Kjeldahl nitrogen (Section 3.3.6).

6.3.2.2 Moisture Content

Moisture content was determined by heating weighed samples as previously described in the Section 3.3.7.

6.3.2.3 DPFGSE-NMR Spectrometry

DPFGSE-NMR (Section 3.3.8) was utilized to determine organic solutes present in liquid from washing and filtration.

6.3.3 Experimental Procedures

6.3.3.1 Production of Protein Concentrate and Valuable Compound Recovery

6.3.3.1.1 Washing Slurry I from Small-scale TSF at 25 °C to Obtain Protein Concentrate

Slurry I (30 mL) from small-scale TSF fermented at 25 °C was centrifuged using a benchtop centrifuge (Allegra X-22R, Beckman Coulter Canada Inc., Mississauga, ON, Canada) at 4 °C and 800 × *g* for 10 min to obtain solid I and liquid II fractions. Solid I was mixed with Reverse osmosis (RO) water at a ratio of 1:1 (v:v) and stirred for 30 min. The solution was then centrifuged at 800 × *g* for 10 min at 4 °C. Supernatant was decanted, hereafter called liquid III. The solid pellet remaining after centrifugation, hereafter called solid II, was washed by suspending in water, centrifugation then decanting. The pellet was washed twice using this procedure. Supernatants recovered after the 2nd and 3rd washes were called liquid IV and liquid V. The pellet after the final wash is called solid IV and it is also referred to as protein concentrate. Liquid II, liquid III, liquid IV, liquid V and solid IV were analyzed to determine nitrogen content, using the Kjeldahl method, and moisture content, using the oven method, as mentioned. In addition, the concentration of organic solutes in liquid II, liquid III, liquid IV, and liquid V were analyzed using DPFGSE-NMR to determine organic solutes.

6.3.3.1.2 Washing Slurry I from Replications of Small-scale TSF to Produce Protein Concentrate

Based on protein content and the concentration of organic solutes from washing slurry I, it was determined that 2 washes were sufficient to increase protein content to approximately 60% (w/w, db) and recover valuable compounds [1,3-PD, acetic acid, and glycerophosphorylcholine (GPC)]. Therefore, slurry I from replications of small-scale TSF were washed only 2 times to

study washing using pilot scale equipment. Washing was conducted at POS Bio-Sciences, SK, Canada. Slurry I samples from 25 L-scale TSF replicates 1 and 2 were passed through a solid-bowl decanter at 3,322 × *g* for particle removal, solid I decanter. Liquid from the decanter was passed through a disc stack desludger centrifuge at 6,540 × *g* to remove fine particles (solid I desludger). The supernatants after decanting and desludging treatments are called liquid I decanter and liquid I desludger, respectively. Solid I decanter and solid I desludger were then mixed with water at a ratio of 1: 1 (w: w) in a mixing tank for approximately 30 min for the 1st wash. The mixture was then passed through the decanter and desludger as previously described. The solids and supernatant from the 1st wash are called solid II decanter, solid II desludger, liquid II decanter, and liquid II desludger, in that order. Solid II decanter and solid II desludger were mixed and washed a 2nd time using the same procedure used for the 1st wash. Solid and supernatant from 2nd wash are called solid III decanter, solid III desludger, liquid III decanter, and liquid III desludger. The mass of each fraction from washing was recorded.

Decanter and desludger feed rates for 1st pass, 1st wash, and 2nd wash are provided in Table 6.3. Equipment used in processing TSF products are shown in Figure 6.1. Samples of all fractions (slurry I, solid I decanter, solid I desludger, liquid I decanter, liquid I desludger, solid II decanter, solid II desludger, liquid II decanter, liquid II desludger, solid III decanter, solid III desludger, liquid III decanter, and liquid III desludger) were collected for determination of protein and moisture content (refer to Sections 3.3.6 and 3.3.7 in Chapter 3). Moreover, the concentration of organic solutes in liquid I decanter, liquid I desludger, liquid II decanter, liquid II desludger, liquid III decanter, and liquid III desludger were determined using DPFGSE-NMR (Section 3.3.8).

6.3.3.1.3 Filtration of Liquid I from TSF, Liquid I Desludger, Liquid II Desludger, and Liquid III Desludger

Liquid I, liquid I desludger, liquid II desludger, and liquid III desludger from replications of small-scale TSF and washing slurry I from replications of small-scale TSF replicate 1 were filtered through a crossflow filter. The flow was provided with a standard industrial three phase motor and pump. The crossflow filter was operated with inlet, permeate and retentate pressures of 173–207, 49 and 69 kPa, respectively, as recommended by the manufacturer. The motor was operated at 1,784 rpm. The permeate and retentate masses and filtration times were recorded. Filtration flux was also determined. Permeate and retentate were analyzed for protein and

Table 6.3 Feed rate of decanter and desludger 1st pass, 1st wash, and 2nd wash for washing slurry I from replications of small-scale TSF

Feed rate (kg/h)	Replicate 1		Replicate 2	
	Decanter	Desludger	Decanter	Desludger
1st pass	300	320	300	300
1st wash	400	400	350	350
2nd wash	400	400	400	400

The feed rate of decanter and desludger was decided based on the presence of particles in the supernatant that bypassed the decanter and desludger.

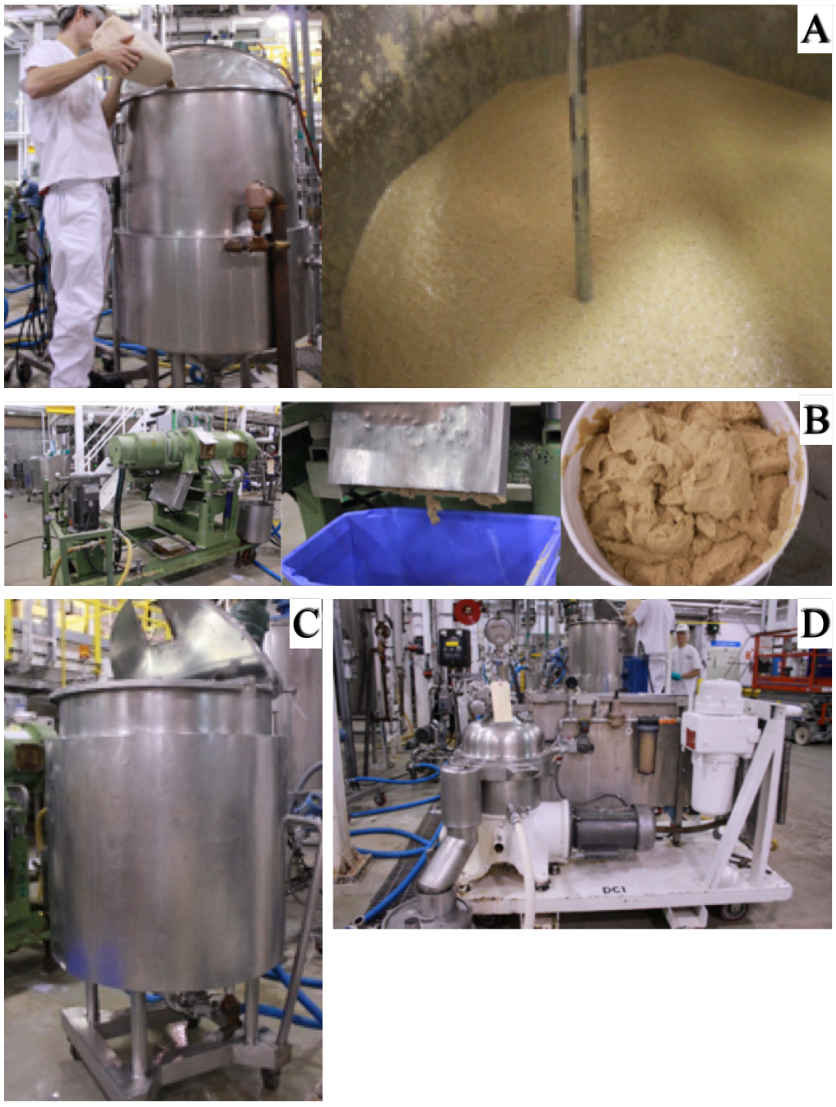

Figure 6.1 Equipment used in processing TSF products (A) mixing tank, (B) decanter and solid from decanter, (C) mixing tank of liquid from decanter before desludger, and (D) desludger

moisture contents according to Kjeldahl and oven methods. The concentration of organic solutes present in permeate and retentate were determined using DPFGSE-NMR.

6.3.3.2 Drying Protein Concentrate

Solids produced from washing slurry I from replications of small-scale TSF were utilized to study drying processes. Spray drying, drum drying, and tray drying were compared for drying protein concentrate samples.

Protein concentrate was mixed with water (ratio 1:1) to aid in flow when fed to a spray dryer or drum dryer. The inlet temperature for spray drying was adjusted to control the outlet temperature while aspirator, % pump, and nozzle cleaning were set at 100, 20, and 4, respectively. The set point inlet temperature was adjusted to 149, 139, and 129 °C. For drum drying, drum rotation speed was adjusted to control drum surface temperature while stream pressure was set at 221 kPa. Drum rotation speed was adjusted to 2 (78 sec/round) and 1 (125 sec/round). Samples were placed in Handi-foil® aluminum steam table pans for tray drying. Wet protein concentrate was added to a depth of 1 cm. The oven temperature was 70 °C. Dried protein concentrate was analyzed for protein (Kjeldahl method) and moisture (oven method) contents, color, and water activity (a_w).

Dried protein concentrate color was determined using a Hunterlab Miniscan XE (Hunter, Reston VA, USA). Results are reported according to the L, a, b color space conventions (Hunter, 1948), L (positive represents white and 0 represents dark), a (positive is red and negative is green), and b (positive is yellow and negative is blue). The a_w of dried protein concentrate was analyzed using a chilled-mirror dew point sensor water activity meter (AquaLab CX-1, Decagon Devices, Inc. Pullman, WA, USA).

Dried protein concentrate from the tray drier was submitted to the Experiment Station Chemical Laboratories, University of Missouri, USA for crude fat (ether extract) (AOAC official method 920.39 A), ash (AOAC official method 942.05), crude fibre (AOAC official method 978.10), acid detergent fibre (AOAC official method 973.18 A-D), Neutral detergent fibre (NDF) (Holst, 1973), amino acid profile analysis (AOAC official method 982.30 a,b,c), lysine availability (AOAC official method 975.44), phosphorus, (AOAC official method 966.01), sulfur (AOAC official method 956.01), calcium, sodium, magnesium, selenium, potassium, iron, zinc, copper, manganese, chromium, arsenic, and cadmium (AOAC official method 990.08).

6.3.4 Statistical Analysis

Duplicate analysis was conducted and analysis of variance (ANOVA) was utilized for mean comparisons. Duncan's multiple-range test was performed using the SPSS program (SPSS 21.0, IBM Corp., Armonk, NY, USA). A *P* value of 0.05 was used as the level of significance.

6.4 Results and Discussion

6.4.1 Washing Slurry I from Small-scale TSF at 25 °C to Obtain Protein Concentrate

Slurry I is a mixture of entrained solution, similar in composition to liquid I, and protein rich particles. As the concentration of protein (db) is higher in slurry I than liquid I, it is reasonable to assume that washing slurry I, to remove the entrained solution surrounding the particles, will produce a product that has greater protein content (db) than dried slurry I (db). The sedimentation rate of particles is increased with centrifugation (Geankoplis, 2003c) and some water surrounding the particles is removed by pressure. This would suggest that slurry produced by centrifugation and not settling might have higher protein content (db) than slurry I prepared by gravity sedimentation. Washing the slurry might improve protein content for use as an animal feed and solution-surrounding particles is released for subsequent solute separation. The protein content of slurry I, which was 46 and 47% (w/w, db) (fermenters 1 and 2 at 172 h of fermentation, Table 5.3) improved with washing to 58 and 60% (fermenters 1 and 2, Table 6.4) for replicate 1 and increased from 48 and 46% (w/w, db) (fermenters 1 and 2 at 94 h of fermentation, Appendix C Table C.1) to 61 and 62% (w/w, db) (fermenters 1 and 2, Appendix D Table D.1) for replicate 2. These results indicate that separating particles from fermentation medium after TSF by washing led to a product with greatly improved protein concentration.

1,3-PD, acetic acid, glycerol, GPC, and lactic acid, were present in the wash water arising from TSF process and repeated washing lowered the content of these compounds (Table 6.5 and Appendix D Table D.2). The organic solutes present in liquid I were also present in wash water.

Wash water fractions (liquid II, liquid III, liquid IV, and liquid V) can be combined with liquid I from TSF and utilized as a source of soluble compounds. These recovery methods are similar to methods for bacterial recovery for DNA extraction (Steffan *et al*., 1988). It was discovered that repeated washing produced very dilute streams of compounds especially after

Table 6.4 Protein and moisture contents of liquid II, liquid III, liquid IV, liquid V, and solid IV from washing slurry I from fermentation at 25 °C

Sample	Fermenter 1			Fermenter 2		
	Protein (%, w/w, wb)	Moisture (%, w/w, wb)	Protein (%, w/w, db)	Protein (%, w/w, wb)	Moisture (%, w/w, wb)	Protein (%, w/w, db)
Liquid II	0.49 ± 0.01	97.02 ± 0.02	16.4 ± 0.5	0.47 ± 0.02	97.09 ± 0.00	16.2 ± 0.7
Liquid III	0.37 ± 0.02	98.39 ± 0.02	23 ± 1	0.35 ± 0.02	98.47 ± 0.02	22.8 ± 0.8
Liquid IV	0.18 ± 0.01	99.26 ± 0.01	24 ± 1	0.19 ± 0.02	99.34 ± 0.01	28 ± 3
Liquid V	0.09 ± 0.00	99.65 ± 0.01	24.1 ± 0.3	0.08 ± 0.00	99.71 ± 0.00	28.0 ± 0.3
Solid IV	7.7 ± 0.1	86.75 ± 0.06	58.4 ± 0.5	7.87 ± 0.02	86.84 ± 0.08	59.8 ± 0.2

Each value is presented as the mean ± standard deviation (SD, $n = 2$).

Table 6.5 Concentration (g/L) of organic solutes of liquid II, liquid III, liquid IV, and liquid V from washing slurry I from fermentation at 25 °C

Compound	Fermenter 1				Fermenter 2			
	Liquid II	Liquid III	Liquid IV	Liquid V	Liquid II	Liquid III	Liquid IV	Liquid V
1,3-PD	5.85	3.27	1.93	1.00	6.18	3.42	1.84	0.94
Acetic acid	5.72	3.00	1.66	0.95	5.78	3.27	1.62	0.84
Glycerol[1]	3.33	2.06	0.99	0.46	3.35	1.85	0.72	0.27
GPC	1.32	0.74	0.38	0.15	1.34	0.79	0.38	0.15
Lactic acid	5.76	3.70	2.25	1.08	5.74	3.77	1.93	0.90

[1]The concentration of glycerol in W-TS samples may be affected by the presence of interfering resonances from carbohydrate and protein.

3 washes. Recovering compounds from such dilute streams produced by later washes is unlikely to occur in an industrial process as the volumes of solution would increase with additional washes. Moreover, organic solute concentrations in the 2nd and 3rd wash did not substantially improve compound yield (Table 6.5 and Appendix D Table D.2). Therefore, for replications of small-scale TSF, 2 washing treatments were used for recovering compounds from slurry I.

6.4.2 Washing Slurry I from Replications of Small-scale TSF to Produce Protein Concentrate

The mass balance of slurry I from replications of small-scale TSF and solid and liquid fraction from washing were recorded throughout the washing (Table 6.6 and Appendix D Table D.3). Protein content of solids (solid III decanter) after washing twice was improved to approximately 60% (w/w) (Table 6.7 and Appendix D Table D.4) from approximately 44–47% on a dry basis in slurry I at the end of TSF (Table 5.7 and Appendix C Table C.5). Trends were similar to those observed when washing slurry I from small-scale TSF (Section 6.4.1). Centrifugation pressed liquid surrounding particles from solids and removed entrained solution. Solutes in liquid surrounding particles were removed producing a solid with elevated protein content (db) when compared with slurry prepared by gravity sedimentation. Moreover, it is likely that centrifuged or washed solids (protein concentrate), would be useful as animal feed ingredients because of their elevated protein content. However, conditions for drying this solid after washing should be explored. The protein content (db), moisture content, a_w, fat content, crude fibre content, acid detergent fibre, NDF, protein digestibility, amino acid profile, lysine availability, and mineral content of dried protein concentrate should be determined prior to use as an animal feed.

As expected, both centrifugation and washing reduced the concentration of organic solutes associated with particles and the concentration of organic solutes from the 2nd wash was lower than that of 1st wash, and 1st pass, in that order (Table 6.8 and Appendix D Table D.5). Therefore, the majority of valuable compounds were recovered from slurry I by centrifugation and washing. The liquid fractions obtained from washing could combine with liquid I from TSF for compound extraction. The results of washing slurry I from replications of small-scale TSF proved that washing and centrifugation could be repeated and scaled-up using industrial equipment.

Table 6.6 Mass balance of clarification of replications of small-scale TSF

Sample	Weight of sample (kg)
Slurry I	237.02
Liquid I	66.28
Solid I decanter	75.09
Solid I desludger	29.57
Liquid I desludger	140.00
Solid II decanter	67.42
Solid II desludger	31.40
Water added for 1st wash	104.66
Liquid II desludger	103.50
Solid III decanter	57.63
Solid III desludger	13.12
Water added for 2nd wash	99.00
Liquid III desludger	117.00

Table 6.7 Protein and moisture contents of fractions from replications of small-scale TSF

Sample	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Slurry I	90.30 ± 0.00	4.54 ± 0.00	46.79 ± 0.05
Solid I decanter	82.03 ± 0.04	10.91 ± 0.06	60.7 ± 0.2
Liquid I decanter	96.61 ± 0.00	0.88 ± 0.00	25.94 ± 0.02
Solid I desludger	93.07 ± 0.02	2.52 ± 0.05	36.3 ± 0.7
Liquid I desludger	96.95 ± 0.01	0.72 ± 0.01	23.6 ± 0.3
Mixture of solid and water before 1st wash	92.96 ± 0.00	3.89 ± 0.07	55 ± 1
Solid II decanter	82.37 ± 0.02	11.29 ± 0.07	64.0 ± 0.3
Liquid II decanter	98.05 ± 0.01	0.70 ± 0.00	35.7 ± 0.3
Solid II desludger	95.74 ± 0.03	1.94 ± 0.01	45.5 ± 0.5
Liquid II desludger	98.63 ± 0.04	0.32 ± 0.02	24 ± 1
Mixture of solid and water before 2nd wash	94.18 ± 0.00	3.39 ± 0.05	58.3 ± 0.9
Solid III decanter	83.60 ± 0.06	10.27 ± 0.00	62.6 ± 0.2
Liquid III decanter	99.15 ± 0.01	0.32 ± 0.01	37.9 ± 0.7
Solid III desludger	98.60 ± 0.02	0.59 ± 0.01	42.5 ± 0.2
Liquid III desludger	99.30 ± 0.01	0.17 ± 0.01	24.2 ± 0.4

Each value is presented as the mean ± SD (n = 2).

Table 6.8 Concentration (g/L) of 1,3-PD, acetic acid, glycerol, GPC, and lactic acid from washing of replications of small-scale TSF

Compound	Slurry			Liquid after decanting			Liquid after desludging		
	1st pass	1st wash	2nd wash	1st pass	1st wash	2nd wash	1st pass	1st wash	2nd wash
1,3-PD	7.50	2.88	1.35	5.77	3.30	1.49	5.84	2.71	1.49
Acetic acid	7.13	2.85	1.40	5.82	3.40	1.48	5.73	2.74	1.40
Glycerol[1]	3.01	1.04	0.45	2.46	1.19	0.47	2.51	1.17	0.65
GPC	1.35	0.62	0.27	1.09	0.70	0.35	1.15	0.57	0.25
Lactic acid	3.23	1.42	0.67	2.73	1.87	0.93	2.97	1.56	0.96

[1]The concentration of glycerol in W-TS samples may be affected by the presence of interfering resonances from carbohydrate and protein.

Table 6.9 Mass balance from filtration replications of small-scale TSF

Sample	Weight of liquid (kg)	Weight of permeate (kg)	Weight of retentate (kg)	Hours of operating filter unit	Flux ($L/m^2/h$)
Liquid I from TSF	66.28	63.09	3.31	1.41	75
Liquid I desludger	140.88	129.82	10.17	3.83	56
Liquid II desludger	103.56	94.06	9.31	3.42	46
Liquid III desludger	116.78	99.55	7.30	3.67	45

6.4.3 Filtration of Liquid I from TSF, Liquid I Desludger, Liquid II Desludger, and Liquid III Desludger

Microfiltration flux of decanted and washed liquids (liquid I, liquid I desludger, liquid II desludger, and liquid III desludger) were determined using a cross-flow microfiltration cell. Mass balance and flux from fractions obtained by washing slurry I from replications of small-scale TSF replicate 1 were determined (Table 6.9). The majority of liquid from TSF and washing could pass through a 0.2 μm membrane without changes in membrane flux due to fouling. This indicated that membrane polarization did not occur and these solutions were not colloidal. The solutions had a low content of particulates with sizes greater than 0.2 μm. Protein content of retentate was higher than that of permeate when filtering liquid I from TSF and liquid from washing slurry I through a microfilter. The majority of protein was concentrated by filtration and recovered in the retentate (Table 6.10). The concentration of organic solutes were higher in the retentate than the permeate (Table 6.11). Therefore, organic solutes were partially concentrated by filtration. Furthermore, it was noticed that in permeate and retentate from filtering liquid I desludger, the concentration of 1,3-PD and acetic acid was higher than in permeate and retentate from filtering liquid I from TSF. It is probable that microorganisms in slurry I are actively metabolising glycerol and lactic acid while liquid I is not in contact with bacteria. Therefore, the concentration of 1,3-PD and acetic acid in liquid I desludger was higher than that in the liquid I from TSF due to metabolism by bacterial concentrates. The concentration of 1,3-PD and acetic acid precursor molecules, glycerol and lactic acid, in liquid I desludger was lower than in liquid I from TSF. This further confirms the ability of the microbial biomass associated with particles to convert these compounds to 1,3-PD and acetic acid.

Summary of mass balance and compounds balance from replications of small-scale TSF

1) Mass balance

Mass balance indicated that mass lost occurred from fermentation until filtration of liquid from replications of small-scale TSF replicate 1 (Figure 6.2). Within the margin of error there was no loss of mass in the separation of slurry I and liquid I. However, during centrifugation and washing treatments small mass losses were recorded. Mass balance was calculated below.

Mass in = mass of TSF + mass of water added in 1^{st} wash + water added in 2^{nd} wash (6.1)

= 303.00 + 104.66 + 99.00 = 506.66 kg

Table 6.10 Protein and moisture contents of filtration fractions from replicates of small-scale TSF

Sample	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Liquid I from TSF	96.76 ± 0.00	0.72 ± 0.03	22.2 ± 0.1
Permeate of liquid I after filtering	97.52 ± 0.01	0.41 ± 0.02	16.5 ± 0.5
Retentate of liquid I after filtering	92.37 ± 0.03	2.31 ± 0.03	30.3 ± 0.3
Liquid I desludger	96.95 ± 0.03	0.72 ± 0.00	23.6 ± 0.3
Permeate of liquid I desludger after filtering	97.70 ± 0.00	0.34 ± 0.01	14.6 ± 0.5
Retentate of liquid I desludger after filtering	90.20 ± 0.02	2.51 ± 0.02	25.6 ± 0.2
Liquid II desludger	98.63 ± 0.04	0.32 ± 0.02	24.0 ± 1.0
Permeate of liquid II desludger after filtering	99.90 ± 0.00	0.08 ± 0.01	8.0 ± 1.0
Retentate of liquid II desludger after filtering	96.52 ± 0.02	1.02 ± 0.00	29.35 ± 0.06
Liquid III desludger	99.30 ± 0.01	0.17 ± 0.01	24.2 ± 0.4
Permeate of liquid III desludger after filtering	99.49 ± 0.01	0.01 ± 0.00	2.7 ± 0.5
Retentate of liquid III desludger after filtering	97.8 ± 0.2	0.76 ± 0.01	34.0 ± 0.6

Each value is presented as the mean ± SD (n = 2).

Table 6.11 Concentration (g/L) of 1,3-PD, acetic acid, glycerol, GPC, and lactic acid of filtration fractions from replications of small-scale TSF

Compound	Liquid I		Liquid I desludger		Liquid II desludger		Liquid III desludger	
	Permeate	Retentate	Permeate	Retentate	Permeate	Retentate	Permeate	Retentate
1,3-PD	4.72	5.44	5.68	5.95	2.73	2.98	1.47	1.56
Acetic acid	4.84	5.59	5.51	5.68	2.62	2.98	1.59	1.58
Glycerol[1]	4.29	6.65	2.07	4.11	1.06	1.59	0.47	0.85
GPC	1.17	1.50	1.14	1.33	0.55	0.70	0.34	0.34
Lactic acid	3.87	4.52	2.27	3.21	1.33	1.70	0.73	0.92

[1]The concentration of glycerol in W-TS samples may be affected by the presence of interfering resonances from carbohydrate and protein.

