ANALYSIS AND ENGINEERING OF METABOLIC PATHWAYS OF *LACTOBACILLUS PANIS* PM1

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ABSTRACT

Lactobacillus panis PM1 is a novel microorganism isolated from thin stillage (TS), a major by-product resulting from bioethanol fermentation, and was selected as the focus of this thesis due to its ability to produce 1,3-propanediol (1,3-PDO) from glycerol. The purpose of this thesis was to understand the central and auxiliary metabolic pathways of *L. panis* PM1 and to metabolically-engineer strain PM1 based on the improved metabolic knowledge for industrial applications. The 16S rRNA sequence and carbohydrate fermentation pattern were used to classify *L. panis* PM1 as belonging to the group III lactobacilli; thus, strain PM1 exclusively fermented glucose to lactate, acetate, and/or ethanol, clearly suggesting that its primary metabolism occurred via the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway. In contrast to typical group III lactobacilli, for fructose fermentation, *L. panis* PM1 utilized both the 6-PG/PK and the Embden-Meyerhof pathways, showing distinct strain-specific characteristics (more lactate, less acetate, no mannitol, and sporadic growth). In the PM1 strain, auxiliary metabolic pathways governed end-product formation patterns along with central metabolism. Under aerobic conditions, a coupled NADH oxidase-NADH peroxidase system was a determinant for NAD^+ regeneration and was regulated by oxygen availability; however, the accumulation of its major end-product, hydrogen peroxide, eventually resulted in oxidative stress. The citrate-to-succinate route was another important auxiliary pathway in *L.* panis PM1. This route was directly connected to central energy metabolism, producing extra ATP for survival during the stationary phase, and was regulated by the presence of citrate, acetate, and succinate and a transcriptional repressor (PocR). *Lactobacilli panis* PM1 produced 1,3-PDO via the glycerol reductive route; however, the absence of the glycerol oxidative route restricted the utilization of glycerol to solely that of electron acceptor. Lower ratio of glucose to glycerol, in combination with PocR, repressed the glycerol reductive route, resulting in less 1,3-PDO production. In an effort to metabolically engineer *L. panis* PM1, an artificial glycerol oxidative pathway was introduced, and the engineered PM1 strain successfully produced a significant amount of important platform chemicals, including 1,3-PDO, lactate, and ethanol, solely from TS. Overall, this thesis reveals the significant feasibility of utilizing *L. panis* PM1 for industrial fermentative applications.

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This thesis is dedicated to
my beloved son, Snowden J. Kang

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1,3-PDO	1,3-propanediol
3-HPA	3-hydroxypropionaldehyde
6-PG/PK	6-phosphogluconate/phosphoketolase
ADH	Alcohol dehydrogenase
AK	Acetate kinase
ALDH	Acetaldehyde dehydrogenase
ATP	Adenosine triphosphate
CL	Citrate lyase
DhaB	Glycerol dehydratase
DhaD	Glycerol dehydrogenase
DhaK	Dihydroxyacetone kinase
DhaT	1,3-propanediol dehydrogenase
DHA	Dihydroxyacetone
DHAP	Dihydroxyacetone phosphate
EDH	Erythritol dehydrogenase
ENO	Enolase
EM	Embden-Meyerhof
EPT	Erythrose 4-phosphate phosphotransferase
FAD	Flavin adenine dinucleotide
FBA	Fructose 1,6-diphosphate aldolase
FDP	Fructose 1,6-diphosphate
FH	Fumarate hydratase
FK	Fructokinase
G3P	Glycerol 3-phosphate
G6P	Glucose 6-phosphate
G6PDH	Glucose 6-phosphate dehydrogenase
GAP	Glyceraldehyde 3-phosphate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLK	Glucokinase

GlpD	Glycerol 3-phosphate dehydrogenase
GlpF	Glycerol facilitator
GlpK	Glycerol kinase
GRAS	Generally recognized as safe
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase
LS	Liquid stillage
MDH	Malate dehydrogenase
ME	Malic enzyme
NAD	Nicotinamide adenine dinucleotide
NOX	NADH oxidase
NPX	NADH peroxidase
PDH	Pyruvate dehydrogenase
PEP	phosphoenolpyruvate
PFK	6-phosphofructokinase
PGDH	6-phosphogluconate dehydrogenase
PGI	Glucose 6-phosphate isomerase
PGK	Phosphoglycerate kinase
PGM	Phosphoglyceromutase
PK	Pyruvate kinase
PMF	Proton motive force
PTA	Phosphotransacetylase
PTS	PEP-dependent sugar phosphotransferase
PYC	Pyruvate carboxylase
RPE	Ribulose 5-phosphate 3-epimerase
SDH	Succinate dehydrogenase
TCA	Tricarboxylic acid cycle
TPI	Triosephosphate isomerase
TS	Thin stillage
XFP	Xylulose 5-phosphate phosphoketolase
YqhD	NADPH dependent aldehyde reductase