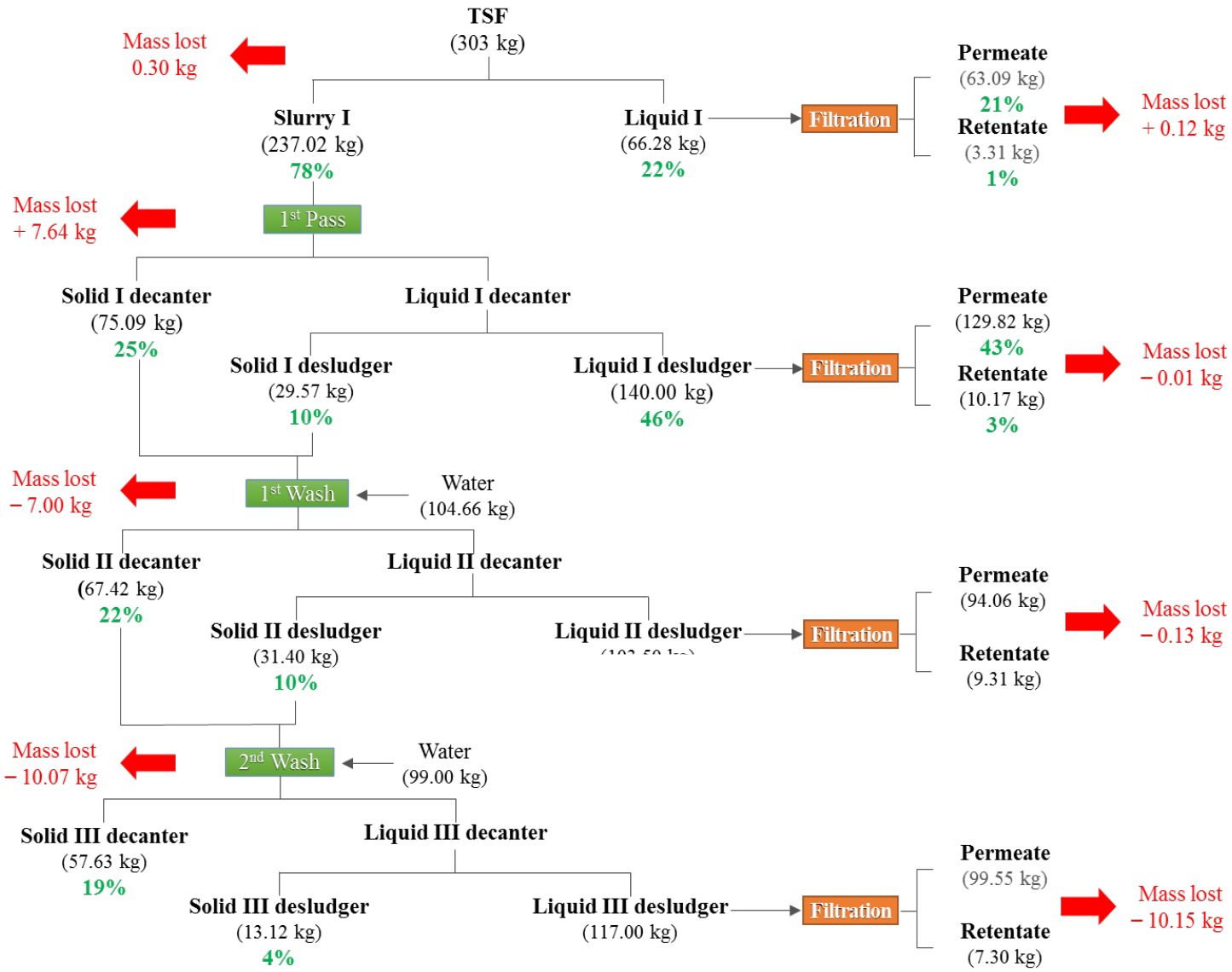

Figure 6.2 Mass balance of replications of small-scale TSF

Mass out = mass of solid III decanter + solid III desludger + permeate and retentate filtering liquid I, liquid I desludger, liquid II desludger, and liquid III desludger (6.2)

= 57.63 + 13.12 + 63.09 + 3.31 + 129.82 + 10.17 + 94.06 + 9.31 + 99.55 + 7.30

= 487.36 kg

Mass lost from entire process = mass in − mass out = 506.66 − 487.36 = 19.30 kg (6.3)

Mass lost from washing = mass lost from 1st pass + mass lost from 1st wash + protein lost from 2nd wash = − 7.64 + 7.00 + 10.07

= 9.43 kg (6.4)

Mass lost from filtration= mass lost from filtering liquid I + mass lost from filtering liquid I desludger + mass lost from filtering liquid II desludger + mass lost from filtering liquid III desludger (6.5)

= − 0.12 + 0.01 + 0.13 + 10.15

= 10.17 kg

From the calculation of mass balance, it was concluded that 19 kg or 3.8% was lost from the processing of 507 kg (mass of TSF and water added). Most of the mass loss occurred during washing. Mass was also lost during filtration (10 kg or 2%). The loss from the entire process is less than 5%. It is likely that this loss can be reduced with an increase in process scale. Percent recovery of mass from washing and filtration was calculated from the Equation below.

$$\% \text{ Recovery} = \frac{\text{kg of mass out from process}}{\text{kg of mass in the process}} \times 100 \quad (6.6)$$

$$= \frac{487.36}{506.66} \times 100$$

= 96%

2) Protein balance

Protein balance from washing and filtration indicated that protein was lost during these processes. No protein was lost from TSF (Figure 6.3). In addition, it was noticed that most of the protein loss occurred in 2nd wash process. This could be the result of solid remaining in the decanter (see mass in 2nd wash lost from Figure 6.2). The mass loss of the 2nd wash step was greater than the mass loss of the 1st pass and 1st wash. Therefore, more solid from the 2nd wash step likely remained in the decanter. Solid III decanter had 63% (w/w, db) protein (Table 6.7). Therefore, if major mass loss occurred during the 2nd wash decanting step, this could explain protein losses in this step.

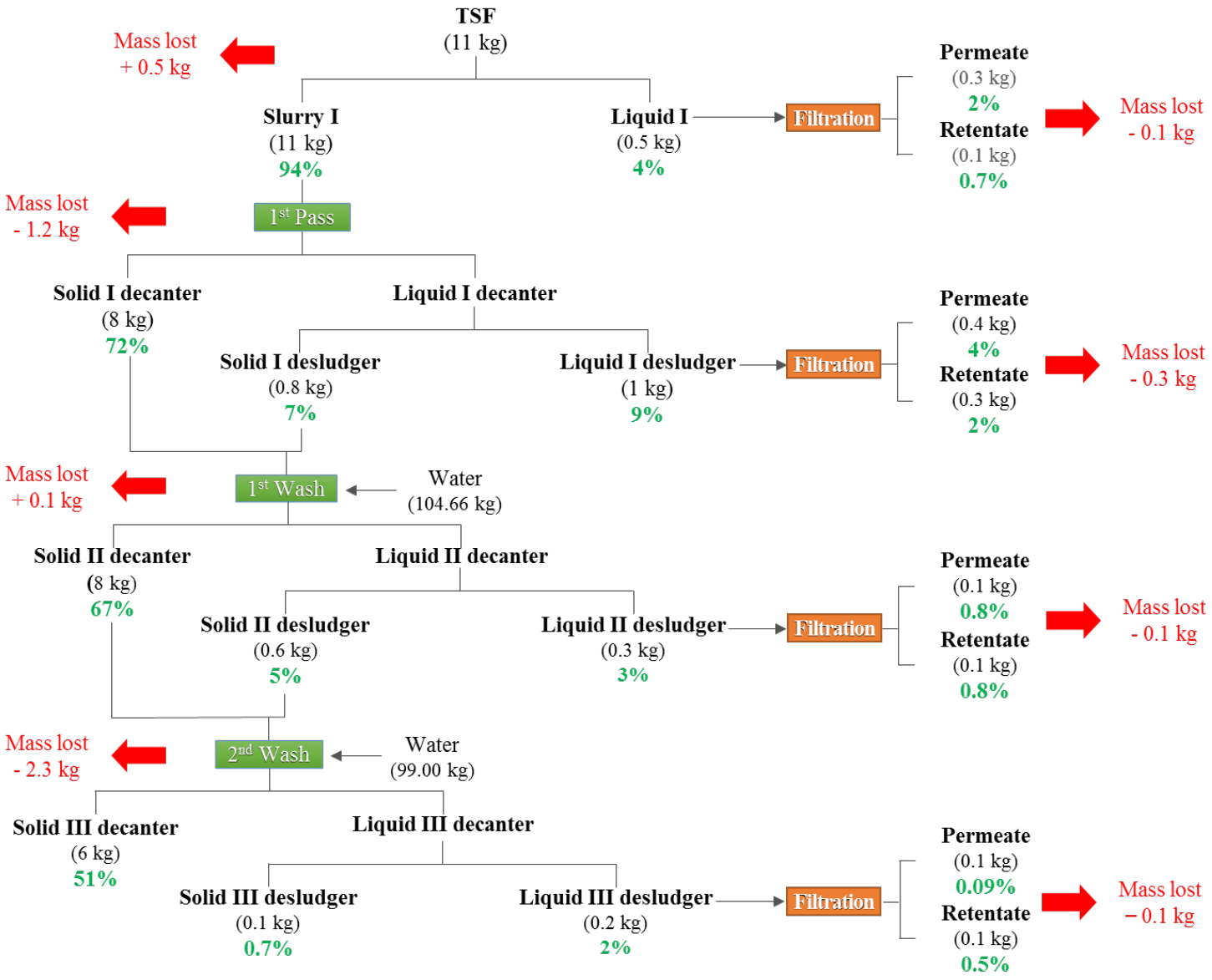

Figure 6.3 Protein balance of replications of small-scale TSF

Protein in = protein in TSF

= 11 kg

Protein out = protein in solid III decanter + solid III desludger + permeate and retentate from filtering liquid I, liquid I desludger, liquid II desludger, and liquid III desludger (6.7)

= 6 + 0.1 + 0.3 + 0.1 + 0.4 + 0.3 + 0.1 + 0.1 + 0.1 + 0.1

= 7.6 kg

Protein lost = protein in − protein out (6.8)

= 11 − 7.6 = 3.4 kg

Protein lost from washing = protein lost from 1st pass + protein lost from 1st wash + protein lost from 2nd wash (6.9)

= 1.2 − 0.1 + 2.3 = 3.4 kg

Protein lost from filtration = protein lost from filtering liquid I + protein lost from filtering liquid I desludger + protein lost from filtering liquid II desludger + protein lost from filtering liquid III desludger (6.10)

= 0.1 + 0.3 + 0.1 + 0.1 = 0.6 kg

Protein lost from 11 kg of protein from TSF was 3.4 kg or 31% of protein lost due to washing, centrifugation and decanting. In addition, it was found that 0.6 kg of protein was lost from filtration.

When calculating losses of the entire process, 31 and 5% protein was lost from washing and filtering compared to protein lost from the entire process, respectively. Protein recovery could be improved if the scale of process is larger as less solid will be retained in process equipment. Percent protein recovery from washing and filtration were calculated from the Equation below.

$$\% \text{ Recovery} = \frac{\text{kg of protein out from process}}{\text{kg of protein slurry I+kg of protein in liquid I}} \times 100 \quad (6.11)$$

$$= \frac{7.6}{11+0.5} \times 100$$

= 66%

3) Glycerol balance

Glycerol balance of TSF, washing, and filtration showed glycerol lost during the process (Figure 6.4). The major glycerol loss, approximately 1,319 g (14 M) of glycerol, occurred during TSF or (56%) glycerol present. Similar losses were observed for replications of small-scale TSF.

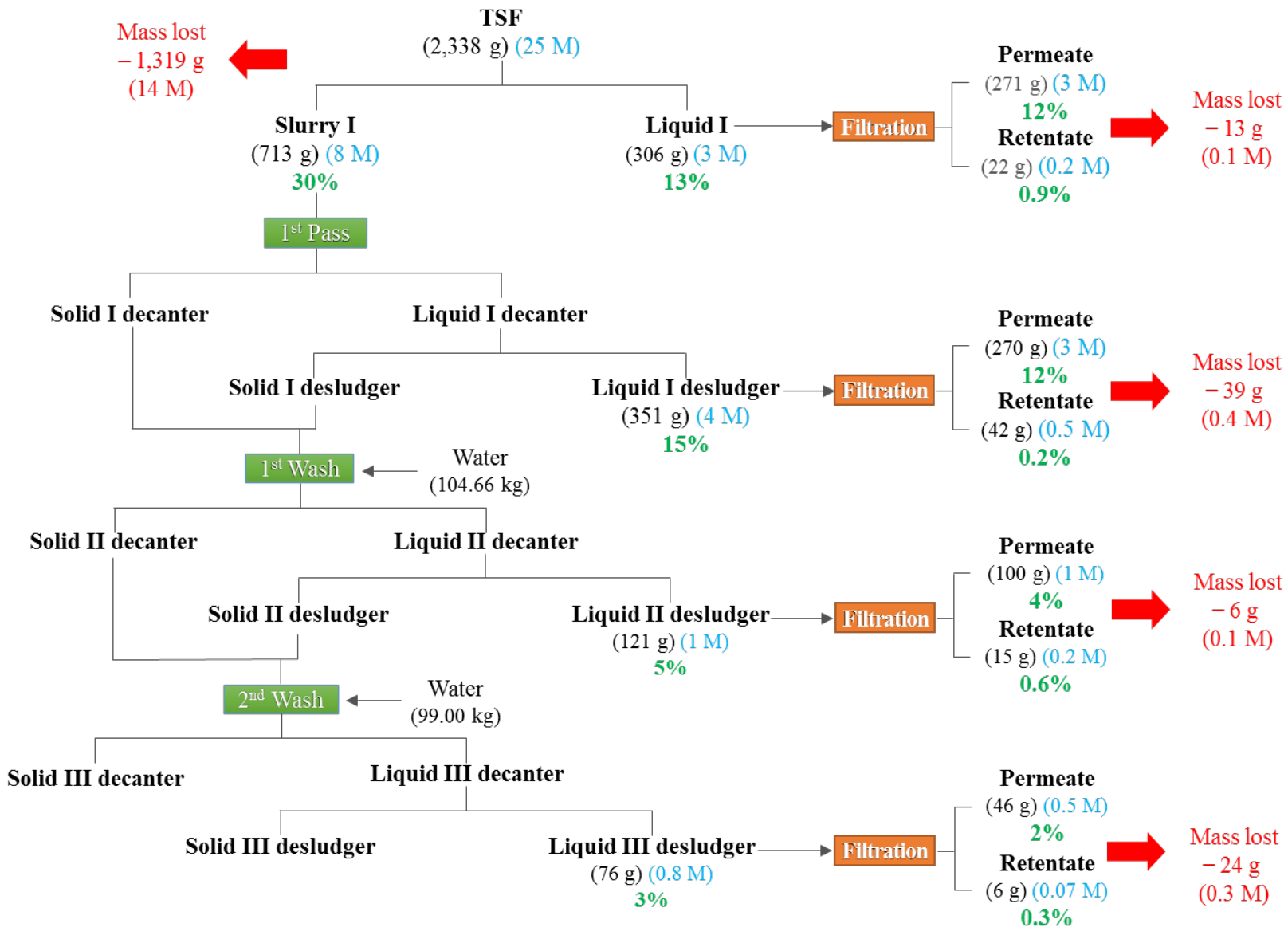

Figure 6.4 Glycerol balance of replications of small-scale TSF

Glycerol fermentation pathways convert 1 M of glycerol to 1 M of 1,3-PD [Figure 2.3 (Kang *et al*., 2014b), Figure 5.8 (Biebl *et al*., 1999), and Figure 6.5 (Nakamura and Whited, 2003)]. Therefore, the major loss of glycerol in TSF is due to endemic bacterial conversion to 1,3-PD. The glycerol balance was calculated below.

Glycerol in = glycerol in TSF
= 2,338 g
= 25 M

Glycerol out = glycerol in permeate and retentate from filtering liquid I, liquid I desludger, liquid II desludger, and liquid III desludger (6.12)
= 271 + 22 + 270 + 42 + 100 + 15 + 46 + 6
= 772 g
= 8 M

Glycerol lost = glycerol in – glycerol out (6.13)
= 2,338 – 772 = 1,566 g
= 17 M

Glycerol lost from filtration = glycerol lost from filtering liquid I + glycerol lost from filtering liquid I desludger + glycerol lost from filtering liquid II desludger + glycerol lost from filtering liquid III desludger (6.14)
= 13 + 39 + 6 + 24 = 82 g
= 0.9 M

Glycerol losses were calculated based on the assumption that no glycerol remained in solid after washing and there was no glycerol in solid III in the decanter.

Glycerol balance calculations show 1,566 g (17 M, 67%) of glycerol lost while processing (Figure 6.4). Moreover, 1,319 g (14 M) of glycerol was lost from TSF or 84% of glycerol lost. The major glycerol losses occurred in TSF from the conversion of glycerol to 1,3-PD as explained previously. In addition, 82 g (0.9 M) of glycerol was lost during filtration resulting in 10% of glycerol lost. The loss of glycerol from filtration could arise from liquid in tubing and the cross-flow filter. Moreover, other glycerol lost from these processes could be the result of liquid and solid lost during washing. Furthermore, it has to be noted that some glycerol likely remained in solids after washing. Glycerol remaining in solid was not determined. Consequently, the glycerol balance is incomplete.

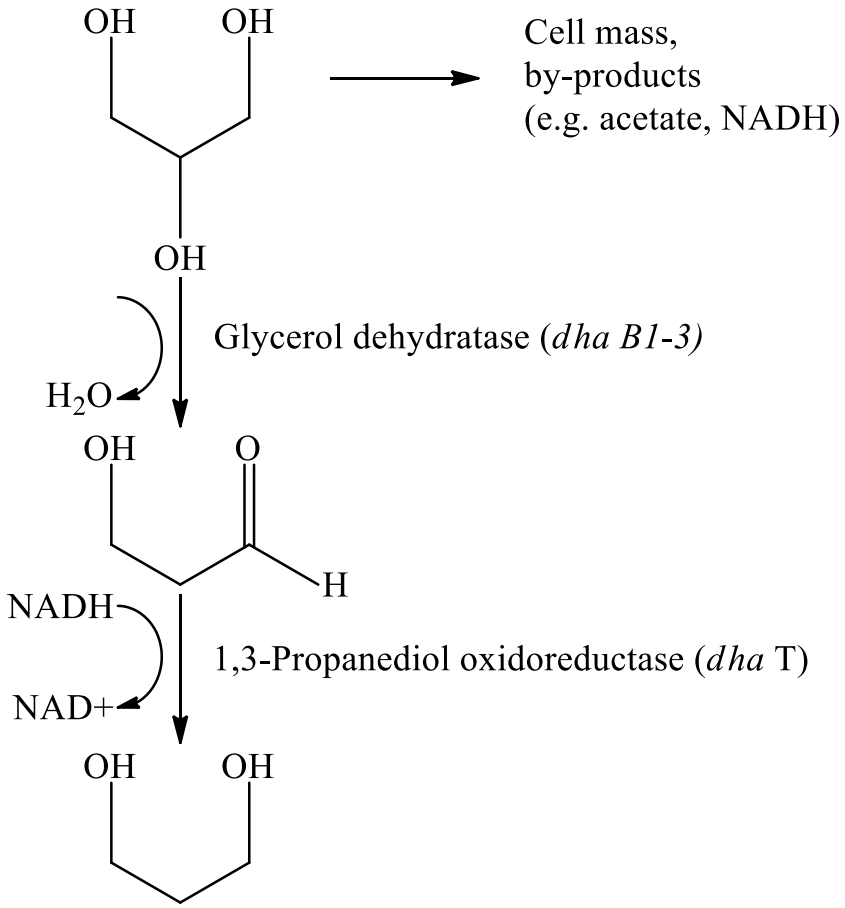

Figure 6.5 1,3-PD production by natural organism in the absence of oxygen (Nakamura and Whited, 2003)

When comparing the quantity of glycerol remaining in liquid I desludger, liquid II desludger, and liquid III desludger, it was found that glycerol concentration in the liquid from the desludger decreased from 1st pass, 1st wash, and 2nd wash, in that order. This was the result of dilution during washing. These findings are in good agreement with the results of washing slurry I (Table 6.5 and Appendix D Table D.2) Percent recoveries for glycerol from washing and filtration steps were calculated from the Equation below.

$$\% \text{ Recovery} = \frac{\text{gram of glycerol in permeate and retentate from filtration}}{\text{gram of glycerol in slurry I+gram of glycerol in liquid I}} \times 100 \quad (6.15)$$

$$= \frac{772}{713+306} \times 100$$

$$= 76\%$$

4) 1,3-PD balance

1,3-PD balance (Figure 6.6) begins with 1,3-PD (17 M or 149%) production during TSF. This is the result of *Lactobacillus* species present in W-TS converting glycerol to 1,3-PD.

When comparing 1,3-PD in slurry I and liquid I from TSF, it was found that the majority of 1,3-PD in TSF was present in slurry I (82%) while only 18% of 1,3-PD was in liquid I. This could be the result of the high ratio of lactobacilli in slurry I comparing to unseparated liquid as previously described in Section 5.4.2 and 5.4.3. *Lactobacillus* species produce EPS that could induce or stabilize particle coalescence and assist formation of slurry I as previously stated. Potentially diffusion in slurries is limited, the bacterial concentration is higher and this may lead to greater conversion of glycerol to 1,3-PD.

The amount of 1,3-PD decreased during washing and 1,3-PD concentrations in liquid I desludger, liquid II desludger, and liquid III desludger reflected that trend (Table 6.5 and Appendix D Table D.2). These results suggested that 1,3-PD present in slurry I might be recovered by washing. Moreover, washing could recover substantial 1,3-PD from TSF.

Based on glycerol balance and 1,3-PD balance (Figures 6.4 and 6.6), it was clear that 14 M of glycerol was lost and 17 M of 1,3-PD produced during TSF. The conversion molar ratio of glycerol to 1,3-PD was approximately 1:1. It cannot be ruled out that additional glycerol was formed in the second stage of fermentation if yeast were present and that this glycerol was converted to 1,3-PD. However, further replication of this study should be conducted to determine the conversion ratio. The results are in good agreement with the metabolism of glycerol to 1,3-PD under anaerobic conditions. The 1,3-PD balance of the process was calculated below.

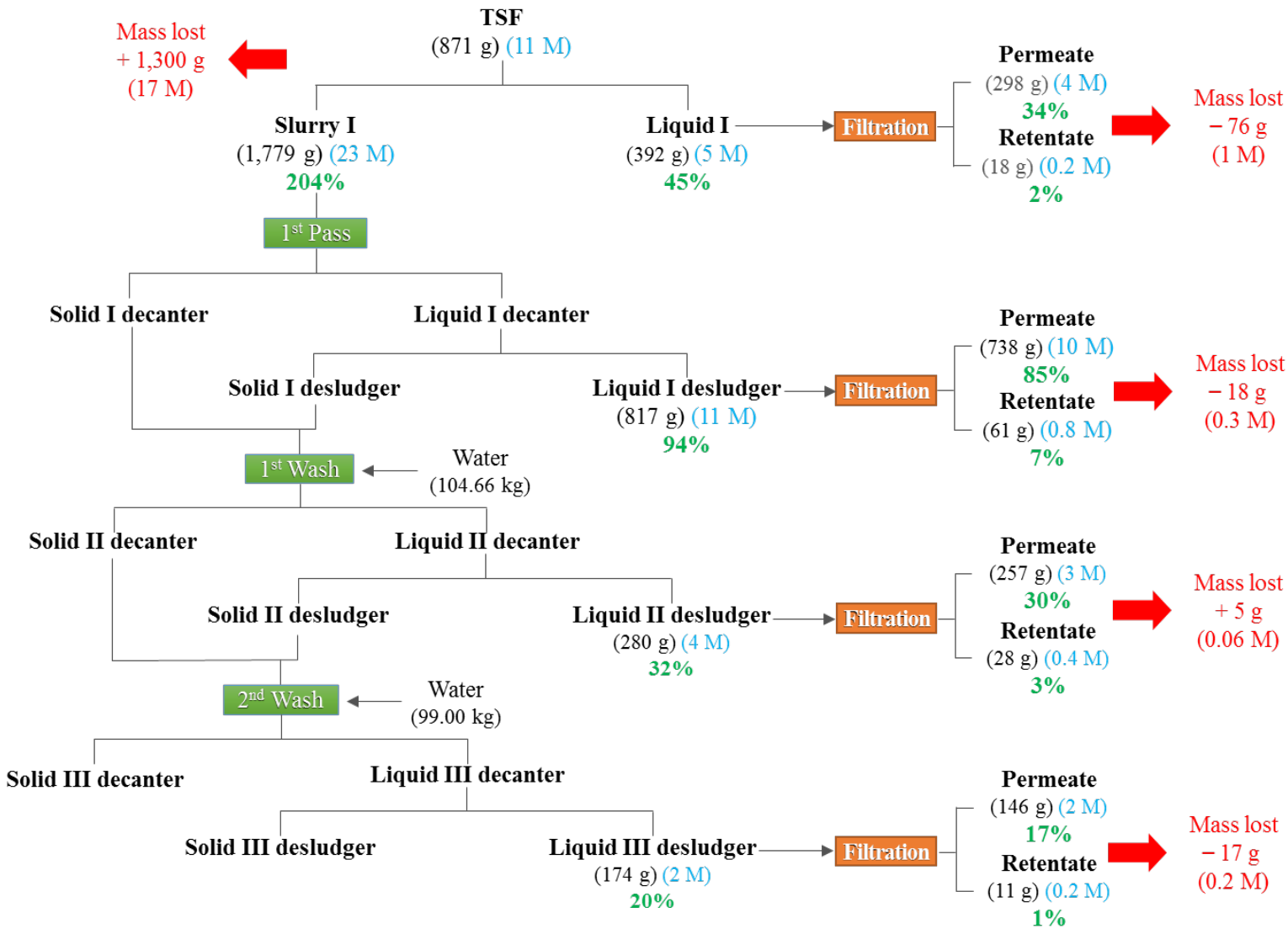

Figure 6.6 1,3-PD balance of replications of small-scale TSF

1,3-PD in = 1,3-PD in TSF

= 871 g

= 11 M

1,3-PD out = 1,3-PD in permeate and retentate from filtering liquid I, liquid I desludger, liquid II desludger, and liquid III desludger (6.16)

= 298 + 18 + 738 + 61 + 257 + 28 + 146 + 11

= 1,557 g

= 20 M

1,3-PD gain = 1,3-PD out – 1,3-PD in (6.17)

= 1,557 – 871

= 686 g = 9 M

1,3-PD lost from filtration = 1,3-PD lost from filtering liquid I + 1,3-PD lost from filtering liquid I desludger + 1,3-PD lost from filtering liquid II desludger + 1,3-PD lost from filtering liquid III desludger (6.18)

= 76 + 18 – 5 + 17

= 106 g

= 1 M

1,3-PD losses were calculated based on the assumption of no 1,3-PD remained in solids after washing. 1,3-PD lost from washing could not be calculated exactly as 1,3-PD in solids was not determined.

There was 1,300 g (17 M, 149%) of 1,3-PD synthesized during TSF. In addition, 106 g (6%) of 1,3-PD was lost during filtration. Fifteen percent of 1,3-PD lost during filtration was smaller than the 1,3-PD gain from the entire process. The 1,3-PD lost from filtration could be explained in terms of liquid remained in tubing and the microfilter apparatus. Furthermore, liquid and solid lost from washing could lead to additional 1,3-PD losses. 1,3-PD in solid after washing could also be considered in calculating 1,3-PD balance. The amount of 1,3-PD remained in the solid was not determined. Percent recovery of 1,3-PD from washing and filtration were calculated from the Equations below.

$$\% \text{ Recovery} = \frac{\text{gram of 1,3-PD in permeate and retentate from filtration}}{\text{gram of 1,3-PD in slurry I + gram of 1,3-PD in liquid I}} \times 100 \quad (6.19)$$

$$= \frac{1{,}557}{1{,}779+392} \times 100$$

= 72%

5) Glycerol and 1,3-PD balance

Glycerol and 1,3-PD balance calculation showed below.

Glycerol and 1,3-PD in = glycerol and 1,3-PD in TSF

= 37 M

Glycerol and 1,3-PD out = glycerol and 1,3-PD in permeate and retentate from filtering liquid I, liquid I desludger, liquid II desludger, and liquid III liquid III desludger (6.20)

= 7 + 0.5 + 13 + 1 + 4 + 0.5 + 2 + 0.2

= 28.2 M

Glycerol and 1,3-PD lost = glycerol and 1,3-PD in – glycerol and 1,3-PD out (6.21)

= 37 – 28.2

= 8.8 M

Glycerol and 1,3-PD lost from filtration = glycerol and 1,3-PD lost from filtering liquid I + glycerol and 1,3-PD lost from filtering liquid I desludger + glycerol and 1,3-PD lost from filtering liquid II desludger + glycerol and 1,3-PD lost from filtering liquid III desludger (6.22)

= 0.5 + 1 + 0.5 + 0.8

= 2.8 M

According to glycerol and 1,3-PD balance diagram (Figure 6.7) and calculations, there were 37 M of glycerol and 1,3-PD at the beginning of the process and 28.2 M of glycerol and 1,3-PD in fractions. Therefore, 8.8 M of glycerol and 1,3-PD were lost (24%) during washing and filtration. In addition, 2.8 M of glycerol and 1,3-PD were lost during filtration. These was calculated as 9% decrease in glycerol and 1,3-PD during filtration, 32% glycerol and 1,3-PD lost from filtration comparing to total glycerol and 1,3-PD lost from entire process, and 8% glycerol and 1,3-PD lost from the entire process. Furthermore, after washing slurry I twice, it was assumed that there was little glycerol and 1,3-PD in the solid fraction. The level of these compounds in solids could be considered in a future study. However, glycerol and 1,3-PD were not determined in this fraction. Percent recovery of glycerol and 1,3-PD from washing and filtration was calculated from the Equation below.

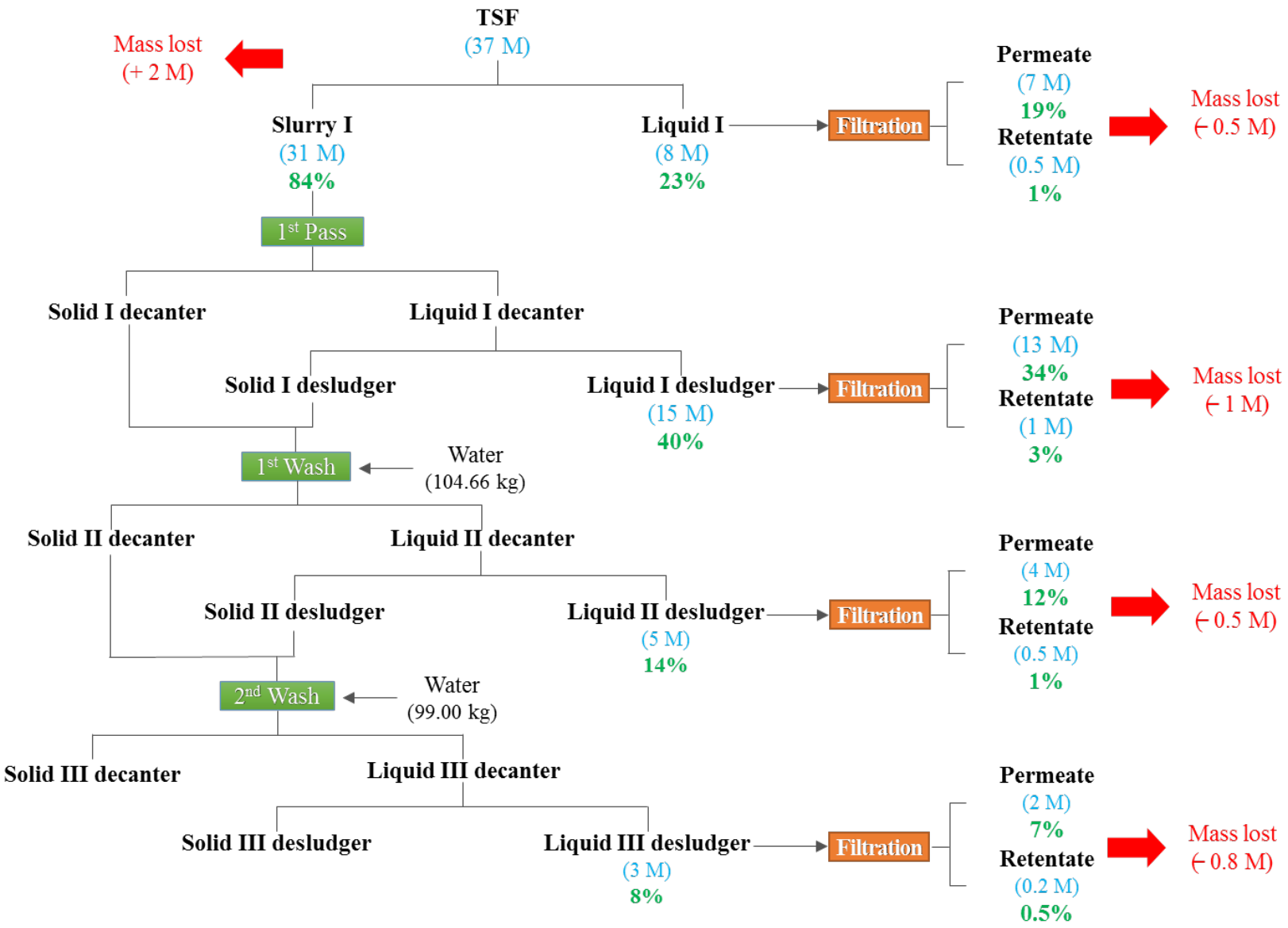

Figure 6.7 Glycerol and 1,3-PD balance in molar basis

$$\%\text{Recovery} = \frac{\text{molar of glycerol and 1,3−PD in permeate and retentate from filtration}}{\text{molar of glycerol and 1,3−PD in slurry I+molar of glycerol and 1,3−PD in liquid I}} \times 100 \quad (6.23)$$

$$= \frac{28.2}{31+8} \times 100 = 72\%$$

6) Lactic acid balance

Lactic acid was lost from TSF (864 g or 10 M) which accounted for 45% of total losses (Figure 6.8). Lactic acid could be converted to acetic acid under anaerobic condition in molar ratio of 1:1 (Oude Elferink *et al*., 2001). Therefore, conversion of lactic acid to acetic acid from the metabolism of *Lactobacillus* species may lead to this loss. Lactic acid balance showed below.

Lactic acid in = lactic acid in TSF

= 1918 g = 21 M

Lactic acid out = lactic acid in permeate and retentate from filtering liquid I, liquid I desludger, liquid II desludger, and liquid III desludger (6.24)

= 244 + 15 + 295 + 33 + 125 + 16 + 73 + 7

= 808 g = 9 M

Lactic acid lost = lactic acid in - lactic acid out (6.25)

= 1,918 − 808

= 1,110 g = 12 M

Lactic acid lost from filtration = lactic acid lost from filtering liquid I + lactic acid lost from filtering liquid I desludger + lactic acid lost from filtering liquid II desludger + lactic acid lost from filtering liquid III desludger (6.26)

= 31 + 88 + 21 + 32 = 172 g = 2 M

Lactic acid losses were calculated based on the assumption of no lactic acid remaining in solids after washing. Specifically, lactic acid balance was calculated assuming that no lactic acid was in solid III decanter. The amounts of lactic acid present in liquid I desludger, liquid II desludger, and liquid III desludger decreased in that order indicating that washing removed lactic acid associated with solids (Table 6.5 and Appendix D Table D.2). Lactic acid balance indicated that there was 1,110 g (12 M) of lactic acid lost which accounted for 58% lactic acid lost from the entire process. Furthermore, there was 864 g (10 M) of lactic acid lost from TSF, which account for 78% of lactic acid lost from the entire process. These results indicated that majority of lost lactic acid occurred during TSF. Moreover, there was 172 g (2 M or 18%) of

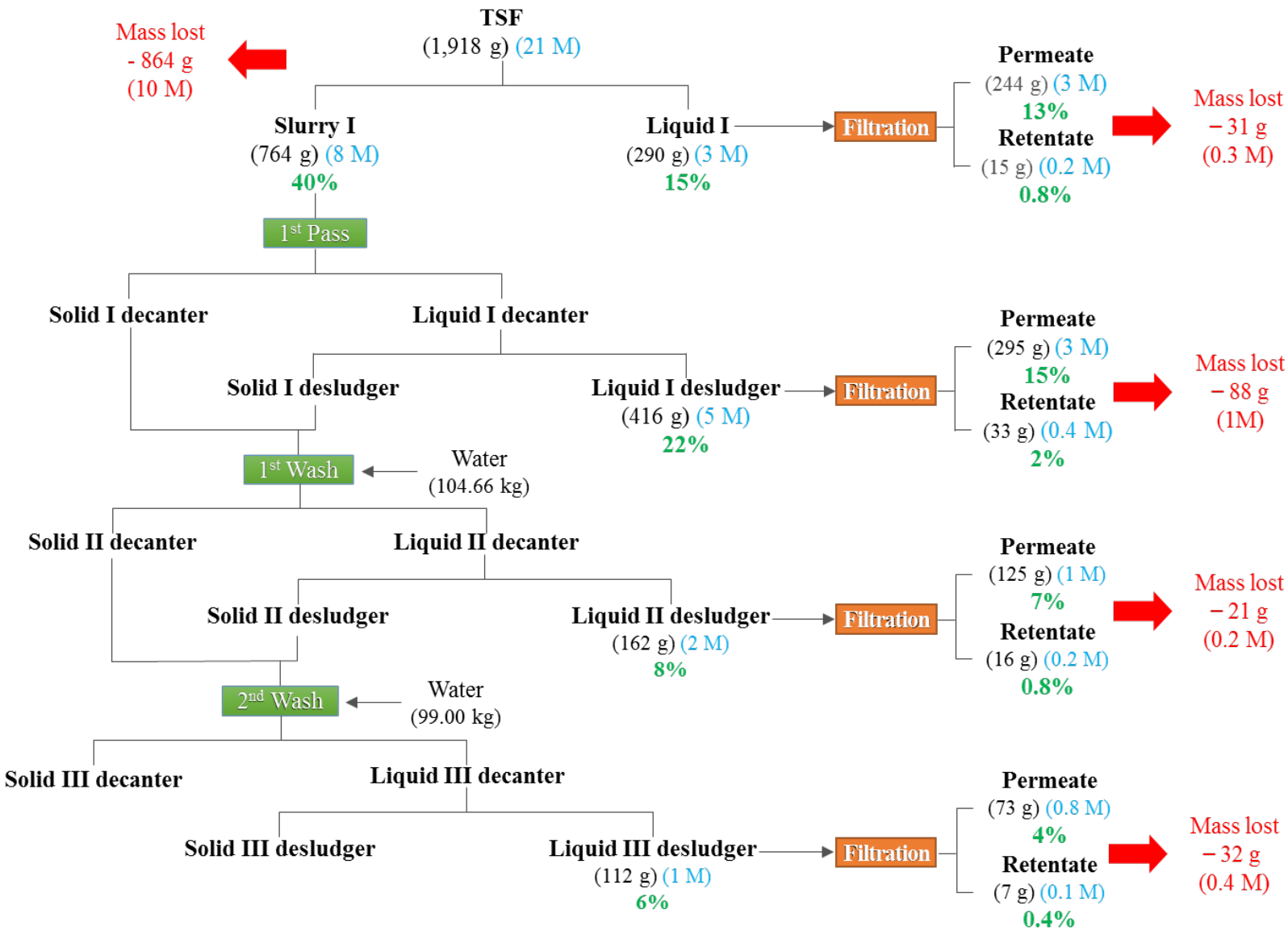

Figure 6.8 Lactic acid balance of replications of small-scale TSF

lactic acid lost during filtration. The liquid remaining in tubing of the microfiltration device may contain lost lactic acid. In addition, other losses of lactic acid could arise from losses during solid washing. It should be noted that there was lactic acid in solids after washing that was not determined. Therefore, lactic acid balance is not complete. Percent recovery of lactic acid from washing and filtration was calculated from the Equation below.

$$\% \text{ Recovery} = \frac{\text{gram of lactic acid in permeate and retentate from filtration}}{\text{gram of lactic acid in slurry I+gram of lactic acid in liquid I}} \times 100 \quad (6.27)$$

$$= \frac{808}{764+290} \times 100$$

$$= 77\%$$

7) Acetic acid balance

According to Figure 2.3, 1 M of lactic acid could be converted to 1 M of acetic acid. However, the results from lactic acid balance (Figure 6.8) indicated that there was 10 M of lactic was lost during TSF, which could lead to approximately 10 M of acetic acid being produced (Figure 6.9). There were 15 M (77%) of acetic acid produced during TSF, which is more than expected from the conversion of lactic acid to acetic acid alone. These results indicated that acetic acid-producing bacteria present in W-TS may utilize other carbon sources to produced acetic acid during TSF. Therefore, the moles of acetic acid produced from TSF is more than the moles of lactic acid lost during TSF.

When comparing the quantity of acetic present in slurry I with the acetic acid from liquid I, it was found that there were 81 and 19% of acetic acid present in slurry I and liquid I, respectively. This distribution could arise from the greater microbial population in slurry I compared to liquid I (as previously mentioned in 1,3-PD balance section). The quantity of acetic acid in liquid from washing slurry I indicated that there was the decrease of acetic acid in the liquid from liquid I desludger, liquid II desludger, and liquid III desludger, in that order (Table 6.5 and Appendix D Table D.2). This indicates that acetic acid could be removed from solids by washing, which could be of benefit for acetic acid extraction and recovery. Acetic acid balance illustrated below.

Acetic acid in = acetic acid in TSF

= 1,173 g

= 20 M

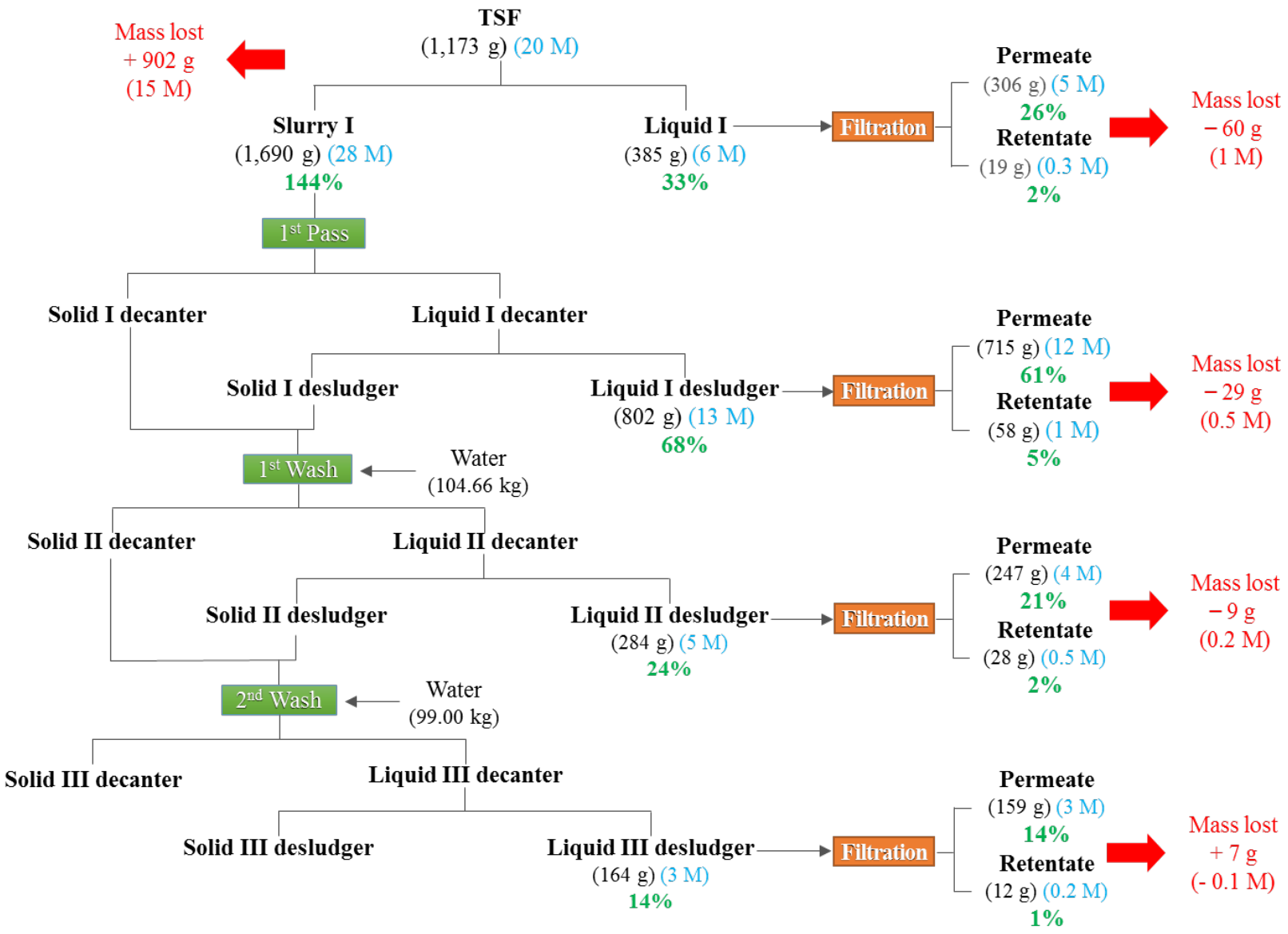

Figure 6.9 Acetic acid balance of replications of small-scale TSF

Acetic acid out = acetic in permeate and retentate from filtering liquid I, liquid I desludger, liquid II desludger, and liquid III desludger (6.28)

= 306 + 19 + 715 + 58 + 247 + 28 + 159 + 12

= 1,544 g = 26 M

Acetic acid gain = acetic acid out − acetic acid in (6.29)

= 1,544 − 1,173

= 371 g = 6 M

Acetic acid lost from filtration = acetic acid lost from filtering liquid I + acetic acid lost from filtering liquid I desludger + acetic acid lost from filtering liquid II desludger + acetic acid lost from filtering liquid III desludger (6.30)

= 60 + 29 + 9 − 7

= 91 g = 2 M

Acetic acid losses were calculated based on the assumption of no acetic acid remained in solid from washing (i.e. there was no acetic acid remaining in solid III decanter). Acetic acid lost from washing was not calculated.

Acetic acid accumulated in TSF process solutions (902 g or 15 M, 77%). In addition, it was found that 91 g (6%) of acetic acid was lost during microfiltration. Liquid remaining in tubing of the microfiltration equipment could have led acetic acid losses. Liquid and solid losses from washing may explain other acetic acid loses. Furthermore, acetic acid balance did not include acetic acid remaining in solid fractions after washing. Percent recovery of acetic acid from washing and filtration was calculated from the Equation below.

$$\% \text{ Recovery} = \frac{\text{gram of acetic acid in permeate and retentate from filtration}}{\text{gram of acetic acid in slurry I+gram of acetic acid in liquid I}} \times 100 \quad (6.31)$$

$$= \frac{1{,}544}{1{,}690+385} \times 100$$

= 74%

8) GPC balance

GPC balance (Figure 6.10) indicated that there was almost no GPC lost from the entire process. GPC balance is calculated by the Equations below.

GPC in = GPC in TSF

= 342 g = 1 M

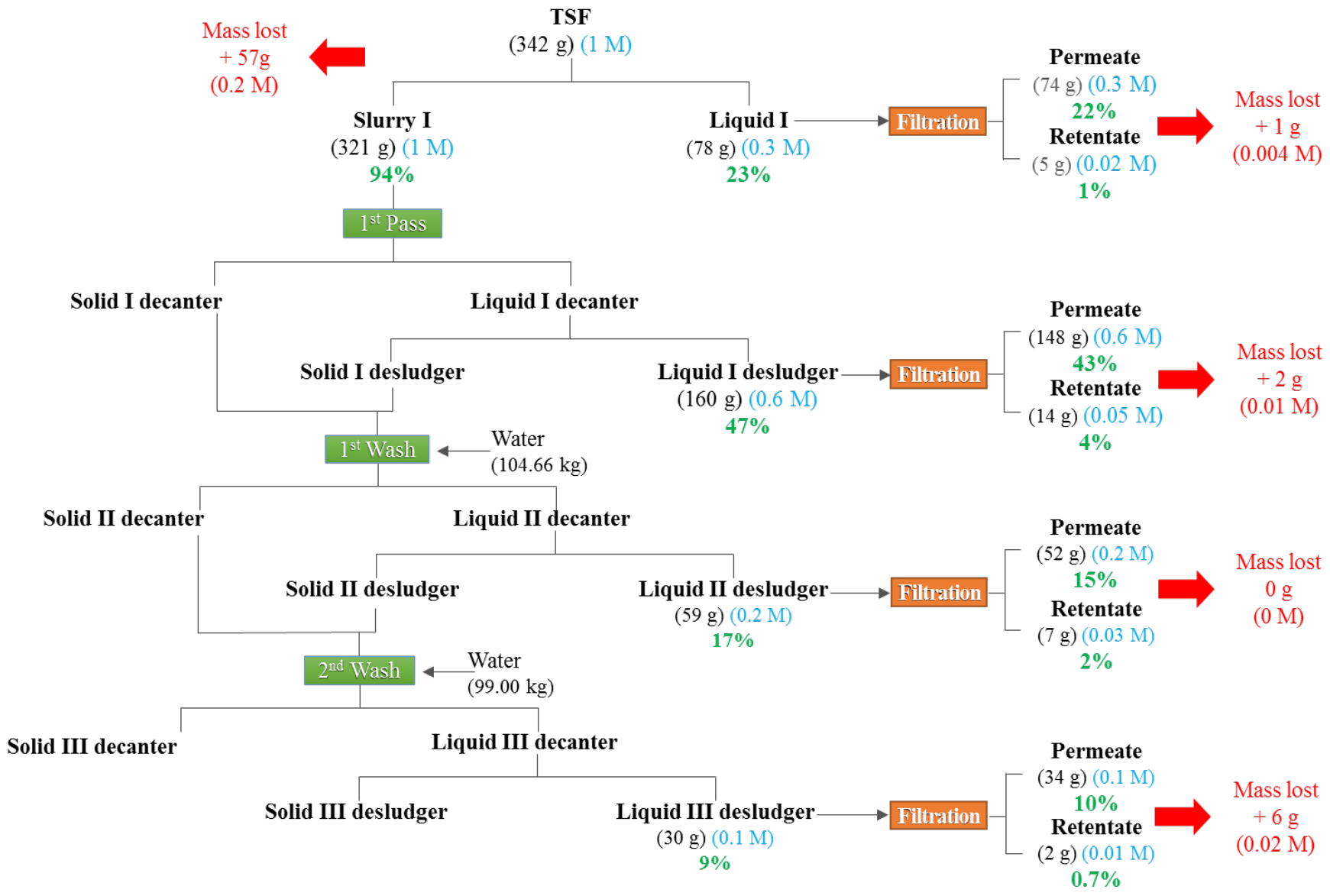

Figure 6.10 GPC balance of replications of small-scale TSF

GPC out = GPC in permeate and retentate from filtering liquid I, liquid I desludger, liquid II desludger, and liquid III desludger (6.32)

= 74 + 5 + 148 + 14 + 52 + 7 + 34 + 2

= 336 g = 1 M

GPC lost = GPC in − GPC out (6.33)

= 342 – 336 = 6 g = 0.02 M

GPC gain from filtration = GPC lost from filtering liquid I + GPC lost from filtering liquid I desludger + GPC lost from filtering liquid II desludger + GPC lost from filtering liquid III desludger (6.34)

= 1 + 2 + 0 + 6 = 9 g = 0.03 M

GPC losses were calculated based on the assumption that no GPC remained in solids from washing and that there was no GPC in solid III decanter. Even though slurry I was washed twice, some GPC remained in the solid fraction. Percent recovery of GPC from washing and filtration was calculated from the Equation below.

$$\% \text{ Recovery} = \frac{\text{gram of GPC in permeate and retentate from filtation process}}{\text{gram of GPC in slurry I+gram of GPC in liquid I}} \times 100 \quad (6.35)$$

$$= \frac{336}{321+78} \times 100 = 84\%$$

6.4.4 Drying Protein Concentrate

Three methods were tested for drying protein concentrate produced by washing slurry I. Spray drying was utilized to dry protein concentrate (Table 6.12). RO water was sprayed to determine outlet temperature without a sample and an outlet temperature of 100 °C was achieved. The inlet temperature of 149 °C was utilized to dry protein concentrate solution, a mixture of protein concentrate and water were mixed in a 1:1 ratio for spraying. It was found that the outlet temperature was 90 °C when a sample was introduced. Particles present in protein concentrate solution might lower the temperature of the outlet stream. However, using an outlet temperature of 90 °C allowed protein concentrate solution to be dried to a powder and accumulate in the dried product reservoir (Figure 6.11). Chaubal and Popescu (2008) reported excessive collection of particles in the spray chamber when operating a spray drier at higher inlet temperatures. In contrast, the lower inlet temperature may result in unacceptably high residual moisture in the product (Chaubal and Popescu, 2008). A major concern when drying protein

Table 6.12 Condition of spray dryer

Sample	Temperature (°C)			Aspirator	% Pump	Nozzle cleaning
	Set point inlet	Actual inlet	Outlet			
Without sample	149	149	100	100	20	4
	149	149	90	100	20	4
With sample	139	139	83	100	20	4
	129	129	75	100	20	4

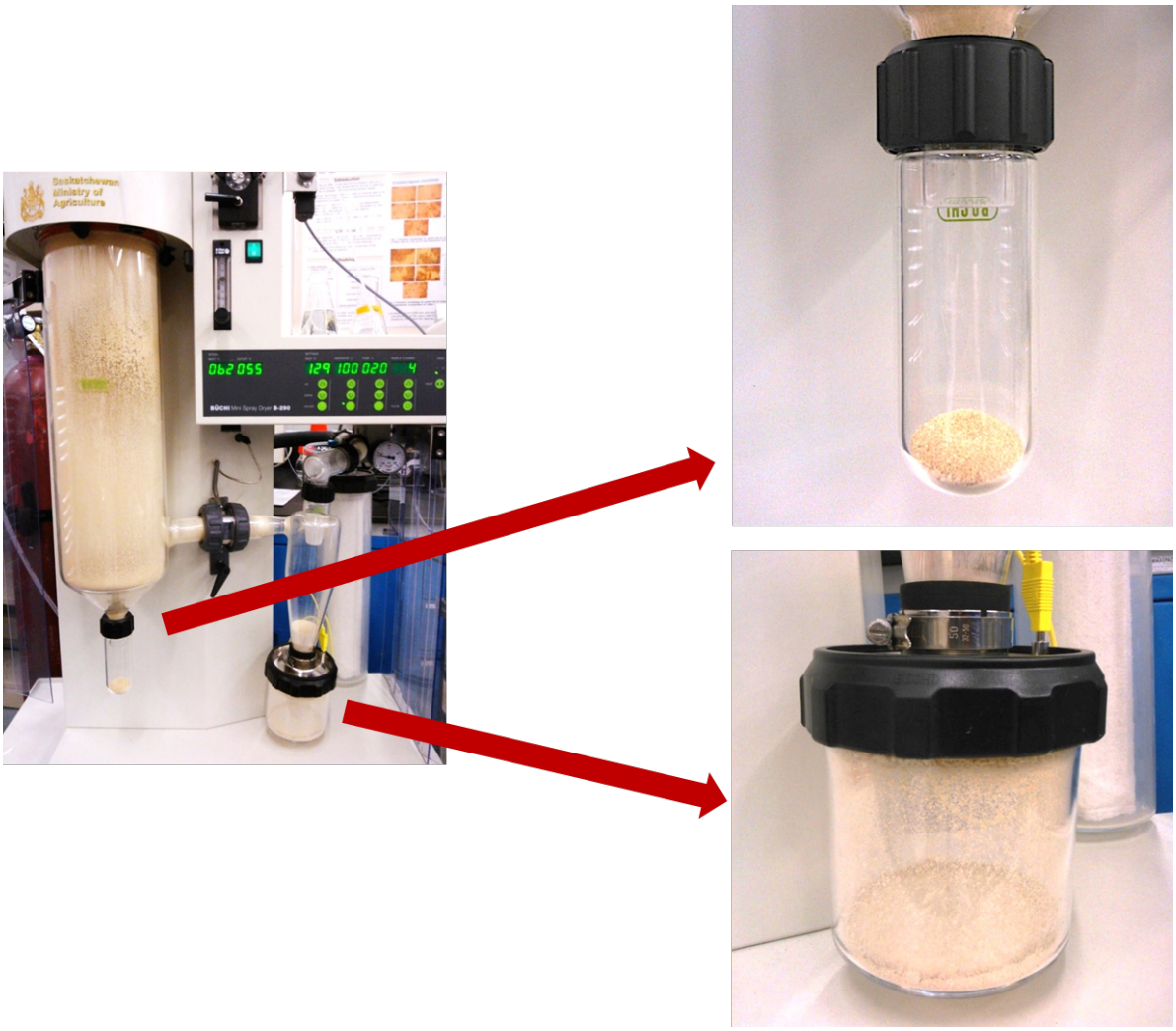

Figure 6.11 Spray dryer

concentrates is the potential for degrading protein and lysine with excessive heat. Consequently, lower set point of inlet temperature was studied to dry protein concentrate solution and to avoid using high drying temperatures to limit heat damage. The inlet set point temperature was adjusted to 139 and 129 °C resulting in temperatures of 83 and 75 °C, respectively. It was discovered that protein concentrate solution dried to a powder when processed at an outlet temperature of 75 °C. Production cost for drying protein concentrate with lower temperature spray drying would be advantageous.

Drum drying was also employed in drying protein concentrate solutions (Figures 6.12A and 6.12B). The drum rotation speed was varied to study temperature effects on drying of the protein concentrate solution (Table 6.13). With a drum rotation speed of 2, the temperature at the surface of the drum was approximately 110 °C and the protein concentrate solution was not fully dried. Apparently the higher drum rotation speed did not allow sufficient drying time. Therefore, the drum rotation speed was reduced to 1 and a higher surface temperature was achieved as stated by Valous *et al*., 2002. The drum surface temperature was approximately 130 °C. This drum rotation speed led higher drum surface temperature drum and adequate drying time. The protein concentrate solution was dried acceptably when using a drum rotation speed setting of 1.

Tray drying was also utilized to dry protein concentrate. The thickness of protein concentrate in the tray determined the rate of protein concentrate drying. The protein concentrate was dried by diffusion of moisture that transports to the surface to be evaporated as stated by Doymaz (2004). The temperature for drying was maintained at 70 °C to avoid damage to protein and lysine. The protein concentrate remained in the air-force oven until completely dry.

Dried protein concentrates from spray drying, drum drying, and tray drying were analyzed for protein and moisture contents. The protein concentrate from spray drying, drum drying, and tray drying were similar, approximately 60% (w/w, db) protein and approximately 3–6% moisture (Table 6.14). Based on protein content of dried protein concentrate and the results from 16S ribosome sequencing (Figures 5.3, 5.5, and 5.6), dried protein concentrate could be utilized as the protein source for animal feed to replace soybean meal and fishmeal. In addition, it could be considered as a safe protein source for animal feed as *L. panis*, *L. helveticus*, and *L. gallinarum* contributed most of the sequences present in slurry I. Moreover, it could benefit to the animals as probiotic due to presence of lactobacilli in wet slurry (Pedersen *et al*., 2004) but dried products might not contain live bacteria. Furthermore, because of its low moisture content,

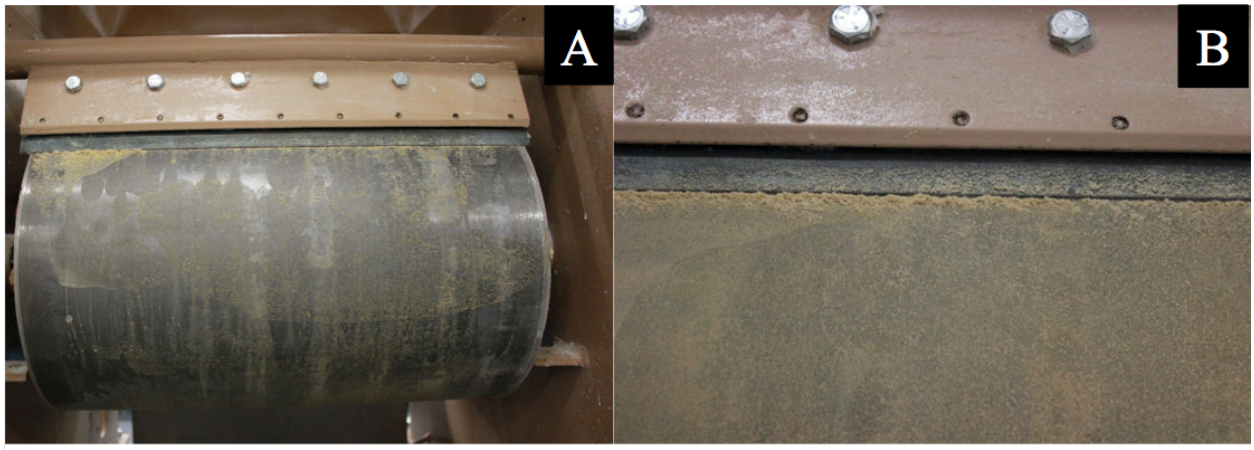

Figure 6.12 (A) drum dryer and (B) drum dryer and dried protein concentrate

Table 6.13 Condition of drum dryer

Steam pressure (kPa)	Actual temperature (°C)		Drum rotation speed	Product
	Drum 1	Drum 2		
221	107	111	2	Wet
221	130	130	1	Dry

Table 6.14 Protein and moisture contents of dried protein concentrate using different devices

Sample	Protein (%, w/w, wb)	Moisture (%, w/w, wb)	Protein (%, w/w, db)
Spray dryer	59 ± 1	3.86 ± 0.04	61 ± 1
Drum dryer	58 ± 1	3.5 ± 0.1	60 ± 1
Tray dryer	59 ± 1	5.76 ± 0.05	63 ± 1

Each value is presented as the mean ± SD (n = 2).
Conversion factor used to calculate protein from nitrogen content was 5.7.

it may be stored at ambient temperature and should provide long shelf-life.

Protein concentrate from spray drying was lightest colored comparing to products prepared by drum drying and tray drying (Figure 6.13). The color of dried protein concentrates obtained from different drying methods were analyzed. Drying method significantly affected dried protein concentrate color ($P < 0.05$) (Table 6.15). Dried protein concentrate produced using a spray dryer was the lightest, the tray dried product was second lightest, and drum dried product was darkest. In addition, dried protein concentrate produced using a drum dryer was reddest following by tray dried and spray dried products, in that order. When considering b value, drum dried product was the yellowest following by tray dried and spray dried products. These findings can be explained in terms of temperature and time for drying. When drying protein concentrates non-enzymatic browning, i.e. the Maillard reaction, could occur due to reactions between primary amines and reducing sugars present. Accumulation of Maillard reaction products is a function of a_w, the nature of amino acids, the nature of reducing sugars, pH, temperature, and time. Temperature and time promote the accumulation of Maillard reaction products (BeMiler and Huber, 2008). The temperature of the drum dryer (130 °C) was higher than the temperature of the spray dryer (75 °C) and the tray dryer (70 °C). The dried protein concentrate produced by drum drying was also darker, redder, and yellower compared to protein concentrate produced by spray and tray drying (Figure 6.13 and Table 6.15). The product produced from spray drying was lighter, less red and yellow than the tray dried product. Even though the spray drying temperature was slightly higher than the drying temperature of the tray dryer, the drying time was much shorter than that of tray drying (approximately 3–4 days). Therefore, there was less accumulation of Maillard reaction products in spray dried materials.

a_w is an important factor associated with degradation of food e.g. hydrolytic chemical reactions and microbial growth. Therefore, safety, stability, and other properties can be predicted from a_w (Reid and Fennema, 2008). All dried protein concentrate had low a_w (Table 6.16). Nevertheless, the methods of drying protein concentrate significantly affected a_w ($P < 0.05$). It was discovered that dried protein concentrate produced by tray drying had the highest a_w followed by drum dried and spray dried products. The temperature utilized for tray drying was low compared to the drying temperatures for spray and drum drying. The a_w of dried protein concentrate produced by spray drying was slightly lower than a_w of dried protein concentrate produced by drum drying. These could be explained by spray dryer efficiency.

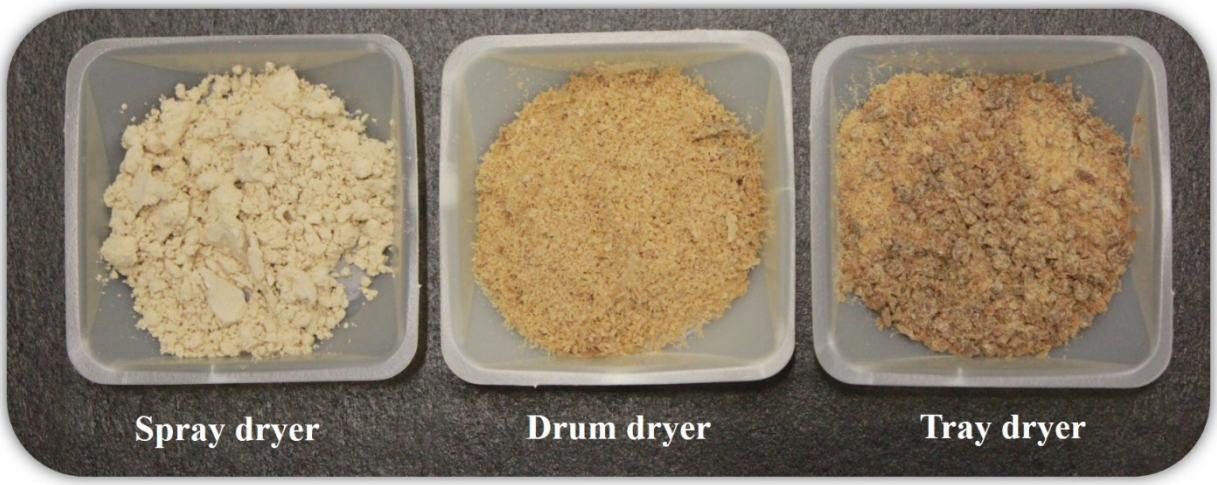

Figure 6.13 Dried protein concentrate using different devices

Table 6.15 Color of dried protein concentrate using different devices

Sample	L	a	b
Spray dryer	66.6 ± 0.2^{a}	6.78 ± 0.09^{c}	18.2 ± 0.2^{c}
Drum dryer	54.8 ± 0.2^{c}	10.36 ± 0.09^{a}	24.8 ± 0.8^{a}
Tray dryer	56.40 ± 0.07^{b}	10.16 ± 0.03^{b}	23.7 ± 0.3^{b}

Each value is presented as the mean ± SD ($n = 2$).
Values with different letters in the same column are significantly different ($P < 0.05$).

Table 6.16 a_w at 22 °C of dried protein concentrate using different devices

Sample	a_w
Spray dryer	0.23 ± 0.00^{c}
Drum dryer	0.25 ± 0.00^{b}
Tray dryer	0.30 ± 0.01^{a}

Each value is presented as the mean ± SD ($n = 2$).
Values with different letters in the same column are significantly different ($P < 0.05$).

The droplets (typically < 500 μm) formed from fluid material by an atomizer and ejected into drying chamber have much greater surface area than the layer applied to the drum drier. The moisture of the droplets was rapidly transferred to the boundary layer surrounding the droplets resulting in very dry particles (Oakley, 2004).

Spray drying was more efficient than drum drying at lowering a_w due to the large drying surface area. The a_w of dried protein concentrate was less than 0.5 (Table 6.16). The products are sufficiently dry to prevent bacterial growth and to have long storage life (Tables 6.1 and 6.2). In addition, Fennema *et al*. (2008) stated that when a_w was below 0.75–0.85, the rate of most reactions tended to decrease. Low a_w could benefit storage of dried protein concentrate by increasing safety and shelf life.

Dried protein concentrate produced from tray drying was analyzed for its nutrients including dry matter, crude protein, crude fat, ash, crude fibre, ADF, NDF, amino acid profile, lysine availability, and major and minor mineral nutrients (Table 6.17). Crude protein content of dried protein concentrate was comparable to soy protein concentrate and much higher than that of western wheat. Western wheat contains substantial carbohydrate while soy and protein concentrates have been treated to deplete total non-protein ingredients. Therefore, soy and protein concentrates have much lower carbohydrates. Moreover, carbohydrates in wheat were saccharified using enzymes and utilized by yeast to produce ethanol. Consequently, dried protein concentrate has higher protein content than the parent grain. Crude fat in dried protein concentrate was higher than that of western wheat and soy protein concentrate. Therefore, the protein concentrate may provide higher energy to animals than western wheat and soy protein concentrate. When considering amino acid profile, amino acids present in dried protein concentrate were higher than those in western wheat. This is due to dilution by carbohydrates present in western wheat as described previously. Much like in wheat (Woychick *et al*., 1961), glutamic acid was the major amino acid in dried protein concentrate. Dried protein concentrate had higher cysteine, methionine, but lower in arginine, isoleucine, lysine, and threonine, than soy protein concentrate. Therefore, lysine should be supplemented when dried protein concentrate is used as an animal feed because lysine plays a major role in building muscle and supporting body weight gain (Tesseraud *et al*., 1992). The growth rate of chickens was reduced when fed lysine deficient diets. Furthermore, pigs fed lower lysine diets gained less muscle and body weight while retaining less nitrogen than pigs fed a higher lysine diet (Noblet *et al*., 1987).

Table 6.17 Nutrients of dried protein concentrate from tray drying compared to western wheat and soy protein concentrate

Nutrient (%, w/w)	Dried protein concentrate	Western wheat[1]	Soy protein concentrate[2,3]
Dry matter	94.2 ± 0.1	76.0	91.0
Crude protein	59.97 ± 0.02	15.1	63.0
Crude fat	6.4 ± 0.2	1.5	0.6
Ash	1.6 ± 0.1	NA[4]	6.5
Crude fibre	7.2 ± 0.4	2.5	4.0
ADF	33 ± 1	NA	NA
NDF	21 ± 1	NA	NA
Amino acid (% as is)			
Taurine	0.04 ± 0.01	NA	NA
Hydroxyproline	0.01 ± 0.01	NA	NA
Aspartic acid	3.25 ± 0.01	NA	NA
Threonine	2.08 ± 0.01	0.49	2.73
Serine	2.70 ± 0.04	NA	NA
Glutamic acid	17.7 ± 0.2	NA	NA
Proline	6.04 ± 0.08	NA	NA
Lanthionine	0.00 ± 0.00	NA	NA
Glycine	2.28 ± 0.01	NA	NA
Alanine	2.39 ± 0.01	NA	NA
Cysteine	1.44 ± 0.00	0.34	0.98
Valine	3.19 ± 0.04	0.84	3.38
Methionine	1.22 ± 0.00	0.24	0.91
Isoleucine	2.60 ± 0.00	0.72	3.19
Leucine	4.94 ± 0.00	1.34	5.20
Tyrosine	1.97 ± 0.00	NA	NA
Phenylalanine	3.17 ± 0.01	0.91	3.45
Hydroxylysine	0.05 ± 0.00	NA	NA
Ornithine	0.07 ± 0.01	NA	NA
Lysine	1.92 ± 0.01	0.45	4.23
Histidine	1.51 ± 0.01	NA	1.82
Arginine	2.92 ± 0.00	0.69	4.94
Tryptophan	0.64 ± 0.01	0.15	0.78
Available lysine	1.82 ± 0.01	NA	NA

[1]Wheat for animal feed (Bell, 2013), [2]Feed ingredient (ADM, 2015a), [3]Soycomil® R (ADM, 2015b). Each value is presented as the mean ± SD (n = 2). [4]NA means not available.

Table 6.17 Nutrients of dried protein concentrate from tray drying method comparing to western wheat and soy protein concentrate (cont'd)

Nutrient (%, w/w)	Dried protein concentrate	Western wheat[1]	Soy protein concentrate[2,3]
Major mineral (%)			
Phosphorus	0.35 ± 0.00	NA[4]	0.8
Sulfur	0.8 ± 0.1	0.18	NA
Calcium	0.20 ± 0.00	0.04	0.35
Sodium	0.04 ± 0.00	0.03	0.011
Magnesium	0.04 ± 0.00	0.36	0.32
Potassium	0.13 ± 0.00	0.40	2.20
Minor mineral (ppm)			
Selenium	< 2 ± 0	0.38	NA
Iron	146 ± 2	46	130
Zinc	< 0.1 ± 0.0	30	35
Copper	17.5 ± 0.7	6	12
Manganese	64.5 ± 0.7	39	NA
Chromium	< 0.1 ± 0.0	NA	NA
Arsenic	< 2 ± 0	NA	NA
Cadmium	< 0.03 ± 0.00	NA	NA

[1]Wheat for animal feed (Bell, 2013), [2]Feed ingredient (ADM, 2015a), [3]Soycomil® R (ADM, 2015b). Each value is presented as the mean ± SD (n = 2). [4]NA means not available.

Dried protein concentrate had excellent lysine availability 1.82 g/100 g of protein as 95%. In addition, the lysine availability of dried protein concentrate was higher than lysine availability of wheat protein, (80–89%) (Sarwar and Bowland, 1975) and wheat flour (84.25% by ileal and 86% by fecal collection) (Sauer *et al*., 1977). The dried protein concentrate had high sulfur content potentially due to the presence of sulfur-containing amino acids (cysteine and methionine). In addition, dried protein concentrate had low metal content (arsenic, cadmium, chromium, and selenium). Subsequently, it is considered as a safe material for animal feed in terms of heavy metal content.

6.5 Conclusion

TSF slurry had higher protein content (approximately 50%, w/w, db) than W-TS protein (approximately 38–43% w/w, db). The protein was concentrated by microbial growth during fermentation. In addition, more than 93% of the microbial population were lactobacilli and largely members of *L. panis*, *L. gallinarum*, and *L. helveticus,* organisms found in sour dough, dairy products, and Swiss cheese. Due to the relatively high protein content and safe microbial constituents, it is likely that slurry I will provide excellent utility as a protein source in animal feed. However, liquid surrounding particles entrains organic solutes in slurry I. Therefore, washing was studied to recover these solutes from TSF and improve protein content. Washing twice was sufficient to recover most solutes from the slurry. Fortuitously, the protein concentrate of solids recovered after washing contained approximately 60% protein (w/w, db). Moreover, tests using pilot-scale equipment show that washing is readily scaled-up to produce a 60% protein product at an industrial level. Furthermore, liquids from TSF and washing were readily filtered through a cross flow microfiltration device with 0.2 μm membrane without indication of flux drop or membrane fouling. Mass balances of solids and organic solutes from TSF followed by three cycles of centrifugation, decanting, and washing was tracked. Losses were recorded for mass (4% of mass lost) and protein (34% lost). Recovery of organic solutes including glycerol (76%), 1,3-PD (72%), lactic acid (77%), acetic acid (74%), and GPC (84%) from washing and filtration were determined. Protein and GPC might be used as markers for processing losses associated with liquids and solids respectively. Protein rich slurries had high moisture contents after decanting (80–90% w/w). These wet materials would be difficult to store due to the potential for further fermentation and shipping would be difficult due to the high wet product

mass. Therefore, spray drying, drum drying, and tray drying were tested to determine the effects of drying on protein concentrate quality. The three drying methods were able to sufficiently dry protein concentrate. In addition, dried protein concentrate produced from these three methods had low moisture content (4–6%) and a_w (approximately 0.3). Chemical reactions and microbial growth will be very slow in these dried products. This property would benefit storing and shipping of the material.

The protein concentrate from tray drying had high glutamic acid and low lysine contents. These should be taken into consideration that even though the lysine availability (95%) was excellent, lysine should be supplemented before using this concentrate as a protein source in animal feed. In addition, it had very low heavy metal content.

CHAPTER 7
GENERAL DISCUSSION

The world ethanol production has been increasing annually and is predicted to reach approximately 135 billion L by the end of 2016 (OECD/ Food and Agriculture Organization of the United Nations, 2014). Grain based ethanol plants produce beers of approximately 10–20% (v/v) (Ingledew *et al*., 2009). Therefore, evaporation of ethanol from the beer will generate 4-10 times as much TS as alcohol. If 135 billion L of ethanol are produced approximately more than 540 billion L of TS will be generated by the ethanol industry in 2016.

W-TS is composed of salts and a number of organic solutes including 1,3-PD, acetic acid, betaine, ethanol, glycerol, GPC, isopropanol, lactic acid, phenethyl alcohol and succinic acid and salts. These solutes arise from bacteria, plant, and yeast metabolites (Ratanapariyanuch, 2009; Ratanapariyanuch *et al*., 2011) and are potentially valuable without modification or as precursors for additional processing. In addition corn-based TS contains solids of approximately 5–10% (Liu and Barrows, 2013) and protein of W-TS is approximately 36.6 and 45.7% (w/w, db) (Mustafa *et al*., 1999; Mustafa *et al*., 2000). Protein arises from microorganisms and undissolved solids from ethanol fermentation present in W-TS. Due to its high protein content, it is possible to recover solid from W-TS for use as a protein source for animal feed. Two objectives were pursued for adding value to W-TS. These are the development of efficient processes for recovery of solutes and the development of processes for protein concentrate production.

Organic solutes present in W-TS, that are potentially worth recovering include 1,3-PD, acetic acid, and GPC. 1,3-PD can be utilized for many synthetic reactions, particularly as a monomer for polycondensation reactions to produce polyesters and polyurethanes (Biebl *et al*., 1999). Recently, 1,3-PD has been used as a monomer for novel polyester production including nylon, and biodegradable plastic (Saxena *et al*., 2009). Moreover, its applications include cosmetics, foods, transport fuels (antifreeze, fuel additives, or lubricants), and medicines (Xiu and Zeng, 2008). The global sales of 1,3-PD are expected to reach approximately 225.9 kilo tonnes by 2022 from 146 kilotonnes (Business Standard, 2015).

Acetic acid is widely used. Vinegar is a solution of water and acetic acid. It is also a precursor to produce polyvinyl acetate. Household-cleaning products also contained acetic acid. In addition, it is employed in the production of terephthalates [raw material for polyethylene terephthalate production] as solvent. Metal acetate produced from acetic acid includes aluminium acetate and iron (II) acetate (mordant for dyes), copper (II) acetate (pigment and fungicide), and sodium acetate (textile industry and a food preservative) etc. (New World Encyclopedia, 2012). Global demand and world market of acetic acid is expected to be approximately 18,000 kilo tonnes and $13.31 billion by 2022 (Grand View Research, 2015).

GPC is a known precursor in the biosynthesis for membrane phospholipids and the neurotransmitter ACh (Sangiorgi *et al*., 1994). Studies have compared the impact of various dietary cholinergic compounds including GPC on the ability to affect cognitive functions. GPC ingestion is associated with a significant improvement in cognitive symptoms that is not apparent with the consumption of other cholinergic precursors such as choline or lecithin (Parmetti *et al*., 2007). GPC may also be considered as a cholinergic precursor in acute cerebrovascular disease and in dementia disorder (Parnetti *et al*., 2001). Moreover, the cellular GPC level increased significantly in several breast cancer cell lines when treated with antimicrotubule drugs including paclitaxel (anticancer drug), vincristine, colchicine, and nocodazole. Furthermore, the level of GPC elevated, and the level of phosphocholine reduced when malignant human mammary epithelial cells were treated with the nonsteroidal anti-inflammatory agent, indomethacin. In addition, aberrant choline phospholipid metabolism was caused by neurological disorders and inflammation-related pathologies including Alzheimer's disease, Gaucher's disease, and multiple sclerosis (Glunde *et al*., 2004). In addition, GPC has been implicated in diverse cellular functions such as inhibition of lysophospholipase activity inhibition of phosphatidyl inositol transfer protein alpha, and maintenance of renal osmolality. GPC is known for its use in moisturizers, elasticizes restituitives, emollients, nutritive creams, pharmaceutical preparations, and topical cosmetics. In addition to cosmetic and pharmaceutical applications, GPC may be indicated in clinical treatments. Lopez *et al*. (1991) investigated the effect of GPC on rats. They found that GPC reversed the amnesia caused by scopolamine and they observed that cleaving α-GPC within the gut mucosal cells produces free choline and glycerolphosphate. In addition, α-GPC can partly enhance brain ACh levels after scopolamine administration in cortex and hippocampus, but not the striatum. Moreover, when stimulating the rats treated with α-GPC with potassium, the

amount of ACh released by hippocampus increased. Parnetti *et al*. (1993) conducted a study with 126 patients who had probable senile dementia (Alzheimer's type) by investigating the effects of GPC on patients with respect to the patients' conditions with a reference drug (ST200). They found that GPC improved most neuropsychological parameters significantly and the effects of GPC lasted longer than ST200 (Parnetti *et al*., 1993). Amenta, *et al*. (2001) stated that α-GPC is degraded by glyceryl-phosphorylcholine diesterase to release choline and a glycerol-1-phosphate. The choline is then utilized for ACh synthesis while glycerol-1-phosphate is reconstituted to phospholipid products including nerve cell membrane components. In addition, they also stated that free choline supplementation in diet increased the level of choline in the brain. They suggested that incorporation and storage of cholinergic precursors into brain phospholipids is important for ACh biosynthesis. A report from AC Immune (2015) stated that there were approximately 44 million people were suffering from Alzheimer's disease in this world and the number is expected to be 135 million in 2050. The market of Alzheimer's disease was estimated to be $5 billion and will increase to be more than $20 billion annually by 2020 (AC Immune, 2015). According to the market of Alzheimer's disease, the demand and market of GPC will grow rapidly.

Unfortunately, 1,3-PD, acetic acid, and GPC cannot be extracted from W-TS easily because W-TS contains high boiling points and hygroscopic solutes e.g. glycerol, lactic acid etc. including colloids and particles. Therefore, simple processes should be developed for manufacturers to recover these valuable compounds. Microorganisms present in W-TS, for example lactobacilli (Ratanapariyanuch, 2009) such as *L. panis* PM1B, converted glycerol and lactic acid to 1,3-PD and acetic acid through its metabolic pathways (Kang *et al*., 2014b; Khan *et al*., 2013; Reaney *et al*., 2013). Consequently, *L. panis* PM1B could be utilized to reduce high boiling point and hygroscopic solutes through TSF. In addition, the concentration of 1,3-PD and acetic acid increase during *L. panis* PM1B metabolism of stillage. Therefore, TSF could be a novel and simple process that can be applied to manufacturing.

Before extracting/recovering valuable compounds, it is necessary to clarify TS. Corn-based TS contains approximately 5–10% dry matter (Liu and Barrows, 2013). Particles present in W-TS could cause equipment fouling and thereby interfere with heat and mass transfer during heating and filtration. Several researchers have been studying methods to clarify TS including clarifying agent, centrifugation, and membrane filtration. These methods are commonly utilized

for industrial applications. For examples, clarifying agents can clarify red wine (Castellari *et al*., 2001), anionic polymer with cross linking agents and inorganic solutes can be used to dewater corn-based stillage solids (Scheimann, 2009) or polyelectrolytes can clarify corn-based TS (Menkhaus *et al*., 2010). Furthermore, nanofiltration and ultrafiltration have also been used to clarify corn-based TS (Arora *et al*., 2010; Arora *et al*., 2009, 2011; Bento and Fleming, 1993). However, pre-treatment/preparation processes are necessary to prevent membrane fouling and pore blockage during W-TS filtration. AGF is a method that has been utilized to treat animal waste e.g. dairy manure, sewage sludge etc. AGF utilizes anoxic gas to concentrate and return organic acids, protein, and partially digested substances to anaerobic digesters (Burke, 1997b, 1998, 2000b). Metabolic pathways of lactobacilli including *L. panis* PM1B (Biebl *et al*., 1999; Kang *et al*., 2013b; Kang *et al*., 2014b; Oude Elferink *et al*., 2001) produce the anoxic gas, CO_2. Therefore, it is possible that anoxic gas could be generated during TSF. In addition, EPSs produced by lactobacilli (Cerning *et al*., 1994; De Vuyst and Degeest, 1999; Hammes and Gänzle, 1997; Tallon *et al*., 2003) could destabilize colloids and particles. Fermentative coagulation could be a novel method for W-TS clarification. Moreover, TSF process does not require the use of a clarifying agent.

The second aim of this research was to produce protein concentrate. As previously stated, W-TS contained protein from wheat particles. In addition, lactobacilli from TSF could improve protein content and may be beneficial as probiotics for animals when utilized it in animal feed. The high protein content of protein concentrate produced from this research (approximately 60% w/w, db) would be considered as an ideal protein source for animal feed. Good quality of fishmeal should contain more than 66% of protein (Feedipedia, 2015). The price of fishmeal in April, 2015 was CAD $2,287/metric ton (Indexmundi, 2015). The price of DDG, wheat DDGS, canola meal, soy meal including the feed meal per metric ton of protein is less expensive than fishmeal (Table 7.1). In addition, the price of feed per metric ton of protein may be compared against the protein content in a range of feed protein sources (Figure 7.1) to show the increase in as value with protein concentration. Feeds with higher protein content, command a greater value ($/metric ton of protein). According to Figure 7.1, it was estimated that the price of protein concentrate (60% protein content) would be approximately $2,500/metric ton of protein.

To determine the economic impact of protein recovery on ethanol production, it is useful to consider a specific example. The Terra Grain Fuels ethanol plant (SK, Canada) produces 150

Table 7.1 Meal price per metric ton

Animal feed	Price ($/metric ton)	% Protein content	Price ($/metric ton of protein)
DDG	173.38[1]	31[5]	559.29
Wheat DDGS	370.71[2]	37[6]	1,001.92
Canola meal	410.70[3]	38[7]	1,080.79
Soy meal	751.17[2]	48[2]	1,564.94
Fish meal	2,287.00[4]	66[8]	3,465.15

The information of price and protein content retrieved from
[1]Capehart *et al*., 2015
[2]Cordero, 2014, calculate from short ton to long ton and conversion rate US $1 = CAD $1.18
[3]Canola Council of Canada, 2015
[4]Indexmundi, 2015
[5]MacDonald *et al*., 2007
[6]Beliveau and McKinnon, 2008
[7]Higgs *et al*., 2014
[8]Feedipedia, 2015

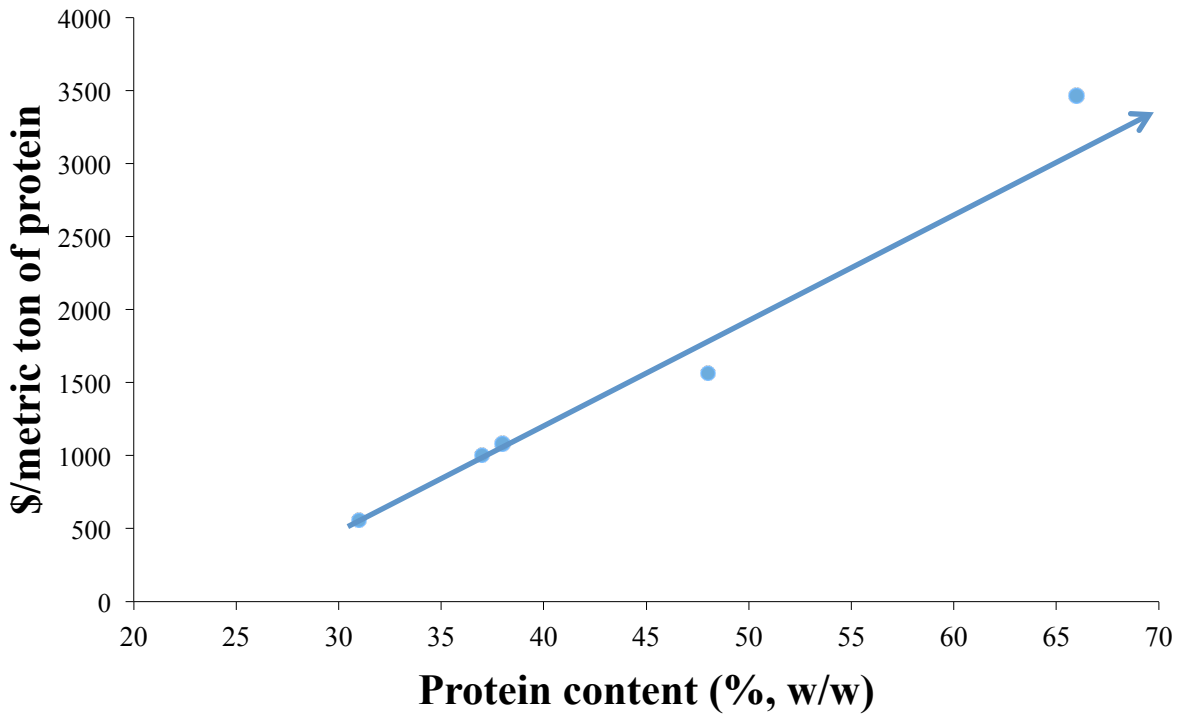

Figure 7.1 Relationship of protein content and price of meal/metric ton of protein in a concentrate. A sixty percent protein content feed ingredient would be approximately $2,500/metric ton of protein.

million litres of ethanol annually from a beer that has less than 15% ethanol. The mass of TS after ethanol production is approximately 6 times the ethanol mass. For the Terra Grains Fuels plant, this would be 900,000 metric tons of W-TS annually. Corn-based TS contained 5–10% solid (Liu and Barrows, 2013). As a result, 900,000 metric tons of W-TS could generate approximately 90,000 metric tons of solid, which will be approximately 41,130 metric tons of protein (calculated from 45.7% protein (db) (Mustafa *et al*., 1999). If the value of the protein was $2,500/metric ton of 60% protein then Terra Gain Fuels could essentially produce $103 million worth of protein concentrate each year. In addition, W-TS contained GPC approximately 0.1% (w/v) (Ratanapariyanuch *et al*., 2011). Stillage from the plant could yield 900,000 kgs of GPC leading to $450 million/year of GPC. However, the values from ethanol, protein concentrate, and GPC are not the net income. Therefore, other costs associated with processing e.g. labour and maintenance should be considered.

Engineering information would be required to determine the feasibility of the proposed processes for conversion of inexpensive W-TS to more valuable products. There are many additional factors to consider if this is to lead to a new process and business venture.

CHAPTER 8
SUMMARY AND CONCLUSIONS

W-TS contained organic solutes from bacteria, wheat, and yeast metabolites. Some of these organic solutes e.g. 1,3-PD, acetic acid, and GPC are valuable and may be recovered. In addition, W-TS also composed of protein from microorganisms and wheat. Therefore, the objectives of this research were 1) organic solute recovery and 2) protein concentrate production. Organic solutes cannot readily be recovered and extracted as W-TS contains high boiling point and hygroscopic solutes, for example glycerol and lactic acid. TSF uses *L. panis* strain PM1B to reduce glycerol and lactic acid concentration and increase the 1,3-PD and acetic acid concentrations. In order to achieve maximum conversion of glycerol and lactic to 1,3-PD and acetic acid, TSF should be conducted under improved conditions. It was discovered that agitation, micronutrients, and pH affected significantly on TSF fermentation. Fermentation temperature 37 °C, freeze-dried W-TS, pH adjusted to 5 daily, ratio of glucose: glycerol of 0.1 M: 1 M, static fermentation conditions, and addition of vitamin (B2, B3, and B12) aided TSF. Furthermore, addition of 1,3-PD and 3-HPA did not impede accumulation of 1,3-PD. TSF was possible at 25 °C and scaled-up to 20 L and 150 L with concentration of 1,3-PD approximately 4 and 2%, respectively in the media after fermentation.

Clarification of the TSF product is an essential process step prior to compound recovery as it helps to prevent equipment fouling and interference of solids with heat transfer and mass transfer from heating and filtration. Several methods were attempted to clarify W-DS solution and W-TS. The use of clarifying agents, W-DWG, and fermentative coagulation were studied for diluted W-DS solution and W-TS clarification. These methods were able to clarify diluted W-DS solution and W-TS. Compared to other clarifying agents bentonite (0.4%, w/v) had the greatest impact on clarity. When using W-DWG to clarify W-TS, turbidity of solution after pressing the mixture of W-DWG from W-B and W-TS with a modified piston screen press was lower than W-TS. In addition, the structure of W-DWG was spongy and could be used repeatedly for pressing. Therefore, this material can be reused for clarification. However, the structure of

W-DWG when absorbing W-TS, pressing, and after pressing should be further explored. In addition, the ability to reuse W-DWG to clarify W-TS with more pressing cycles should also be tested. The use of fermentative coagulation to clarify W-TS was also studied. It was discovered that as fermentation progressed, CO_2 and EPS produced from the metabolic pathways of lactobacilli resulted particle separation producing a liquid (liquid I) and slurry (slurry I). It was found that protein content and dry matter content of slurry I increased as fermentation progressed while the opposite trend occurred for liquid I. Moreover, the concentration of glycerol and lactic acid and the concentration of 1,3-PD and acetic acid in slurry I were lower and higher than that of liquid I, in that order. These could be the results of biofilm formation from EPS produced from lactobacilli in slurry I. These phenomenon would prevent the diffusion of lactobacilli to liquid I resulting better conversion in slurry I.

TSF (25 L scale) was conducted at 25 °C. It was discovered that as fermentation time increased, glycerol and lactic acid concentrations decreased and the concentrations of 1,3-PD and acetic acid increased. Furthermore, protein and dry matter contents of slurry I increased and *vice versa* with respect to liquid I. In addition, approximately 22–56% of liquid I separation could be achieved at the end of TSF. This phenomenon occurred because of the metabolism of endemic flora including lactobacilli and TSF. It was also found that liquid I obtained from TSF was not colloidal and could be filtered using ultrafiltration membranes without flux drop and membrane pore blocking. Moreover, the result from 16S ribosome sequencing of slurry I organisms showed that lactobacilli contributed more than 93% to microbial populations with members of *L. panis* being the predominant bacterial species. Due to its high protein content and the results of microbial population, slurry I could be useful as a protein source for animal feed and could have probiotic effect. When comparing fermentation at 25 and 37 °C, the results showed the reduction of fermentation time in terms of glycerol depletion and increase of 1,3-PD from TSF at 37 °C.

Slurry I is a mixture of entrained solution similar in composition to liquid I and protein rich particles. Therefore, centrifugation and washing processes are necessary to recover the valuable solutes and improve protein content. Slurry I from 25 L scale conducted at 25 °C was studied for washing processes. It was discovered that protein contents of solids after washing (protein concentrate) increased to approximately 60% (w/w, db). In addition, the concentration of valuable compounds in water from washing decreased when number of washing increased. These findings suggest that washing processes could improve protein content and recovery of

valuable solutes entrained in slurry I. However, two washes were sufficient to recover these solutes.

Replication of 25 L scale TSF was conducted to obtain sufficient slurry I to study washing processes using a solid-bowl decanter followed by a disk stack desludger centrifuge. Two washing treatments were utilized. It was found that protein content of the solid, protein concentrate, after washing was approximately 60% (w/w, db). In addition, the concentration of solutes in liquid from washing was reduced when the number of washes increased. Moreover, the liquid from TSF and washing could be filtered through an industrial-scale microfiltration unit without the indication of flux drop and membrane blocking. From overall processing (TSF, washing, and filtration), there were 4% of mass lost, 34% of protein lost, 76% glycerol recovery, 72% 1,3-PD recovery, 77% of lactic acid recovery, 74% of acetic acid recovery, and 84% of GPC recovery. Moreover, protein and GPC were suitable markers for solid and liquid processing fractions respectively.

Because of high moisture content of protein concentrate, drying processes are necessary to reduce the moisture to prevent spoilage and reduce transportation costs. Spray drying, drum drying, and tray drying were studied. The results from these three methods showed that they could be utilized to produce dry protein concentrates. In addition, the moisture content was reduced from 80–90% (w/w) to approximately 4–6% (w/w). Moreover, the a_w of dried protein concentrates were low (approximately 0.3) indicating that the dried protein concentrate could have a long shelf-life. Moreover, nutrients of dried protein concentrate produced from tray drying were analyzed. The amino acids were rich in glutamic acid and low in lysine. Even though the lysine availability was approximately 95%, lysine should be supplemented prior to use a protein source for animal feed. In addition, the heavy metal content was low. These suggested that dried protein concentrate is a safe material in terms of heavy metal exposure.

REFERENCES

Abbad-Andaloussi, S., Manginot-Durr, C., Amine, J., Petitdemange, E., & Petitdemange, H. (1995). Isolation and characterization of *Clostridium butyricum* DSM 5431 mutants with increased resistance to 1,3-propanediol and altered production of acids. *Applied and Environmental Microbiology*, *61*(12), 4413–4417.

AC Immune. (2015). Alzheimer's disease: Incident and market. Retrieved on May 29, 2015 from http://www.acimmune.com/content/?p=22.

ADM. (2015a). Feed ingredients catalog. Retrieved on May 21, 2015 from http://www.adm.com/en-US/products/Documents/ADM-Feed-Ingredients-Catalog.pdf

ADM. (2015b). Soycomil R. Retrieved on May 21, 2015 from http://www.adm.com/en-US/Products/_layouts/ProductDetails.aspx?productId=430.

Akiyama, D. M., Dominy, W. G., & Lawrence, A. L. (1992). Penaeid shrimp nutrition. In Fast, A. W., & Lester, L. J. (Eds.) *Marine shrimp culture: Principles and practices* (pp 535–568), Amsterdam, The Netherlands: Elsevier Science B.V.

Amenta, F., Parnetti, L., Gallai, V., & Wallin, A. (2001). Treatment of cognitive dysfunction associated with Alzheimer's disease with cholinergic precursors. Ineffective treatments or inappropriate approaches?. *Mechanisms of Ageing and Development, 122*(16), 2025–2040.

Anderson, J., Schingoethe, D., Kalscheur, K., & Hippen, A. (2006). Evaluation of dried and wet distillers grains included at two concentrations in the diets of lactating dairy cows. *Journal of Dairy Science, 89*(8), 3133–3142.

AOAC. (2006). In Horwitz, W. & Latimer, G. W. (Eds.) *Official methods of analysis of AOAC international*, *Eighteen Edition*, Gathersburg, USA: AOAC International.

Arendt, E. K., Ryan, L. A., & Dal Bello, F. (2007). Impact of sourdough on the texture of bread. *Food Microbiology*, *24*(2), 165–174.

Arora, A., Dien, B. S., Belyea, R. L., Singh, V., Tumbleson, M. E., & Rausch, K. D. (2010). Nutrient recovery from the dry grind process using sequential micro and ultrafiltration of thin stillage. *Bioresource Technology*, *101*(11), 3859–3863.

Arora, A., Dien, B. S., Belyea, R. L., Wang, P., Singh, V., Tumbleson, M. E., & Rausch, K. D. (2009). Thin stillage fractionation using ultrafiltration: Resistance in series model. *Bioprocess and Biosystems Engineering*, *32*(2), 225–233.

Arora, A., Dien, B. S., Belyea, R. L., Wang, P., Singh, V., Tumbleson, M. E., & Rausch, K. D. (2011). Ultrafiltration of thin stillage from conventional and e-mill dry grind processes. *Applied Biochemistry and Biotechnology*, *164*(1), 58–67.

Badel, S., Bernardi, T., & Michaud, P. (2011). New perspectives for lactobacilli exopolysaccharides. *Biotechnology Advances*, *29*(1), 54–66.

Baker, R. W. (2012). Overview of membrane science and technology *Membrane technology and applications*, *Third Edition*: pp 1–14, West Sussex, UK: John Wiley and Sons Ltd.

Beliveau, R., & McKinnon, J. (2008). Effect of graded levels of wheat-based dried distillers' grains with solubles on performance and carcass characteristics of feedlot steers. *Canadian Journal of Animal Science*, *88*(4), 677–684.

Bell, B. (2013). Wheat for animal feed. Retrieved on May 21, 2015 from http://www.omafra.gov.on.ca/english/livestock/beef/facts/wheat.htm

Belyea, R., Eckhoff, S., Wallig, M., & Tumbleson, M. (1998). Variability in the nutritional quality of distillers solubles. *Bioresource Technology*, *66*(3), 207–212.

BeMiler, J. N., & Huber, K. C. (2008). Carbohydrats. In Damodaran, S., Parkin, K. L. & Fennema, O. R. (Eds.) *Fennema's food chemistry*, *Fourth Edition*: pp 83–154, Boca Raton, USA: Taylor & Francis Group.

Benke, M. B., Mermut, A. R., & Chatson, B. (1998). Carbon-13 CP/MAS NMR and DR-FTIR spectroscopic studies of sugarcane distillery waste. *Canadian Journal of Soil Science*, *78*(1), 227–236.

Bento, J. M. A., & Fleming, H. L. (1993). Membrane-based process for the recovery of lactic acid and glycerol from a "corn thin stillage" stream. US Patent 5250182 A.

Beuchat, L. R. (1981). Microbial stability as affected by water activity. *Cereal Foods World*, *26*(7), 345–349.

Biebl, H., Marten, S., Hippe, H., & Deckwer, W.-D. (1992). Glycerol conversion to 1,3-propanediol by newly isolated clostridia. *Applied Microbiology and Biotechnology*, *36*(5), 592–597.

Biebl, H., Menzel, K., Zeng, A.-P., & Deckwer, W.-D. (1999). Microbial production of 1,3-propanediol. *Applied Microbiology and Biotechnology*, *52*(3), 289–297.

Bisson, L. F. (1993). Yeast-metabolism of sugars. In Fleet, G. H. (Ed) *Wine microbiology and biotechnology* (pp 55–75), New York, USA: Taylor & Francis Inc.

Boenigk, R., Bowien, S., & Gottschalk, G. (1993). Fermentation of glycerol to 1,3-propanediol in continuous cultures of *Citrobacter freundii*. *Applied Microbiology and Biotechnology*, *38*(4), 453–457.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254.

Bremford, D. J., & Müller-Steinhagen, H. M. (1996). Multiple effect evaporator performance for black liquor. Part II: Development of improved steady state models. *Appita Journal*, *49*(5), 337–346.

Bremford, D. J., & Müller-Steinhagen, H. (1999). Multiple-effect evaporator performance for black liquor-Part III: The effects of fouling on evaporator performance. *Appita Journal*, *52*(1), 30–36.

Buckner, C. D., Erickson, G. E., Mader, T. L., Colgan, S. L., Karges, K. K., & Gibson, M. L. (2007). Optimum levels of dry distillers grains with solubles for finishing beef steers. *Nebraska Beef Cattle Reports*, *68*, 34–38.

Burke, D. A. (1997a). *Anaerobic digestion of sewage sludge using the anoxic gas flotation (AGF) process.* Paper presented at the 8th International conference on anaerobic digestion, Sendai Japan.

Burke, D. A. (1997b). *Pilot plant operation of the AGF (Anoxic Gas Flotation) stabilization process at potato processing facilities.* Paper presented at the International conference on anaerobic digestion, Sendai Japan.

Burke, D. A. (1998). Nothing wasted. *Civil Engineering-ASCE*, *68*(6), 62–64.

Burke, D. A. (2000a). Anaerobic treatment process with removal of inorganic material. US Patent 6,113,786.

Burke, D. A. (2000b). *Application of the AGF (Anoxic Gas Flotation) process.* Paper presented at the The 4th International Conference: Flotation in water and waste water treatment, Helsinki Finland, Finnish water and waste water works association.

Business Standard. (2015). Global propanediol market to reach $776 mn by 2022. Retrieved on May 29, 2015 from http://www.business-standard.com/content/b2b-chemicals/global-propanediol-market-to-reach-776-mn-by-2022-115042400730_1.html.

Cameron, D. C., Altaras, N. E., Hoffman, M. L., & Shaw, A. J. (1998). Metabolic engineering of propanediol pathways. *Biotechnology Progress*, *14*(1), 116–125.

Canola Council of Canada. (2015). Current canola oil, meal, and seed prices. Retrieved on May 26, 2015 from http://www.canolacouncil.org/markets-stats/statistics/current-canola-oil,-meal,-and-seed-prices/.

Capehart, T., Allen, E., & Bond, J. K. (2015). Feed Outlook: December 2014. Retrieved on May 26, 2015 from http://www.ers.usda.gov/publications/fds-feed-outlook/fds-14l.aspx.

Castellari, M., Versari, A., Fabiani, A., Parpinello, G. P., & Galassi, S. (2001). Removal of ochratoxin A in red wines by means of adsorption treatments with commercial fining agents. *Journal of Agricultural and Food Chemistry*, *49*(8), 3917–3921.

Cerning, J., Renard, C. M. G. C., Thibault, J. F., Bouillanne, C., Landon, M., Desmazeaud, M., & Topisirovic, L. (1994). Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. *Applied and Environmental Microbiology*, *60*(11), 3914–3919.

Chadwick, T. H., & Schroeder, E. D. (1973). Characterization and treatability of pomace stillage. *Journal (Water Pollution Control Federation)*, *45*(9), Annual Conference Issue (September, 1973), 1978–1984.

Challa, R. K. (2015). Characteristics of heat transfer fouling of thin stillage using model thin stillage and evaporator concentrates. Ph.D. thesis. University of Illinois at Urbana-Champaign. Champaign, Illinois, USA.

Chatzifragkou, A., Papanikolaou, S., Dietz, D., Doulgeraki, A. I., Nychas, G. J. E., & Zeng, A. P. (2011). Production of 1,3-propanediol by *Clostridium butyricum* growing on biodiesel-derived crude glycerol through a non-sterilized fermentation process. *Applied Microbiology and Biotechnology*, *91*(1), 101–112.

Chaubal, M. V., & Popescu, C. (2008). Conversion of nanosuspensions into dry powders by spray drying: A case study. *Pharmaceutical Research*, *25*(10), 2302–2308.

Chen, X., Xiu, Z., Wang, J., Zhang, D., & Xu, P. (2003). Stoichiometric analysis and experimental investigation of glycerol bioconversion to 1,3-propanediol by *Klebsiella pneumoniae* under microaerobic conditions. *Enzyme and Microbial Technology*, *33*(4), 386–394.

Cheng, K.-K., Zhang, J.-A., Liu, D.-H., Sun, Y., Liu, H.-J., Yang, M.-D., & Xu, J.-M. (2007). Pilot-scale production of 1,3-propanediol using *Klebsiella pneumoniae*. *Process Biochemistry*, *42*(4), 740–744.

Cheng, K.-K., Zhang, J.-A., Liu, D.-H., Sun, Y., Yang, M.-D., & Xu, J.-M. (2006). Production of 1,3-propanediol by *Klebsiella pneumoniae* from glycerol broth. *Biotechnology Letters*, *28*(22), 1817–1821.

Chirife, J. (1993). Physicochemical aspects of food preservation by combined factors. *Food Control*, *4*(4), 210–215.

Cordero, A. A. (2014). Exports of DDGS in 2013/2014. Retrieved on September 9, 2015 from http://www.distillersgrains.org/files/symposium/2014Presentations/cordero_DGTC__2014.pdf.

Corrigan, W. E., & Mass, R. A. (2009). Utilisation of distillers' dried grains with solubles (DDGS) by cattle. In Ingledew, W. M., Kelsall, D. R., Austin, G. D. & Kluhspies, C. (Eds.) *The alcohol textbook*, *Fifth Edition*: pp 323–336, Nottingham, UK: Nottingham University Press.

Corsetti, A., & Settanni, L. (2007). Lactobacilli in sourdough fermentation. *Food Research International*, *40*(5), 539–558.

Crittended, J. C., Trussell, R. R., Hand, D. W., Howe, K. J., & Tchobanoglous, G. (2005). Coagulation, mixing, and floculation *Water treatment: Princiles and design*, *Second Edition*: pp 643–778, Hoboken, USA: John Wiley & Sons, Inc.

Davis, L., Jeon, Y. J., Svenson, C., Rogers, P., Pearce, J., & Peiris, P. (2005). Evaluation of wheat stillage for ethanol production by recombinant *Zymomonas mobilis*. *Biomass and Bioenergy*, *29*(1), 49–59.

De Man, J. C., Rogosa, D., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of applied Bacteriology*, *23*(1), 130–135.

De Vuyst, L., & Degeest, B. (1999). Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiology Reviews*, *23*(2), 153–177.

De Vuyst, L., & Vancanneyt, M. (2007). Biodiversity and identification of sourdough lactic acid bacteria. *Food Microbiology*, *24*(2), 120–127.

Deegan, R. D. (2000). Pattern formation in drying drops. *Physical Review E*, *61*(1), 475–485.

Deegan, R. D., Bakajin, O., Dupont, T. F., Huber, G., Nagel, S. R., & Witten, T. A. (2000). Contact line deposits in an evaporating drop. *Physical Review E*, *62*(1), 756–765.

Dowd, M. K., Johansen, S. L., Cantarella, L., & Reilly, P. J. (1994). Low molecular weight organic composition of ethanol stillage from sugarcane molasses, citrus waste, and sweet whey. *Journal of Agricultural and Food Chemistry*, *42*(2), 283–288.

Dowd, M. K., Reilly, P. J., & Trahanovsky, W. S. (1993). Low molecular weight organic composition of ethanol stillage from corn. *Cereal Chemistry*, *70*(2), 204–209.

Doymaz, I. (2004). Convective air drying characteristics of thin layer carrots. *Journal of Food Engineering*, *61*(3), 359–364.

Edzwald, J. K. (1995). Principles and applications of dissolved air flotation. *Water Science and Technology*, *31*(3), 1–23.

Edzwald, J. K. (2010). Dissolved air flotation and me. *Water Research*, *44*(7), 2077–2106.

El-Dessouky, H., Ettouney, H., & Al-Juwayhel, F. (2000). Multiple effect evaporation vapour compression desalination processes. *Chemical Engineering Research and Design*, *78*(4), 662–676.

Fallah, H., Fallah, A., Rahmani, A., Afkhami, M., & Ahmadi, A. (2012). Size exclusion mechanism, suspension flow through porous medium. *International Journal of Modern Nonlinear Theory and Application*, *1*(04), 113.

Feedipedia. (2015). Fish meal. Retrieved on May 26, 2015 from http://www.feedipedia.org/node/208.

Fennema, O. R., Demodaran, S., & Parkin, K. L. (2008). Introduction to food chemistry. In Damodaran, S., Parkin, K. L. & Fennema, O. R. (Eds.) *Fennema's food chemistry*, *Fourth Edition*: pp 1–14, Boca Raton, USA: Taylor & Francis Group.

Ferreira, T. F., Saab, V. d. S., de Matosb, P. M., Ribeiroc, C. M. S., & Coelhob, M. A. Z. (2014). Evaluation of 1,3-propanediol production from glycerine by *Clostridium butyricum* NCIMB 8082. *Chemical Engineering Transactions*, *38*, 475–480.

French, C. S., & Milner, H. W. (1955). Disintegration of bacteria and small particles by high-pressure extrusion. In Colowick, S. P. & Kaplan, N. O. (Eds.) *Methods in enzymology* (Vol. 1 pp 64–67), New York, USA: Academic Press Inc.

Fuchs, R., Ryu, D. D., & Humphrey, A. E. (1971). Effect of surface aeration on scale-up procedures for fermentation processes. *Industrial & Engineering Chemistry Process Design and Development*, *10*(2), 190–196.

Fujiwara, S., Seto, Y., Kimura, A., & Hashiba, H. (2001). Establishment of orally-administered *Lactobacillus gasseri* SBT2055SR in the gastrointestinal tract of humans and its influence on intestinal microflora and metabolism. *Journal of Applied Microbiology*, *90*(3), 343–352.

Gaggìa, F., Mattarelli, P., & Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. *International Journal of Food Microbiology*, *141*, S15–S28.

Geankoplis, C. J. (2003a). Evaporation *Transport process and separation process principles (Includes unit operation)*, *Fouth Edition*: pp 527–558, Upper saddle river, USA: Prentice Hall Professional Technical Reference.

Geankoplis, C. J. (2003b). Mebrane separation processes *Transport process and separation process principles (Includes unit operation)*, *Fouth Edition*: pp 840–902, Upper saddle river, USA: Prentice Hall Professional Technical Reference.

Geankoplis, C. J. (2003c). Mechanical-physical separation processes *Transport process and separation process principles (Includes unit operation)*, *Fouth Edition*: pp 903–954, Upper saddle river, USA: Prentice Hall Professional Technical Reference.

Geankoplis, C. J. (2003d). Principle of steady-state heat transfer *Transport process and separation process principles (Includes unit operation)*, *Fouth Edition*: pp 235–356, Upper saddle river, USA: Prentice Hall Professional Technical Reference.

Geankoplis, C. J. (2003e). Vapor-liquid separation processes. *Transport process and separation process principles (Includes unit operation)*, *Fouth Edition*: pp 696–759, Upper saddle river, USA: Prentice Hall Professional Technical Reference.

Ginn, T. R. (2000). Comment on "Stochastic analysis of virus transport in aquifers," by Linda L. Campbell Rehmann, Claire Welty, and Ronald W. Harvey. *Water Resources Research*, *36*(7), 1981–1982.

Giraffa, G., Chanishvili, N., & Widyastuti, Y. (2010). Importance of lactobacilli in food and feed biotechnology. *Research in Microbiology*, *161*(6), 480–487.

Glunde, K., Jie, C., & Bhujwalla, Z. M. (2004). Molecular causes of the aberrant choline phospholipid metabolism in breast cancer. *Cancer Research*, *64*(12), 4270–4276.

Grahame, D. A. S., Kang, T. S., Khan, N. H., & Tanaka, T. (2013). Alkaline conditions stimulate the production of 1,3-propanediol in *Lactobacillus panis* PM1 through shifting metabolic pathways. *World Journal of Microbiology and Biotechnology*, *29*(7), 1207–1215.

Grand View Research. (2015). Acetic acid market worth $13.31 billion by 2022: Grand View Research, Inc. Retrieved on August 5, 2015 from http://www.prnewswire.com/news-releases/acetic-acid-market-worth-1331-billion-by-2022-grand-view-research-inc-515264261.html.

Günzel, B., Yonsel, S., & Deckwer, W. D. (1991). Fermentative production of 1,3-propanediol from glycerol by *Clostridium butyricum* up to a scale of 2 m^3. *Applied microbiology and Biotechnology*, *36*(3), 289–294.

Ham, G., Stock, R., Klopfenstein, T., Larson, E., Shain, D., & Huffman, R. (1994). Wet corn distillers byproducts compared with dried corn distillers grains with solubles as a source of protein and energy for ruminants. *Journal of Animal Science*, *72*, 3246–3246.

Hammes, W., & Gänzle, M. (1997). Sourdough breads and related products. In Wood, B. J. B. (Ed) *Microbiology of fermented foods*, *Second Edition*: pp 199–216, London, UK: Blackie Academic and Professional.

Hao, J., Wang, W., Tian, J., Li, J., & Liu, D. (2008). Decrease of 3-hydroxypropionaldehyde accumulation in 1,3-propanediol production by over-expressing dhaT gene in *Klebsiella pneumoniae* TUAC01. *Journal of Industrial Microbiology & Biotechnology*, *35*(7), 735–741.

Hao, J., Xu, F., Liu, H., & Liu, D. (2006). Downstream processing of 1,3-propanediol fermentation broth. *Journal of Chemical Technology and Biotechnology*, *81*(1), 102–108.

Held, A. M. (1996). The fermentation of glycerol to 1,3-propanediol by *Klebsiella pneumoniae.* M.Sc. thesis. University of Wisconsin Madison. Medison, Wisconsin, USA.

Higgs, D. A., Prendergast, A. F., & Dosanjh, B. S. (2014). Canola protein offer hope for efficient salmon production. Retrieved on September 6, 2015 from http://fishphysiology.org/wp-content/uploads/2014/02/higgs.pdf.

Himmi, E. H., Bories, A., & Barbirato, F. (1999). Nutrient requirements for glycerol conversion to 1,3-propanediol by *Clostridium butyricum*. *Bioresource Technology*, *67*(2), 123–128.

Hoffman, P. C. (2008). Impact of NDF Digestibility on Energy Content of Forages and Dairy Rations. Retrieved on May 4, 2015 from http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.414.6757&rep=rep1&type=pdf

Holst, D. (1973). Holst filtration apparatus for vansoest detergent fiber analyses. *Journal of the Association of Official Analytical Chemists*, *56*(6), 1352–1356.

Hoppe, K. (2010). Source and price for delected co-products produced in North Dakota. Retrieved on September 20, 2015 from https://www.ag.ndsu.edu/cattledocs/backgrounding-cattle-breakeven-calculations/files/fileinnercontentproxy.2010-10-15.8952439134.

Hunter, R. S. (1948). Photoelectric color-difference meter. *Journal of the Optical Society of America* In Proceedings of the winter meeting of the optical society of America, *38*(7), 661.

Indexmundi. (2015). Fishmeal price. Retrieved on May 26, 2015 from http://www.indexmundi.com/commodities/?commodity=fish-meal&currency=cad.

Ingledew, W. M. (2009a). Yeast stress in the fermentation. In Ingledew, W. M., Kelsall, D. R., Austin, G. D. & Kluhspies, C. (Eds.) *The alcohol textbook*, *Fifth Edition*: pp 115–126, Nottingham, UK: Nottingham University Press.

Ingledew, W. M. (2009b). Yeast: Physiology, nutrition and ethanol production. In Ingledew, W. M., Kelsall, D. R., Austin, G. D. & Kluhspies, C. (Eds.) *The alcohol textbook*, *Fifth Edition*: pp 101–114, Nottingham, UK: Nottingham University Press.

Ingledew, W. M., Austin, G. D., Kelsall, D. R., & Kluhspies, C. (2009). The alcohol industry: How has it changed and matured. In Ingledew, W. M., Kelsall, D. R., Austin, G. D. & Kluhspies, C. (Eds.) *The alcohol textbook*, *Fifth Edition*: pp 1–6, Nottingham, UK: Nottingham University Press.

International Feed Industry Federation. (2015a). The global feed industry. Retrieved on September 6, 2015 from http://www.ifif.org/pages/t/The+global+feed+industry.

International Feed Industry Federation. (2015b). The global feed production. Retrieved on September 6, 2015 from http://www.ifif.org/pages/t/Global+feed+production.

Jirjis, B. F., & Luque, S. (2010). Practical aspects of membrane system design in food and bioprocessing applications. In Cui, Z. F. & Muralidhara, H. S. (Eds.) *Membrane technology* (pp 179–212), Burlington, USA: Elsevier Ltd.

Junker, B. H. (2004). Scale-up methodologies for *Escherichia coli* and yeast fermentation processes. *Journal of Bioscience and Bioengineering*, *97*(6), 347–364.

Kalbfuss, B., Wolff, M., Morenweiser, R., & Reichl, U. (2007). Purification of cell culture-derived human influenza A virus by size-exclusion and anion-exchange chromatography. *Biotechnology and Bioengineering, 96*(5), 932–944.

Kang, T. S., Korber, D. R., & Tanaka, T. (2013a). Contributions of citrate in redox potential maintenance and ATP production: Metabolic pathways and their regulation in *Lactobacillus panis* PM1. *Applied Microbiology and Biotechnology*, *97*(19), 8693–8703.

Kang, T. S., Korber, D. R., & Tanaka, T. (2013b). Glycerol and environmental factors: effects on 1,3-propanediol production and NAD^+ regeneration in *Lactobacillus panis* PM1. *Journal of Applied Microbiology*, *115*(4), 1003–1011.

Kang, T. S., Korber, D. R., & Tanaka, T. (2013c). Influence of oxygen on NADH recycling and oxidative stress resistance systems in *Lactobacillus panis* PM1. *AMB Express*, *3*(1), 1–9.

Kang, T. S., Korber, D. R., & Tanaka, T. (2013d). Regulation of dual glycolytic pathways for fructose metabolism in heterofermentative *Lactobacillus panis* PM1. *Applied and Environmental Microbiology*, *79*(24), 7818–7826.

Kang, T. S., Korber, D. R., & Tanaka, T. (2014a). Bioconversion of glycerol to 1,3-propanediol in thin stillage-based media by engineered *Lactobacillus panis* PM1. *Journal of Industrial Microbiology & Biotechnology*, *41*(4), 629–635.

Kang, T. S., Korber, D. R., & Tanaka, T. (2014b). Metabolic engineering of a glycerol-oxidative pathway in *Lactobacillus panis* PM1 for utilization of bioethanol thin stillage: potential to produce platform chemicals from glycerol. *Applied and environmental microbiology*, *80*(24), 7631–7639.

Kang, T. S., Korber, D. R., & Tanaka, T. (2014c). Transcriptional repressor role of PocR on the 1,3-propanediol biosynthetic pathway by *Lactobacillus panis* PM1. *Biotechnology Letters*, *36*(6), 1263–1269.

Kee, H. L., Dishinger, J. F., Lynne Blasius, T., Liu, C.-J., Margolis, B., & Verhey, K. J. (2012). A size-exclusion permeability barrier and nucleoporins characterize a ciliary pore complex that regulates transport into cilia. *Nature Cell Biology*, *14*(4), 431–437.

Kelsall, D. R., & Piggot, R. (2009). Grain milling and cooking for alcohol production: Designing for the options in dry milling. In Ingledew, W. M., Kelsall, D. R., Austin, G. D. & Kluhspies, C. (Eds.) *The alcohol textbook*, *Fifth Edition*: pp 161–176, Nottingham, UK: Nottingham University Press.

Khan, N. H., Kang, T. S., Grahame, D. A. S., Haakensen, M. C., Ratanapariyanuch, K., Reaney, M. J., Korber, D. R., Tanaka, T. (2013). Isolation and characterization of novel 1,3-propanediol-producing *Lactobacillus panis* PM1 from bioethanol thin stillage. *Applied Microbiology and Biotechnology*, *97*(1), 417–428.

Khoury, F. M. (2015). Liquid-liquid extraction and supercritical extraction. In Khoury, F. M. (Ed) *Multistage separation processes*, *Fourth Edition*: pp 355–380, Boca Raton, USA: Taylor & Francis group.

Kim, D.-S. (2003). Measurement of point of zero charge of bentonite by solubilization technique and its dependence of surface potential on pH. *Environmental Engineering Research*, *8*(4), 222–227.

Kim, Y., Mosier, N. S., Hendrickson, R., Ezeji, T., Blaschek, H., Dien, B., Cotta, M., Dale, B., & Ladisch, M. R. (2008). Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage. *Bioresource Technology*, *99*(12), 5165–5176.

Kingsly, A. R. P., Ileleji, K. E., Clementson, C. L., Garcia, A., Maier, D. E., Stroshine, R. L., & Radcliff, S. (2010). The effect of process variables during drying on the physical and chemical characteristics of corn dried distillers grains with solubles (DDGS)–Plant scale experiments. *Bioresource Technology*, *101*(1), 193–199.

Kirk, J. L., Klironomos, J. N., Lee, H., & Trevors, J. T. (2005). The effects of perennial ryegrass and alfalfa on microbial abundance and diversity in petroleum contaminated soil. *Environmental Pollution*, *133*(3), 455–465.

Klassen, D. R., Elmer, C. M., & Nickerson, M. T. (2011). Associative phase separation involving canola protein isolate with both sulphated and carboxylated polysaccharides. *Food Chemistry*, *126*(3), 1094–1101.

Kleinschmit, D., Schingoethe, D., Kalscheur, K., & Hippen, A. (2006). Evaluation of various sources of corn dried distillers grains plus solubles for lactating dairy cattle. *Journal of Dairy Science*, *89*(12), 4784–4794.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2012). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*, 1–11.

Klopfenstein, T. J., Erickson, G. E., & Bremer, V. R. (2008). Board-invited review: Use of distillers by-products in the beef cattle feeding industry. *Journal of Animal Science*, *86*(5), 1223–1231.

Kohl, S. (2009). Wet milling and mash preparation. In Ingledew, W. M., Kelsall, D. R., Austin, G. D. & Kluhspies, C. (Eds.) *The alcohol textbook*, *Fifth Edition*: pp 177–192, Nottingham, UK: Nottingham University Press.

Kwiatkowski, J. R., McAloon, A. J., Taylor, F., & Johnston, D. B. (2006). Modeling the process and costs of fuel ethanol production by the corn dry-grind process. *Industrial Crops and Products*, *23*(3), 288–296.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685.

Lebeer, S., Verhoeven, T. L., Vélez, M. P., Vanderleyden, J., & De Keersmaecker, S. C. (2007). Impact of environmental and genetic factors on biofilm formation by the probiotic strain *Lactobacillus rhamnosus* GG. *Applied and Environmental Microbiology*, *73*(21), 6768–6775.

Li, Z., Jiang, B., Zhang, D., & Xiu, Z. (2009). Aqueous two-phase extraction of 1,3-propanediol from glycerol-based fermentation broths. *Separation and Purification Technology*, *66*(3), 472–478.

Li, Z., Teng, H., & Xiu, Z. (2011). Extraction of 1,3-propanediol from glycerol-based fermentation broths with methanol/phosphate aqueous two-phase system. *Process Biochemistry*, *46*(2), 586–591.

Liu, H., Xu, Y., Zheng, Z., & Liu, D. (2010). 1,3-propanediol and its copolymers: research, development and industrialization. *Biotechnology Journal*, *5*(11), 1137–1148.

Liu, H. J., Zhang, D. J., Xu, Y. H., Mu, Y., Sun, Y. Q., & Xiu, Z. L. (2007). Microbial production of 1,3-propanediol from glycerol by *Klebsiella pneumoniae* under micro-aerobic conditions up to a pilot scale. *Biotechnology Letters*, *29*(8), 1281–1285.

Liu, K., & Barrows, F. T. (2013). Methods to recover value-added coproducts from dry grind processing of grains into fuel ethanol. *Journal of Agricultural and Food Chemistry*, *61*(30), 7325–7332.

LoCascio, M. B., & Dunbar, R. K. (2014). Thin stillage clarification. US Patent Application 20140319066 A1.

Lopez, C., Govoni, S., Battaini, F., Bergamaschi, S., Longoni, A., Giaroni, C., & Trabucchi, M. (1991). Effect of a new cognition enhancer, alpha-glycerylphosphorylcholine, on scopolamine-induced amnesia and brain acetylcholine. *Pharmacology Biochemistry and Behavior*, *39*(4), 835–840.

Lovell, T. (1998). Nonnutrient diet components. In Lovell, T. (Ed) *Nutrition and feeding of fish*, *Second Edition*: pp 95–108, New York, USA: Springer Science+Business Media, LLC.

Lucarini, A. C., & Kilikian, B. V. (1999). Comparative study of lowry and bradford methods: interfering substances. *Biotechnology Techniques*, *13*(2), 149–154.

Luo, H., Wang, Q., Zhang, T. C., Tao, T., Zhou, A., Chen, L., & Bie, X. (2014). A review on the recovery methods of draw solutes in forward osmosis. *Journal of Water Process Engineering, 4*, 212–223.

Lüthi-Peng, Q., Dileme, F., & Puhan, Z. (2002). Effect of glucose on glycerol bioconversion by *Lactobacillus reuteri*. *Applied Microbiology and Biotechnology*, *59*(2–3), 289–296.

Ma, B. B., Xu, X. L., Zhang, G. L., Wang, L. W., Wu, M., & Li, C. (2009). Microbial production of 1,3-propanediol by *Klebsiella pneumoniae* XJPD-Li under different aeration strategies. *Applied Biochemistry and Biotechnology*, *152*(1), 127–134.

MacDonald, J., Klopfenstein, T. J., Erickson, G. E., & Griffin, W. (2007). Effects of dried distillers grains and equivalent undegradable intake protein or ether extract on performance and forage intake of heifers grazing smooth bromegrass pastures. *Journal of Animal Science, 85*(10), 2614–2624.

Macfarlane, A., Prestidge, R., Farid, M., & Chen, J. (2009). Dissolved air flotation: A novel approach to recovery of organosolv lignin. *Chemical Engineering Journal*, *148*(1), 15–19.

Madson, P. W. (2009). Ethanol distillation: The fundamentals. In Ingledew, W. M., Kelsall, D. R., Austin, G. D. & Kluhspies, C. (Eds.), *The alcohol textbook*, *Fifth Edition*: pp 289–302, Nottingham, UK: Nottingham University Press.

Maier, U., Losen, M., & Büchs, J. (2004). Advances in understanding and modeling the gas–liquid mass transfer in shake flasks. *Biochemical Engineering Journal*, *17*(3), 155–167.

Manning, J. H., & Hutten, I. M. (1992). Synthetic fiber paper having a permanent crepe. US Patent 5094717.

Martin, J. L., Cupp, A. S., Rasby, R. J., Hall, Z., & Funston, R. N. (2007). Utilization of dried distillers grains for developing beef heifers. *Journal of Animal Science*, *85*(9), 2298–2303.

Menkhaus, T. J., Anderson, J., Lane, S., & Waddell, E. (2010). Polyelectrolyte flocculation of grain stillage for improved clarification and water recovery within bioethanol production facilities. *Bioresource Technology*, *101*(7), 2280–2286.

Menzel, K., Zeng, A. P., & Deckwer, W. D. (1997). High concentration and productivity of 1,3-propanediol from continuous fermentation of glycerol by *Klebsiella pneumoniae*. *Enzyme and Microbial Technology*, *20*(2), 82–86.

Meredith, J. (2003). Dryhouse design: Focusing on reliability and return on investment. In Jacques, K. A., Lyons, T. P. & Kelsall, D. R. (Eds.) *The alcohol textbook*, *Fourth Edition*: pp 363–376, Nottingham, UK: Nottingham University Press.

Metsoviti, M., Zeng, A.-P., Koutinas, A. A., & Papanikolaou, S. (2013). Enhanced 1,3-propanediol production by a newly isolated *Citrobacter freundii* strain cultivated on biodiesel-derived waste glycerol through sterile and non-sterile bioprocesses. *Journal of Biotechnology, 163*(4), 408–418.

Miles, R. D., & Chapman, F. A. (2005). The benefits of fish meal in aquaculture diets. Retrieved on November 4, 2015 from https://www.edis.ifas.ufl.edu/pdffiles/FA/FA12200.pdf.

Moghaddam, S., & Ohadi, M. M. (2005). Effect of electrode geometry on performance of an EHD thin-film evaporator. *Journal of Microelectromechanical Systems*, *14*(5), 978–986.

Monceaux, D. A., & Kuehner, D. (2009). Dryhouse technologies and DDGS production. In Ingledew, W. M., Kelsall, D. R., Austin, G. D. & Kluhspies, C. (Eds.) *The alcohol textbook*, *Fifth Edition*: pp 303–321, Nottingham, UK: Nottingham University Press.

Mu, Y., Teng, H., Zhang, D.-J., Wang, W., & Xiu, Z.-L. (2006). Microbial production of 1,3-propanediol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparations. *Biotechnology Letters*, *28*(21), 1755–1759.

Mu, Y., Xiu, Z. L., & Zhang, D. J. (2008). A combined bioprocess of biodiesel production by lipase with microbial production of 1,3-propanediol by *Klebsiella pneumoniae*. *Biochemical Engineering Journal*, *40*(3), 537–541.

Müller-Steinhagen, H., Malayeri, M., & Watkinson, A. (2011). Heat exchanger fouling: Mitigation and cleaning strategies. *Heat Transfer Engineering*, *32*(3–4), 189–196.

Mustafa, A. F., McKinnon, J. J., & Christensen, D. A. (1999). Chemical characterization and in vitro crude protein degradability of thin stillage derived from barley- and wheat-based ethanol production. *Animal Feed Science and Technology*, *80*(3–4), 247–256.

Mustafa, A. F., McKinnon, J. J., Ingledew, M. W., & Christensen, D. A. (2000). The nutritive value for ruminants of thin stillage and distillers' grains derived from wheat, rye, triticale and barley. *Journal of the Science of Food and Agriculture*, *80*(5), 607–613.

Najder, L. E. (1964). Thin film evaporation. *Industrial & Engineering Chemistry*, *56*(2), 26–30.

Nakamura, C. E., & Whited, G. M. (2003). Metabolic engineering for the microbial production of 1,3-propanediol. *Current Opinion in Biotechnology*, *14*(5), 454–459.

Naylor, R. L., Hardy, R. W., Bureau, D. P., Chiu, A., Elliott, M., Farrell, A. P., Forster, I., Gatlin D. M., Goldburg, R. J., Hua, K. & Nichols, P. D. (2009). Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences*, *106*(36), 15103–15110.

New World Encyclopedia. (2012). Acetic acid. Retrieved on August 4, 2015 from http://www.newworldencyclopedia.org/entry/Acetic_acid.

Noblet, J., Henry, Y., & Dubois, S. (1987). Effect of protein and lysine levels in the diet on body gain composition and energy utilization in growing pigs. *Journal of Animal Science*, *65*(3), 717–726.

Nout, M. J. R. (1992). Upgrading traditional biotechnological processes. In *Applications of biotechnology to traditional fermented foods* (pp 11–19), Washington D.C., USA: National Academy Press.

Oakley, D. E. (2004). Spray dryer modeling in theory and practice. *Drying Technology*, *22*(6), 1371–1402.

OECD/Food and Agriculture Organization of the United Nations. (2014). OECD-FAO Agricultural Outlook 2014–2023. Retrived on September 6, 2015 from http://www.oecd-ilibrary.org/docserver/download/5114041e.pdf?expires=1442683541&id=id&accname=ocid177637&checksum=E1C190A6B73B280EE5C01EA97377C58C.

Ojowi, M. O., Christensen, D. A., McKinnon, J. J., & Mustafa, A. F. (1996). Thin stillage from wheat-based ethanol production as a nutrient supplement for cattle grazing crested wheatgrass pastures. *Canadian Journal of Animal Science*, *76*(4), 547–553.

Okonkowski, J., Kizer-Bentley, L., Listner, K., Robinson, D., & Chartrain, M. (2005). Development of a robust, versatile, and scalable inoculum train for the production of a DNA vaccine. *Biotechnology Progress*, *21*(4), 1038–1047.

Olin, H. L., & Peterson, H. W. (1937). The use of bentonite as a coagulant in water treatment. *Journal American Water Works Association*, *29*(4), 513–522.

Oude Elferink, S. J. W., Krooneman, J., Gottschal, J. C., Spoelstra, S. F., Faber, F., & Driehuis, F. (2001). Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. *Applied and Environmental Microbiology*, *67*(1), 125–132.

Pan America Environmental Inc. (2015). Dissolved air flotation theory. Retrieved on June 9, 2015 from http://www.dissolved-air-flotation.com/dissolved-air-flotation-theory.html

Pandiyan, T., de Bazua, C. D., Ilangovan, K., & Noyola, A. (1999). ^{13}C NMR Studies on vinasses effluent treated with iron. *Water Research*, *33*(1), 189–195.

Papanikolaou, S., Fakas, S., Fick, M., Chevalot, I., Galiotou-Panayotou, M., Komaitis, M., Marc, I. & Aggelis, G. (2008). Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl esters) manufacturing process: Production of 1,3-propanediol, citric acid and single cell oil. *Biomass and Bioenergy*, *32*(1), 60–71.

Papanikolaou, S., Ruiz-Sanchez, P., Pariset, B., Blanchard, F., & Fick, M. (2000). High production of 1,3-propanediol from industrial glycerol by a newly isolated *Clostridium butyricum* strain. *Journal of Biotechnology*, *77*(2), 191–208.

Parnetti, L., Abate, G., Bartorelli, L., Cucinotta, D., Cuzzupoli, M., Maggioni, M., Villardita, C., & Senin, U. (1993). Multicentre study of l-α-Glyceryl-Phosphorylcholine vs ST200 among patients with Probable senile dementia of Alzheimer's type. *Drugs & Aging*, *3*(2), 159–164.

Parnetti, L., Amenta, F., & Gallai, V. (2001). Choline alphoscerate in cognitive decline and in acute cerebrovascular disease: An analysis of published clinical data. *Mechanisms of Ageing and Development*, *122*(16), 2041–2055.

Parnetti, L., Mignini, F., Tomassoni, D., Traini, E., & Amenta, F. (2007). Cholinergic precursors in the treatment of cognitive impairment of vascular origin: ineffective approaches or need for re-evaluation?. *Journal of the Neurological Sciences*, *257*(1), 264–269.

Pedersen, C., Jonsson, H., Lindberg, J., & Roos, S. (2004). Microbiological characterization of wet wheat distillers' grain, with focus on isolation of lactobacilli with potential as probiotics. *Applied and Environmental Microbiology*, *70*(3), 1522–1527.

Pereira, D. D., Rocha, S. D. F., & Mansur, M. B. (2007). Recovery of zinc sulphate from industrial effluents by liquid–liquid extraction using D2EHPA (di-2-ethylhexyl phosphoric acid). *Separation and Purification Technology*, *53*(1), 89–96.

Petitdemange, E., Dürr, C., Andaloussi, S. A., & Raval, G. (1995). Fermentation of raw glycerol to 1,3-propanediol by new strains of *Clostridium butyricum*. *Journal of Industrial Microbiology*, *15*(6), 498–502.

Pflugmacher, U., & Gottschalk, G. (1994). Development of an immobilized cell reactor for the production of 1,3-propanediol by *Citrobacter freundii*. *Applied Microbiology and Biotechnology*, *41*(3), 313–316.

Pflügl, S., Marx, H., Mattanovich, D., & Sauer, M. (2012). 1,3-Propanediol production from glycerol with *Lactobacillus diolivorans*. *Bioresource Technology*, *119*, 133–140.

Plessas, S., Fisher, A., Koureta, K., Psarianos, C., Nigam, P., & Koutinas, A. A. (2008). Application of Kluyveromyces marxianus, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *L. helveticus* for sourdough bread making. *Food Chemistry*, *106*(3), 985–990.

Porter, M. C. (1972). Concentration polarization with membrane ultrafiltration. *Industrial & Engineering Chemistry Product Research and Development*, *11*(3), 234–248.

Poznanskaja, A. A., Tanizawa, K., Soda, K., Toraya, T., & Fukui, S. (1979). Coenzyme B12-dependent diol dehydrase: Purification, subunit heterogeneity, and reversible association. *Archives of Biochemistry and Biophysics*, *194*(2), 379–386.

Pretorius, I. S. (2000). Tailoring wine yeast for the new millennium: Novel approaches to the ancient art of winemaking. *Yeast*, *16*(8), 675–729.

Prevost, J. & Hammond, N. (2004). Treatment of thin spillage resulting from the production of ethanol from cereal grains. US Patent Application. 2004/0082044 A1.

Putman, R. E. (2000). Mechanical cleaning of heat-exchanger tubes. In Müller-Steinhagen, H. (Ed) *Heat exchanger fouling: Mitigation and cleaning techniques* (pp 55–68), Warwickshire, UK: Institution of Chemical Engineers.

Raghavan, B., Ramalakshmi, K., Borse, B. B., Ramesh, M. N., & Prakash, V. (2004). Preparing ready-to-dilute sugarcane juice beverage powder using a vacuum shelf dryer. US Patent 6783785 B1.

Rand, M. C. (1959). General principles of chemical coagulation. *Sewage and Industrial Wastes*, *31*(7), 863–871.

Ratanapariyanuch, K. (2009). Protein extraction from mustard (*Brassica juncea* (L.) Czern) meal using thin stillage. M.Sc. thesis. University of Saskatchewan. Saskatoon, Saskatchewan, Canada.

Ratanapariyanuch, K., Shen, J., Jia, Y., Tyler, R. T., Shim, Y. Y., & Reaney, M. J. T. (2011). Rapid NMR method for the quantification of organic compounds in thin stillage. *Journal of Agricultural and Food Chemistry*, *59*(19), 10454–10460.

Ratanapariyanuch, K., Tyler, R. T., Shim, Y. Y., & Reaney, M. J. T. (2012). Biorefinery process for protein extraction from oriental mustard (*Brassica juncea* (L.) Czern.) meal using ethanol stillage. *AMB Express*, 2, 5.

Ratanapariyanuch, K., Shim, Y. Y., Tyler, R. T., & Reaney, M. J. T. (2014). Industrial food process water: Quality and reuse opportunities for biorefinery purposes. In iConcept

Press. (Ed) *Protein Purification and Analysis III - Methods and Applications*, *First Edition*: pp 263–283, Kowloon, Hong Kong: iConcept Press Ltd.

Reaney, M. J. T., Haakensen, M., Korber, D., Tanaka, T., & Ratanapariyanuch, K. (2013). Process for the conversion of glycerol to 1,3-propanediol. US Patent Application 2013/0316417 A1.

Reaney, M. J. T., Shen, J., Jia, Y., & Ratanapariyanuch, K. (2014). Recovery of multiple compounds and recyclable water from thin stillage. US Patent 8835665 B2.

Reid, D. S., & Fennema, O. R. (2008). Water and ice. In Damodaran, S., Parkin, K. L. & Fennema, O. R. (Eds.) *Fennema's food chemistry*, *Fourth Edition*: pp 17–82, Boca Raton, USA: Taylor & Francis Group.

Reimann, A., & Biebl, H. (1996). Production of 1,3-propanediol by *Clostridium butyricum* DSM 5431 and product tolerant mutants in fedbatch culture: Feeding strategy for glycerol and ammonium. *Biotechnology Letters*, *18*(7), 827–832.

Roy, D., Ward, P., Vincent, D., & Mondou, F. (2000). Molecular identification of potentially probiotic lactobacilli. *Current Microbiology*, *40*(1), 40–46.

Rubio, J., Souza, M. L., & Smith, R. W. (2002). Overview of flotation as a wastewater treatment technique. *Minerals Engineering*, *15*(3), 139–155.

Saint-Amans, S., Perlot, P., Goma, G., & Soucaille, P. (1994). High production of 1,3-propanediol from glycerol by *Clostridium butyricum* VPI 3266 in a simply controlled fed-batch system. *Biotechnology Letters*, *16*(8), 831–836.

Saito, T. (2004). Selection of useful probiotic lactic acid bacteria from the *Lactobacillus acidophilus* group and their applications to functional foods. *Animal Science Journal, 75*(1), 1–13.

Sangiorgi, G. B., Barbagallo, M., Giordano, M., Meli, M., & Panzarasa, R. (1994). α-Glycerophosphocholine in the mental recovery of cerebral ischemic attacks. *Annals of the New York Academy of Sciences*, *717*(1), 253–269.

Sarwar, G., & Bowland, J. (1975). Availability of amino acids in wheat cultivars used in diets for weanling rats. *Canadian Journal of Animal Science*, *55*(4), 579–586.

Sauer, W., Stothers, S., & Parker, R. (1977). Apparent and true availabilities of amino acids in wheat and milling by-products for growing pigs. *Canadian Journal of Animal Science*, *57*(4), 775–784.

Saxena, R. K., Anand, P., Saran, S., & Isar, J. (2009). Microbial production of 1,3-propanediol: Recent developments and emerging opportunities. *Biotechnology Advances*, *27*(6), 895–913.

Scheimann, D. W. (2009). Method of dewatering grain stillage solids. US Patent 7566469 B2.

Schmidt, F. (2005). Optimization and scale up of industrial fermentation processes. *Applied Microbiology and Biotechnology*, *68*(4), 425–435.

Schneider, Z., Larsen, E. G., Jacobson, G., Johnson, B. C., & Pawelkiewicz, J. (1970). Purification and properties of glycerol dehydrase. *Journal of Biological Chemistry*, *245*(13), 3388–3396.

Shim, M. Y., Pesti, G. M., Bakalli, R. I., Tillman, P. B., & Payne, R. L. (2011). Evaluation of corn distillers dried grains with solubles as an alternative ingredient for broilers. *Poultry Science*, *90*(2), 369–376.

Skinner-Nemec, K. A., Nichols, N. N., & Leathers, T. D. (2007). Biofilm formation by bacterial contaminants of fuel ethanol production. *Biotechnology Letters*, *29*(3), 379–383.

Sosulski, F. W., & Imafidon, G. I. (1990). Amino acid composition and nitrogen-to-protein conversion factors for animal and plant foods. *Journal of Agricultural and Food Chemistry*, *38*(6), 1351–1356.

Soto, R., Russell, I., Narendranath, N., Power, R., & Dawson, K. (2005). Estimation of ethanol yield in corn mash fermentations using mass of ash as a marker. *Journal of the Institute of Brewing*, *111*(2), 137–143.

Spellman, F. R. (2014a). Wastewater treatment operations *Water and wastewater treatment plant operations*, *Third Edition*: pp 681–834, Boca Raton, USA: Tyler & Fransis Group.

Spellman, F. R. (2014b). Water treatment operations *Water and wastewater treatment plant operations*, *Third Edition*: pp 621–677, Boca Raton, USA: Tyler & Fransis Group.

Steffan, R. J., Goksøyr, J., Bej, A., & Atlas, R. (1988). Recovery of DNA from soils and sediments. *Applied and Environmental Microbiology*, *54*(12), 2908–2915.

Swain, R. L. B. (2009). Molecular sieve dehydrators: Why they became the industry standard and how they work. In Ingledew, W. M., Kelsall, D. R., Austin, G. D. & Kluhspies, C. (Eds.) *The alcohol textbook*, *Fifth Edition*: pp 379–384, Nottingham, UK: Nottingham University Press.

Świątkiewicz, S., & Koreleski, J. (2008). The use of distillers dried grains with solubles (DDGS) in poultry nutrition. *World's Poultry Science Journal*, *64*(02), 257–266.

Tag, C. G. (1990). Mikrobielle Herstellung von 1,3-propandiol. Ph.D thesis. University of Oldenburg. Oldenburg, Germany.

Tallon, R., Bressollier, P., & Urdaci, M. C. (2003). Isolation and characterization of two exopolysaccharides produced by *Lactobacillus plantarum* EP56. *Research in Microbiology*, *154*(10), 705–712.

Tan, H., Abdul Aziz. A. R., & Aroua, M. K. (2013). Glycerol production and its applications as a raw material: A review. *Renewable and Sustainable Energy Reviews*, *27*, 118–127.

Tang, J., Feng, H., & Shen, G.-Q. (2003). Drum drying. In Heldman, D. R. *Encyclopedia of agricultural, food, and biology engineering* (pp 211–214), New York, USA: Marcel Dekker Inc.

Tesseraud, S., Larbier, M., Chagneau, A., & Geraert, P. (1992). Effect of dietary lysine on muscle protein turnover in growing chickens. *Reproduction Nutrition Development*, *32*(2), 163–175.

Thacker, P. A., & Widyaratne, G. P. (2007). Nutritional value of diets containing graded levels of wheat distillers grains with solubles fed to broiler chicks. *Journal of the Science of Food and Agriculture*, *87*(7), 1386–1390.

Thomas, K., & Ingledew, W. (1992). Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes. *Journal of Industrial Microbiology*, *10*(1), 61–68.

Tobajas, M., Mohedano, A. F., Casas, J. A., & Rodríguez, J. J. (2009). Unstructured kinetic model for reuterin and 1,3-propanediol production by *Lactobacillus reuteri* from glycerol/glucose cofermentation. *Journal of Chemical Technology and Biotechnology, 84*(5), 675–680.

U.S. Food and Drug Administration. (2015). Corn sugar (dextrose). Retrieved on June 19, 2015 from http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/SCOGS/ucm261263.htm.

U.S. Grains Council. (2012). A guide to distiller's dried grains with solubles (DDGS). Retrieved on June 4, 2015 from http://www.grains.org/sites/default/files/ddgs-handbook/Complete%202012%20DDGS%20Handbook.pdf.

Valence, F., Deutsch, S. M., Richoux, R., Gagnaire, V., & Lortal, S. (2000). Autolysis and related proteolysis in Swiss cheese for two *Lactobacillus helveticus* strains. *Journal of Dairy Research*, *67*(2), 261–271.

Valous, N., Gavrielidou, M., Karapantsios, T., & Kostoglou, M. (2002). Performance of a double drum dryer for producing pregelatinized maize starches. *Journal of Food Engineering*, *51*(3), 171–183.

Van Gansbeghe, F., Bogaert, J.-C., Malhaize, E., Van Gansberghe, M., & Wolff, F. (2002). Method for purifying lactic acid. US Patent 6489508 B1.

Van Leeuwen, J., Khanal, S. K., & Pometto, A. L. (2008). Purification of thin stillage from dry-grind corn milling with fungi. US Patent Application 2008/0153149 A1.

Visser, J., & Jeurnink, T. J. (1997). Fouling of heat exchangers in the dairy industry. *Experimental Thermal and Fluid Science*, *14*(4), 407–424.

Waldherr, F. W., Meissner, D., & Vogel, R. F. (2008). Genetic and functional characterization of *Lactobacillus panis* levansucrase. *Archives of Microbiology*, *190*(4), 497–505.

Wang, Z., Cerrate, S., Coto, C., Yan, F., & Waldroup, P. (2007). Utilization of distillers dried grains with solubles (DDGS) in broiler diets using a standardized nutrient matrix. *International Journal Poultry Science*, *6*(7), 470–477.

Wilkens, E., Ringel, A. K., Hortig, D., Willke, T., & Vorlop, K. D. (2012). High-level production of 1,3-propanediol from crude glycerol by *Clostridium butyricum* AKR102a. *Applied Microbiology and Biotechnology*, *93*(3), 1057–1063.

Wilkins, M. R., Singh, V., Belyea, R. L., Buriak, P., Wallig, M. A., Tumbleson, M. E., & Rausch, K. D. (2006). Effect of pH on fouling characteristics and deposit compositions in dry-grind thin stillage. *Cereal chemistry*, *83*(3), 311–314.

Wine, E., Gareau, M. G., Johnson-Henry, K., & Sherman, P. M. (2009). Strain-specific probiotic (*Lactobacillus helveticus*) inhibition of *Campylobacter jejuni* invasion of human intestinal epithelial cells. *FEMS Microbiology Letters*, *300*(1), 146–152.

Woychik, J., Boundy, J. A., & Dimler, R. (1961). Wheat gluten proteins, amino acid composition of proteins in wheat gluten. *Journal of Agricultural and Food Chemistry*, *9*(4), 307–310.

Wu, Y. V. (1988). Recovery of stillage soluble solids from corn and dry-milled corn fractions by high-pressure reverse osmosis and ultrafiltration. *Cereal Chemistry*, *65*(4), 345–348.

Wu, Y. V. (1990). Recovery of protein-rich byproducts from oat stillage after alcohol distillation. *Journal of Agricultural and Food Chemistry*, *38*(2), 588–592.

Xiu, Z.-L., & Zeng, A.-P. (2008). Present state and perspective of downstream processing of biologically produced 1,3-propanediol and 2,3-butanediol. *Applied Microbiology and Biotechnology*, *78*(6), 917–926.

Yang, F. C. (1998). Drying trials of thin stillage from the manufacture of rice spirit. *Bioresource Technology*, *65*(1), 163–165.

Yang, G., Tian, J., & Li, J. (2007). Fermentation of 1,3-propanediol by a lactate deficient mutant of *Klebsiella oxytoca* under microaerobic conditions. *Applied Microbiology and Biotechnology*, *73*(5), 1017–1024.

Yildiz, F. H., & Schoolnik, G. K. (1999). *Vibrio cholerae* O1 El Tor: Identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proceedings of the National Academy of Sciences*, *96*(7), 4028–4033.

Zhao, Y. N., Chen, G., & Yao, S. J. (2006). Microbial production of 1,3-propanediol from glycerol by encapsulated *Klebsiella pneumoniae*. *Biochemical Engineering Journal*, *32*(2), 93–99.

Zheng, Y. (2013). Effects of compositional variables on fouling behavior of thin stillage. M.Sc. thesis. University of Illinois at Urbana-Champaign. Champaign, Illinois, USA.

Zheng, Y. M., & Yu, H. Q. (2007). Determination of the pore size distribution and porosity of aerobic granules using size-exclusion chromatography. *Water Research*, *41*(1), 39–46.

Zheng, Z. M., Xu, Y. Z., Liu, H. J., Guo, N. N., Cai, Z. Z., & Liu, D. H. (2008). Physiologic mechanisms of sequential products synthesis in 1,3-propanediol fed-batch fermentation by *Klebsiella pneumoniae*. *Biotechnology and Bioengineering*, *100*(5), 923–932.

APPENDIX A

LIST OF CHEMICALS

Purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA)

H_2SO_4	GR ACS grade, Cat. No. SX-5124
NaOH	GR ACS grade, Cat. No. SX0590-7
Boric acid	GR ACS grade, Cat. No. BX0865
Titristar N point indicator	Cat. No. NX0847/3
N,N dimethylformamide	Cat. No. DX1726-6
Glycerol	GR ACS grade, Cat. No. GX0185-2
Acetic acid	GR ACS grade, Cat. No. AX0073
Methanol	HPLC grade, Cat. No. MX0488-1
Celite 545	Cat. No. CX0574-1
Silica 60 30–60 μm	Cat. No. 1.11567

Purchased from Sigma-Aldrich (St. Louis, MO, Canada)

1,3-propanediol	99.8% ACS grade, Cat. No. P50404-500G
Glucose	Bio Reagent ≥ 99.5%, Cat. No. G7021
Bromophenol Blue salt for electrophoresis	Cat. No. B5525-25G
Bovine Serum Albumin	200 mg/mL, Cat. No. P5369-10ML
Vitamin B12	Bio Reagent ≥ 98%, Cat. No. V6629-250MG
Nicotinic acid	Bio Reagent ≥ 98%, Cat. No.N0761-100G
3-hydroxypropanoic acid	Cat. No. CDS000335-500 MG
Glucose hexokinase assay kit	Cat. No. G3293-50ML
Cellulose from cotton liners	Cat. No. C6288
Gelatin from porcine skin type A	Cat. No. G2500
Gelatin from bovine skin type B	Cat. No. G9391
Pectin from citrus esterified ≥ 85%	Cat. No. P9561-25G
Pectin from citrus esterified ≤ 26%	Cat. No. P9135

Purchased from VWR International (West Chester, PA, USA)

Kjeldahl digestion mixture # 200	Cat. No. K310-1000
HCl	1 ± 0.002N, Cat. No. BDH7202-2

Purchased from Bio-rad laboratories (Hercules, CA, USA)

0.5 M Tris-HCl buffer pH 6.8	Cat. No. 161-0799
1.5 M Tris-HCl buffer pH 8.8	Cat. No. 161-0798
10× Tris/Glycine/SDS buffer	Cat. No. 161-0732
40% Acrylamide/Bis Solution	Electrophoresis Purify Reagent, Cat. No. 161-0146
N,N,N,N-Tetramethyllethalenediamine	Cat. No. 161-0801

Purchased from Becton Dickinson and Company (Franklin Lakes, NJ, USA)

Peptone	Cat. No. 211677
Agar, granulated	Cat. No. 214530

Purchased from HiMedia laboratories Pvt, Ltd. (Mumbai, India)

Lactobacillus Heteroform Screen broth	Cat. No. M1164-500G

Purchased from J.L. Baker Chemical Co. (Phillipsburg, NJ, USA)

Sodium phosphate dibasic heptahydrate	ACS grade, Cat. No. 3824-1

Purchased from Supelco (Bellefonte, PA, USA)

Riboflavin	Neat, Cat. No. 47861

Purchased from GE Healthcare Bio Science Corp. (Princeton, NJ, USA)

SDS	Minimum assay 99%, Cat. No. 17-1313-01

Purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA)

Deuterium oxide	99.9%, Cat. No. DML-4-100

Purchased from Mallinkrodt Baker, Inc. (Phillipsburg, NJ, USA)

Coomasie blue-G250	Cat. No. F789-03

Purchased from Fisher Scientific (Fair Lawn, NJ, USA)

Yeast extract	Cat. No. BP1422-500
Sodium phosphate monobasic	99.2%, Cat. No. S-369
Bentonite	Cat. No. B235-500
PageRuler™ Prestained Protein Ladder	Cat. No. SM0671
β-mercaptoethanol	Minimum assay 98%, Cat. No.BP176-100

Purchased from Fluka Chemical Corporation (Ronkonkoma, NY, USA)

κ-Carrageenan	Cat. No. 22048

Purchased from CP Kelco (Lille Skensved, Denmark)

GENU@ pectin type D slow set z	Not applicable

Purchased from Alfa Aesar (Ward Hill, MA, USA)

Tannic acid	Cat. No. 36410

APPENDIX B
DATA FROM REPLICATE 2 CHAPTER 3

Table B.1 Protein and moisture contents of W-TS fermentation fractions replicate 2

Fermentation time (h)	Sample	Weight of material (kg)	Moisture (%, w/w, wb)	Dry matter content	Protein[1] (%, w/w, wb)	Protein[1] (%, w/w, db)	OD_{600}
0	W-TS	ND	93.73 ± 0.03	6.27 ± 0.03	2.51 ± 0.07	40.2 ± 0.1	1.56 ± 0.05
43-67	F	0.22	86.3 ± 0.2	13.69 ± 0.03	6.2 ± 0.1	45.1 ± 0.9	ND
	M	ND					ND
	F	1.14					ND
	M	ND					ND
92	F	0.69	85.31 ± 0.03	14.69 ± 0.03	7.03 ± 0.00	47.8 ± 0.1	ND
	M	ND	94.19± 0.01	5.81 ± 0.01	1.91 ± 0.01	32.8 ± 0.2	1.219 ± 0.009
116	F	0.65	87.63 ± 0.05	12.37 ± 0.05	5.73 ± 0.06	46.4 ± 0.3	ND
	M	ND	94.37 ± 0.00	5.63 ± 0.00	1.80 ± 0.02	32.1 ± 0.4	1.13 ± 0.01
139	F	0.45	89.85 ± 0.01	10.15 ± 0.01	4.5 ± 0.1	45.0 ± 1.0	ND
	M	ND	94.50 ± 0.01	5.50 ± 0.01	1.84 ± 0.01	33.4 ± 0.2	1.108 ± 0.001
161	F	0.44	89.28 ± 0.02	10.72 ± 0.02	4.94 ± 0.03	46.1 ± 0.2	ND
	M	ND	94.72 ± 0.02	5.28 ± 0.02	1.16 ± 0.03	22.1 ± 0.7	0.99 ± 0.02
185	F	0.29	88.22 ± 0.02	11.78 ± 0.02	5.46 ± 0.04	46.4 ± 0.4	ND
	M	ND	94.67 ± 0.02	5.33 ± 0.02	1.55 ± 0.04	29.0 ± 0.8	1.03 ± 0.02
209	F	0.03	91.49 ± 0.01	8.51 ± 0.01	3.13 ± 0.05	36.8 ± 0.7	ND
	M	11.37	96.32 ± 0.01	3.68 ± 0.01	0.45 ± 0.04	12.0 ± 1.0	0.130 ± 0.004
	B	8.90	92.86 ± 0.01	7.14 ± 0.01	2.62 ± 0.07	37.0 ± 1.0	1.57 ± 0.05

Each value except weight of material is presented as the mean ± SD (n = 2).
Abbreviation: F = Floating material; M = middle layer; B = bottom layer; ND = not determined.
[1]Protein content of floating material and bottom layer was not corrected for the nitrogen content of GPC and betaine as no data was available for this material.

APPENDIX C

DATA FROM REPLICATE 2 CHAPTER 5

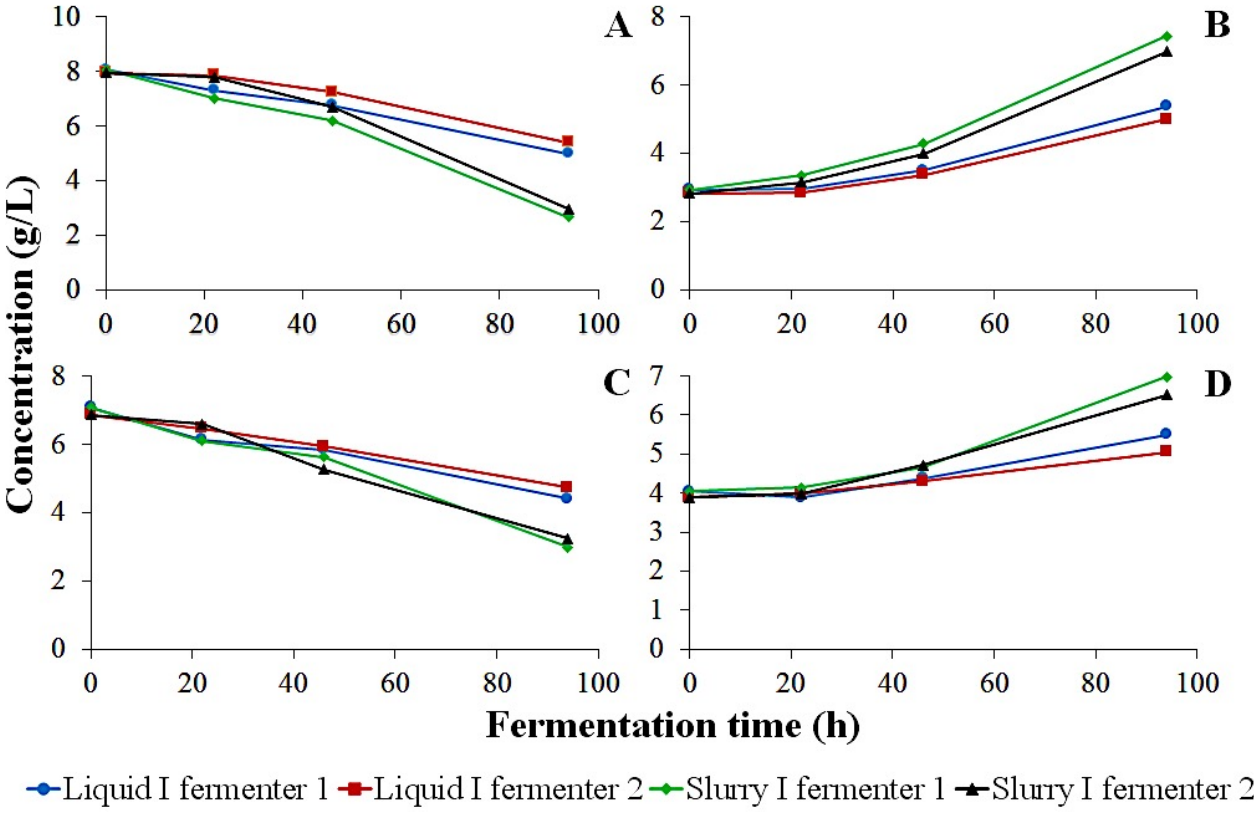

Figure C.1 Metabolisms of (A) glycerol, (B) 1,3-PD, (C) lactic acid, and (D) acetic acid from small-scale TSF replicate 2

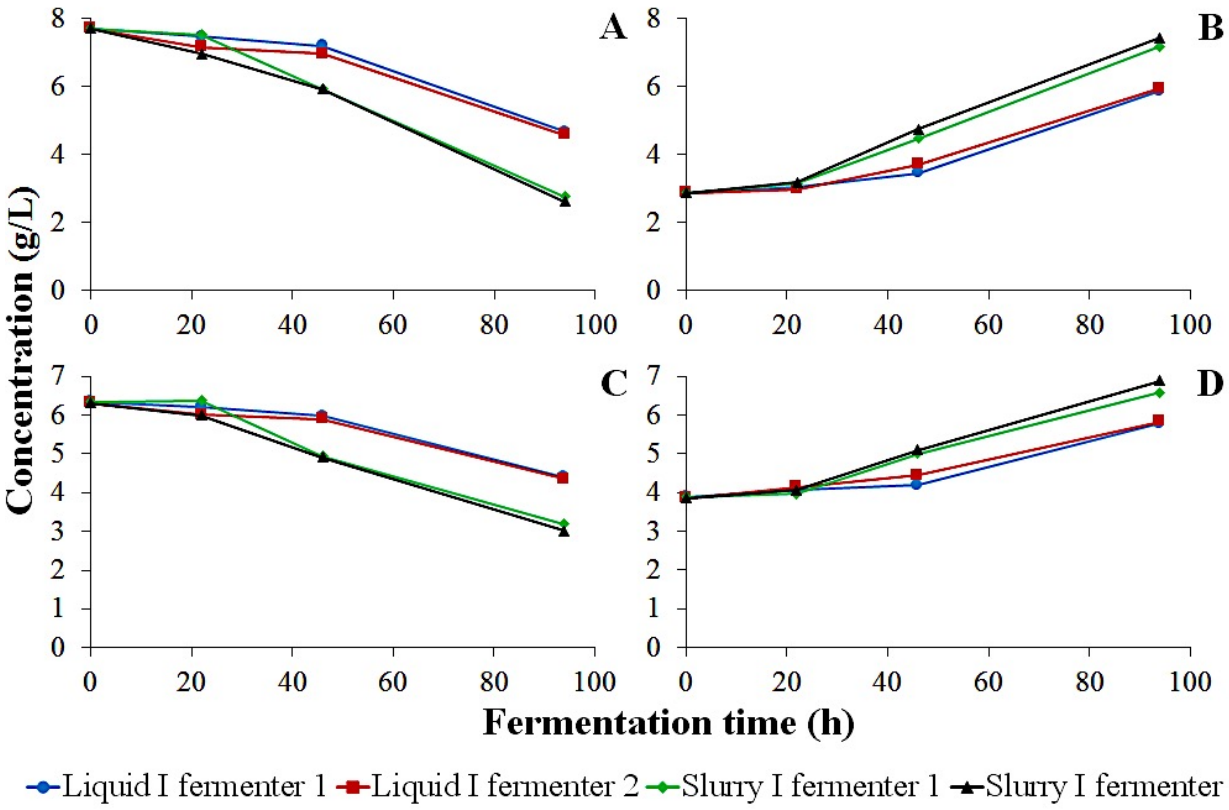

Figure C.2 Metabolisms of (A) glycerol, (B) 1,3-PD, (C) lactic acid, and (D) acetic acid from replications of small-scale TSF replicate 1

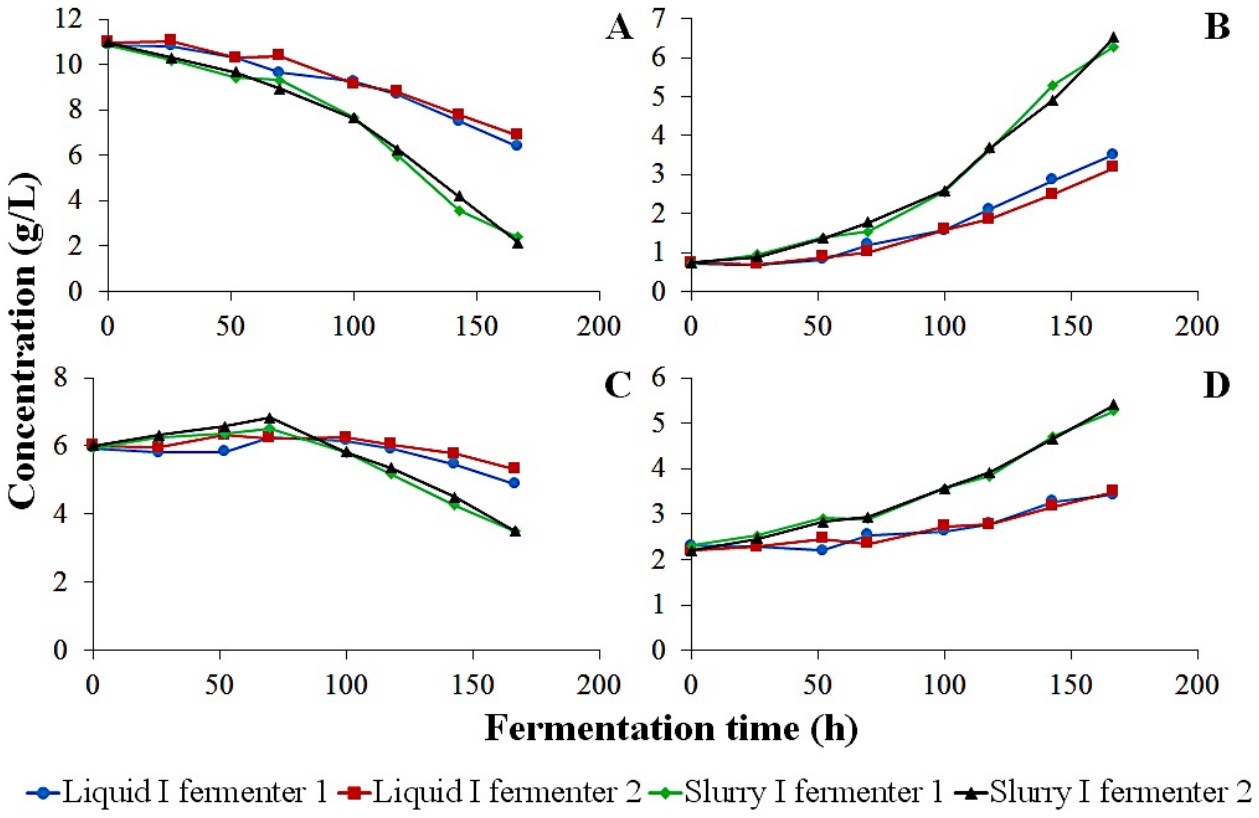

Figure C.3 Metabolisms of (A) glycerol, (B) 1,3-PD, (C) lactic acid, and (D) acetic acid from replications of small-scale TSF replicate 2

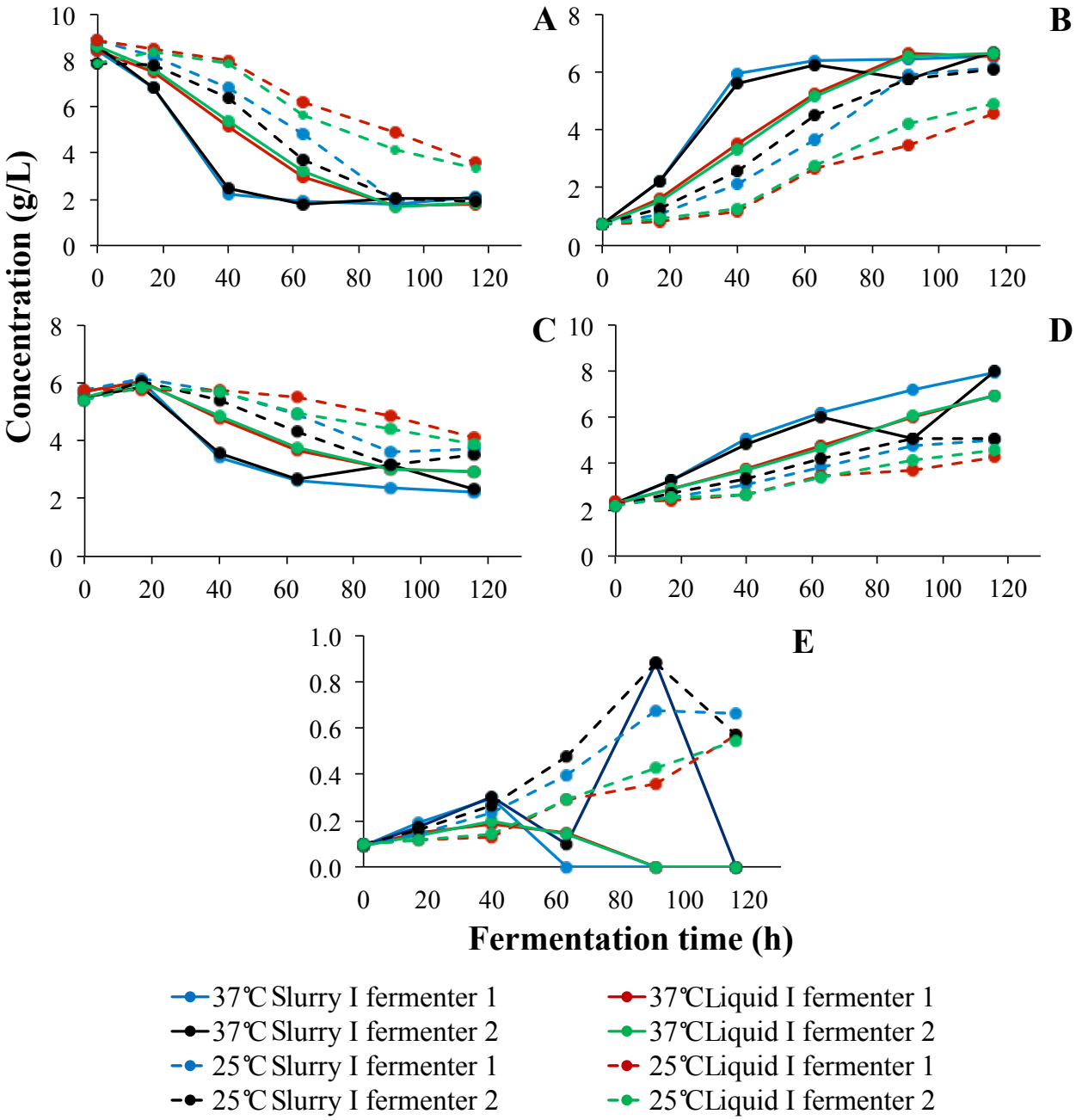

Figure C.4 Concentration of small solutes in duplicate 25 L fermentation (A) glycerol, (B) 1,3-PD, (C) lactic acid, (D) acetic acid, and (E) 3-HPA when fermenting at 25 and 37 °C replicate 2

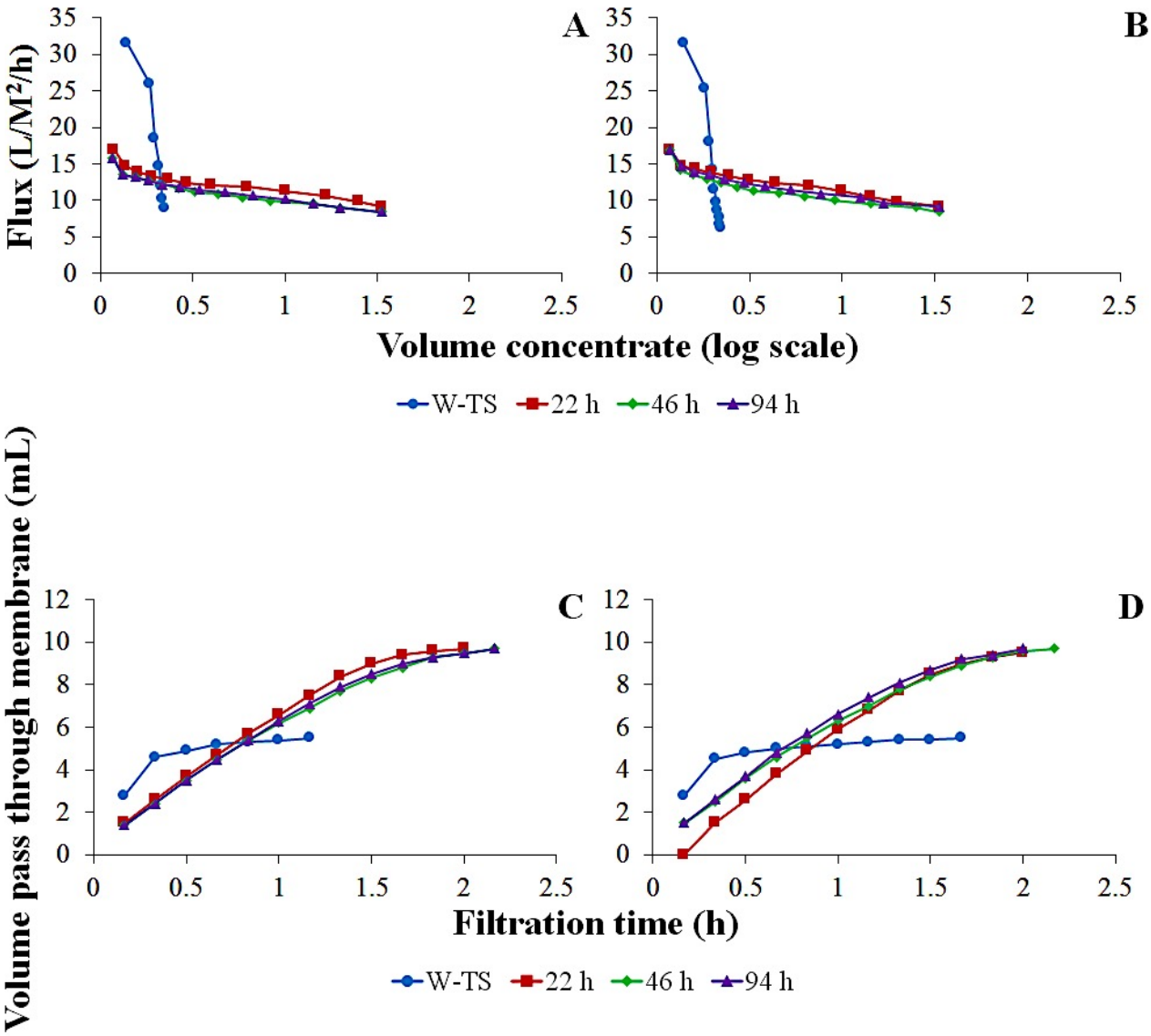

Figure C.5 Transmembrane (10 kDa MWCO) flux of liquid I from (A) fermenter 1 and (B) fermenter 2 replicate 2. Filtered volume of liquid I passed through a 10 kDa MWCO membrane of liquid I from (C), fermenter 1 and (D) fermenter 2 replicate 2. The time in the legend presents the fermentation time.

Table C.1 Protein and moisture contents of TSF products from fermentation at 25 °C of small-scale fermentation replicate 2

Sample	Hour of fermentation	Fermenter 1				Fermenter 2			
		Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)	Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Fermentation medium (W-TS)	0	0	91.20 ± 0.03	3.79 ± 0.08	43 ± 1	0	91.17 ± 0.02	3.66 ± 0.05	41.5 ± 0.6
Slurry I	22	1.5	90.74 ± 0.00	3.93 ± 0.00	42.48 ± 0.01	1.5	90.64 ± 0.00	3.88 ± 0.04	41.5 ± 0.5
Liquid I			95.68 ± 0.01	1.10 ± 0.02	25.5 ± 0.5		96.36 ± 0.00	0.84 ± 0.00	23.12 ± 0.03
Slurry I	46	9	87.91 ± 0.00	5.58 ± 0.09	46.2 ± 0.8	5	89.47 ± 0.01	4.69 ± 0.04	44.5 ± 0.3
Liquid I			95.96 ± 0.02	0.98 ± 0.05	24 ± 1		96.08 ± 0.00	1.04 ± 0.03	26.5 ± 0.8
Slurry I	94	8	88.86 ± 0.01	5.31 ± 0.02	47.7 ± 0.3	7	89.15 ± 0.02	5.00 ± 0.01	46.1 ± 0.2
Liquid I			96.49 ± 0.00	0.84 ± 0.03	24.0 ± 0.7		96.48 ± 0.01	0.83 ± 0.01	23.6 ± 0.2

Each value except volume of liquid I is presented as the mean ± SD (n = 2).
There was no sampling at 77 h of fermentation as the solution was settling at this time. Therefore, the fermentation medium was left for 17 h to let the slurry I precipitate.

Table C.2 Protein content (g/L) of filtrate from ultrafiltration of liquid I from fermentation at 25 °C of small-scale fermentation replicate 2 at different fermentation time

Container	0 h	22 h	46 h	94 h
Fermenter 1	0.30 ± 0.01	0.46 ± 0.01	0.41 ± 0.00	0.49 ± 0.00
Fermenter 2	0.28 ± 0.01	0.42 ± 0.00	0.48 ± 0.01	0.44 ± 0.01

Each value is presented as the mean ± SD (n = 2).

Table C.3 Protein and moisture contents of TSF products from fermentation at 25 °C replicate 2

Sample	Hour of fermentation	Fermenter 1				Fermenter 2			
		Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)	Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Fermentation medium (W-TS)	0	0	91.94 ± 0.00	3.09 ± 0.06	38.4 ± 0.7	0	92.07 ± 0.00	3.10 ± 0.01	39.1 ± 0.2
Slurry I	17	5	92.22 ± 0.01	3.10 ± 0.02	39.8 ± 0.2	3	91.71 ± 0.01	3.37 ± 0.08	41.0 ± 1.0
Liquid I			96.56 ± 0.01	0.60 ± 0.00	17.45 ± 0.00		96.60 ± 0.01	0.66 ± 0.01	19.5 ± 0.4
Slurry I	40	9	91.18 ± 0.01	4.2 ± 0.1	48 ±1	8	91.11 ± 0.01	3.84 ± 0.03	43.3 ± 0.3
Liquid I			95.94 ± 0.00	0.98 ± 0.02	24.0 ± 0.5		96.23 ± 0.03	0.84 ± 0.00	22.4 ± 0.2
Slurry I	63	10	88.81 ± 0.02	5.09 ± 0.09	45.5 ± 0.7	9	88.34 ± 0.01	5.25 ± 0.03	45.0 ± 0.2
Liquid I			96.42 ± 0.02	0.84 ± 0.01	23.4 ± 0.3		96.34 ± 0.01	0.93 ± 0.05	25.0 ± 1.0
Slurry I	91	10	88.25 ± 0.01	5.50 ± 0.03	46.8 ± 0.2	9	88.69 ± 0.02	5.15 ± 0.02	45.49 ± 0.09
Liquid I			96.45 ± 0.01	0.88 ± 0.00	24.87 ± 0.02		96.53 ± 0.01	1.34 ± 0.06	39.0 ± 2.0
Slurry I	116	10	90.60 ± 0.04	4.40 ± 0.03	46.77 ± 0.09	9	90.70 ± 0.03	4.27 ± 0.03	45.9 ± 0.2
Liquid I			96.89 ± 0.01	0.57 ± 0.01	18.4 ± 0.2		96.98 ± 0.01	0.57 ± 0.04	19.0 ± 1.0

Each value except volume of liquid I is presented as the mean ± SD (n = 2).

Table C.4 Protein and moisture contents of TSF products from fermentation at 37 °C replicate 2

Sample	Hour of fermentation	Fermenter 1				Fermenter 2			
		Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)	Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Fermentation medium (W-TS)	0	0	92.02 ± 0.00	3.06 ± 0.00	38.31 ± 0.00	0	92.00 ± 0.00	3.08 ± 0.03	38.5 ± 0.3
Slurry I	17	4.5	92.12 ± 0.02	3.23 ± 0.05	41.0 ± 0.5	4.5	91.32 ± 0.01	3.71 ± 0.08	42.7 ± 0.9
Liquid I			96.76 ± 0.01	0.64 ± 0.00	19.63 ± 0.03		96.72 ± 0.01	0.64 ± 0.04	20 ± 1
Slurry I	40	4	91.00 ± 0.01	4.12 ± 0.01	45.8 ± 0.2	4	91.24 ± 0.02	3.87 ± 0.02	44.2 ± 0.1
Liquid I			96.86 ± 0.01	0.68 ± 0.02	21.6 ± 0.7		96.80 ± 0.02	0.61 ± 0.06	19 ± 2
Slurry I	63	8	90.18 ± 0.03	4.66 ± 0.01	47.43 ± 0.05	9	91.43 ± 0.02	3.83 ± 0.02	44.65 ± 0.09
Liquid I			96.95 ± 0.00	0.67 ± 0.00	22.05 ± 0.01		96.95 ± 0.00	0.58 ± 0.02	19.2 ± 0.8
Slurry I	91	8	91.51 ± 0.02	4.60 ± 0.09	54.2 ± 0.9	9	92.22 ± 0.00	3.8 ± 0.1	47 ± 2
Liquid I			97.14 ± 0.00	0.74 ± 0.01	25.7 ± 0.2		97.12 ± 0.01	0.67 ± 0.01	23.4 ± 0.5
Slurry I	116	8	92.06 ± 0.01	4.2 ± 0.2	53 ± 3	9	92.76 ± 0.01	3.17 ± 0.04	43.8 ± 0.5
Liquid I			97.23 ± 0.00	0.63 ± 0.01	22.8 ± 0.4		97.24 ± 0.01	0.91 ± 0.01	32.8 ± 0.6

Each value except volume of liquid I is presented as the mean ± SD (n = 2).

Table C.5 Protein and moisture contents of small-scale TSF of twelve fermenters replicate 2

Sample	Hour of fermentation	Fermenter 1				Fermenter 2			
		Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)	Liquid I volume (L)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Fermentation medium (W-TS)	0	3.5	91.31 ± 0.01	3.6 ± 0.1	41 ± 1	0	91.99 ± 0.01	3.21 ± 0.05	40.1 ± 0.7
Slurry I	26[1]	ND	89.97 ± 0.01	4.30 ± 0.00	42.83 ± 0.09	4	89.92 ± 0.04	4.51 ± 0.02	44.3 ± 0.4
Liquid I			96.40 ± 0.01	0.88 ± 0.02	24.5 ± 0.4		96.35 ± 0.01	0.78 ± 0.03	21.3 ± 0.8
Slurry I	52	5	90.22 ± 0.01	4.28 ± 0.01	43.6 ± 0.2	4	90.13 ± 0.01	4.39 ± 0.02	44.5 ± 0.2
Liquid I			96.40 ± 0.01	0.74 ± 0.00	20.84 ± 0.07		96.46 ± 0.00	0.76 ± 0.00	21.33 ± 0.07
Slurry I	70	5	90.81 ± 0.01	3.98 ± 0.03	43.3 ± 0.3	5	89.93 ± 0.00	4.57 ± 0.00	45.35 ± 0.01
Liquid I			96.49 ± 0.00	0.80 ± 0.01	22.8 ± 0.1		96.49 ± 0.01	0.80 ± 0.02	22.9 ± 0.7
Slurry I	100	6	90.21 ± 0.00	4.2 ± 0.1	42 ± 1	6	89.94 ± 0.01	4.49 ± 0.01	44.66 ± 0.09
Liquid I			96.53 ± 0.00	0.81 ± 0.01	23.4 ± 0.2		96.43 ± 0.00	0.81 ± 0.01	22.6 ± 0.2
Slurry I	118	8	89.89 ± 0.03	4.52 ± 0.04	44.7 ± 0.3	7	89.84 ± 0.01	4.51 ± 0.00	44.40 ± 0.02
Liquid I			96.58 ± 0.02	0.76 ± 0.05	22.3 ± 0.3		96.47 ± 0.01	0.85 ± 0.02	24.2 ± 0.8
Slurry I	143	8	90.32 ± 0.02	4.37 ± 0.08	45.2 ± 0.8	7	90.60 ± 0.02	4.18 ± 0.03	44.4 ± 0.3
Liquid I			96.61 ± 0.00	0.79 ± 0.00	23.15 ± 0.09		96.48 ± 0.00	0.85 ± 0.02	24.0 ± 0.5
Slurry I	167	8.5	90.53 ± 0.00	4.35 ± 0.04	45.9 ± 0.4	7	89.28 ± 0.01	5.04 ± 0.02	47.0 ± 0.1
Liquid I			96.61 ± 0.01	0.76 ± 0.02	22.5 ± 0.6		96.39 ± 0.00	0.81 ± 0.03	22.5 ± 0.8

[1]The fermentation medium of fermenter at 26 h of fermentation went cloudy. Therefore, the volume of liquid I could not be recorded. Each value except volume of liquid I is presented as the mean ± SD (n = 2).

APPENDIX D
DATA FROM REPLICATE 2 CHAPTER 6

Table D.1 Protein and moisture contents of liquid II, liquid III, liquid IV, liquid V, and solid IV from washing slurry I from fermentation at 25 °C replicate 2

Sample	Fermenter 1			Fermenter 2		
	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Liquid II	96.62 ± 0.01	0.71 ± 0.00	20.89 ± 0.08	96.63 ± 0.01	0.70 ± 0.00	20.8 ± 0.2
Liquid III	98.37 ± 0.01	0.42 ± 0.03	26 ± 2	98.36 ± 0.00	0.32 ± 0.02	20 ± 1
Liquid IV	99.30 ± 0.00	0.17 ± 0.01	24 ± 1	99.27 ± 0.00	0.22 ± 0.01	33 ± 2
Liquid V	99.68 ± 0.01	0.12 ± 0.00	37 ± 2	99.67 ± 0.00	0.08 ± 0.00	24.3 ± 0.9
Solid IV	87.43 ± 0.02	7.68 ± 0.07	61.1 ± 0.7	87.41 ± 0.02	7.8 ± 0.2	62 ± 1

Each value is presented as the mean ± SD (n = 2).

Table D.2 Concentration (g/L) of organic solutes of liquid II, liquid III, liquid IV, and liquid V from washing slurry I from fermentation at 25 °C replicate 2

Compound	Fermenter 1				Fermenter 2			
	Liquid II	Liquid III	Liquid IV	Liquid V	Liquid II	Liquid III	Liquid IV	Liquid V
1,3-PD	7.42	3.60	1.87	1.03	7.23	3.67	1.85	0.99
Acetic acid	7.18	2.97	1.54	0.76	7.08	3.69	1.86	0.92
Glycerol[1]	2.91	1.40	0.73	0.37	2.92	1.59	0.76	0.35
GPC	1.29	0.73	0.37	0.15	1.37	0.67	0.36	0.20
Lactic acid	2.78	1.72	1.03	0.67	3.14	1.88	1.12	0.60

[1]The concentration of glycerol in W-TS samples may be affected by the presence of interfering resonances from carbohydrate and protein.

Table D.3 Mass balance of clarification of replications of small-scale TSF replicate 2

Sample	Weight of sample (kg)
Slurry I	227.23
Liquid I	ND
Solid I decanter	74.45
Solid I desludger	29.29
Liquid I desludger	142.00
Solid II decanter	60.47
Solid II desludger	9.05
Water added for 1st wash	103.00
Liquid II desludger	113.00
Solid III decanter	63.30
Solid III desludger	11.70
Water added for 2nd wash	70.00
Liquid III desludger	63.50

ND = not determined.

Table D.4 Protein and moisture contents of fractions from replications of small-scale TSF replicate 2

Sample	Moisture (%, w/w, wb)	Protein (%, w/w, wb)	Protein (%, w/w, db)
Slurry I	90.26 ± 0.01	4.74 ± 0.02	48.7 ± 0.2
Solid I decanter	81.04 ± 0.07	11.75 ± 0.04	62.0 ± 0.5
Liquid I decanter	96.75 ± 0.00	0.87 ± 0.02	26.8 ± 0.6
Solid I desludger	92.87 ± 0.01	2.71 ± 0.00	38.02 ± 0.05
Liquid I desludger	97.18 ± 0.00	0.66 ± 0.01	23.4 ± 0.5
Mixture of solid and water before 1st wash	92.68 ± 0.01	4.16 ± 0.05	56.8 ± 0.7
Solid II decanter	81.68 ± 0.03	11.4 ± 0.1	62.3 ± 0.6
Liquid II decanter	98.48 ± 0.00	0.47 ± 0.01	31.1 ± 0.8
Solid II desludger	93.48 ± 0.00	2.87 ± 0.02	44.1 ± 0.3
Liquid II desludger	98.63 ± 0.02	0.32 ± 0.01	23.2 ± 0.5
Mixture of solid and water before 2nd wash	92.62 ± 0.00	4.53 ± 0.05	61.4 ± 0.7
Solid III decanter	81.82 ± 0.00	11.6 ± 0.2	64.0 ± 1.0
Liquid III decanter	99.16 ± 0.00	0.30 ± 0.00	36.3 ± 0.5
Solid III desludger	96.64 ± 0.00	1.65 ± 0.02	49.16 ± 0.06
Liquid III desludger	99.41 ± 0.02	0.18 ± 0.01	31.0 ± 3.0

Each value is presented as the mean ± SD (n = 2).

Table D.5 Concentration (g/L) of 1,3-PD, acetic acid, glycerol, GPC, and lactic acid from washing of replications of small-scale TSF replicate 2

Compound	Slurry			Liquid after decanting			Liquid after desludging		
	1st pass	1st wash	2nd wash	1st pass	1st wash	2nd wash	1st pass	1st wash	2nd wash
1,3-PD	6.91	2.55	1.05	5.33	2.61	1.25	5.12	2.66	1.26
Acetic acid	5.69	2.49	1.11	4.75	2.46	1.11	4.51	2.31	1.09
Glycerol[1]	2.21	1.05	0.41	1.80	0.91	0.47	1.80	1.05	0.41
GPC	1.24	0.51	0.17	1.02	0.62	0.20	0.96	0.51	0.22
Lactic acid	3.65	1.64	0.66	3.24	1.82	0.91	3.21	1.81	0.94

[1]The concentration of glycerol in W-TS samples may be affected by the presence of interfering resonances from carbohydrate and protein.