1. INTRODUCTION

Lactobacilli have played a key role in human diets as ubiquitous agents of fermentation and food preservation since ancient times. More importantly, they are known to reside as harmless commensal organisms on the mucosal membranes of the gastrointestinal tracts of healthy individuals. Thus, they are considered to be "generally recognized as safe" (GRAS) microorganisms which may freely be used in the food industry. Historically, Lactobacilli have seen widespread use in the food industry, and more recently, with the aid of an immense accumulation of physiological knowledge and highly-developed genetic tools, this utilization area has been extended from dairy products to high value metabolites. *Lactobacillus panis* strain PM1 was originally isolated from thin stillage (TS) that remained after bioethanol production (Khan et al. 2013); its ability to produce 1,3-propanediol (1,3-PDO) from glycerol in TS suggests the possible utilization of this strain as a cell factory for the production of value-added chemicals. Thus, *L. panis* PM1 was selected as a candidate biotechnological organism, and a variety of aspects of its physiology, metabolism (e.g., pathway elucidation and redox balance), genetics, and fermentation conditions were accordingly elucidated or optimized in this thesis.

1.1 Hypothesis

Lactobacillus panis PM1 is a safe bacterial candidate that can be used as a cell factory for the production of value-added chemicals from low-cost glycerol. However, the fact that this strain cannot use glycerol as the sole energy source is a considerable drawback in terms of potential industrial applications. Thus, the following hypotheses were investigated in this thesis:

i) Understanding of the basic microbial characterizations and central metabolic pathways of *L. panis* PM1 can build a good knowledge base for the further study (Chapters 3 and 4). Sugar fermentation and end product patterns by strain PM1 can classify this strain into group I, II, or III lactobacilli in detail. Furthermore, the quantification of end products, intermediates and activity of enzymes involved in the primary sugar metabolism can elucidate central metabolic pathways and their regulation in depth.

ii) The role of NAD^+/NADH recycling in *L. panis* PM1 plays a key role in defining end product profiles; thus, elucidation of auxiliary metabolic pathways, including oxygen, citrate, and glycerol metabolism, can offer key information about how this strain produces certain end-products (e.g., 1,3-PDO) under certain conditions (e.g., in the presence of glycerol) (Chapters 5, 6, 7, and 8).

iii) The regulatory mechanism of each auxiliary pathway at transcription and/or protein level can reveal the metabolic network of *L. panis* PM1, suggesting rational approaches for metabolic engineering of this strain (Chapters 5, 6, 7, and 8).

iv) The activity of 1,3-PDO dehydrogenase (DhaT) plays a key role in NADH recycling as well as 1,3-PDO production; thus, expression of an *Escherichia coli* YqhD gene (showing DhaT activity) contributes to an increase in 1,3-PDO production by *L. panis* PM1 (Chapter 9).

v) An artificial glycerol oxidative pathway that connects glycerol to the central metabolic pathway enables *L. panis* PM1 to produce valuable chemicals solely from thin stillage, a major by-product after bioethanol production (Chapters 10).

1.2 Technical objectives

The technical objectives of this thesis were to:

i) Develop analytical methods that can quantify major end-products in fermentation samples by *L. panis* PM1.

ii) Develop genetic tools, including transformation method and expression vector system, for the genetic manipulation of *L. panis* PM1.

ii) Elucidate central and auxiliary metabolic pathways of *L. panis* PM1 and their regulatory systems and eventually understand how the PM1 strain utilizes these pathways under a wide spectrum of environmental and nutritive conditions.

iii) Metabolically-engineer *L. panis* PM1 on the basis of the improved understanding of metabolic pathways.

iv) Test the ability of engineered strains to produce value-added chemicals, including ethanol, lactate, and 1,3-PDO, from TS.

2. LITERATURE REVIEW

2.1 Genus *Lactobacillus*

Lactic acid bacteria (LAB) constitute multiple genera within the order Lactobacilliales and have been utilized in human diets as ubiquitous agents of fermentation and food preservation since ancient times due to their ability to rapidly ferment carbohydrates to lactic acid. *Lactobacillus* is one of the most well-characterized LAB and has been widely found in dairy, meat, fermented products, as well as human gut; thus, various *Lactobacillus* species are considered as generally recognized as safe (GRAS) and industrially utilized in food and agricultural fermentations. The lactobacilli constitute a large genus of Gram-positive bacteria, including over 180 species (Bull et al. 2013). Kandler and Weiss (1986) described the genus *Lactobacillus* as "a highly diverse group of Gram-positive, microaerophilic bacteria that microscopically appear as long to short rods or even coccobacillia" in Bergey's Manual of Systematic Bacteriology. Lactobacilli have been classified into three groups based on their fermentative characteristics (Kandler and Weiss 1986): Group I; obligatory homofermentative, Group II; facultatively heterofermentative, and Group III; obligatory heterofermentative (Table 2.1). Together with the molecular characteristics, such as mol% G+C content and ribosomal RNA sequence data, this classical classification has been further elaborated since the 1980s, and the first phylogenetic analysis of lactobacilli was conducted by Collins et al. (1991). The genus *Lactobacillus* shows 35 to 55% mol% G + C content, and is distinguished from the Bifidobacteria which has over 55 mol% G + C content (Stiles and Holzapfel 1997). The history of *L. acidophilus* is an example of this classification scheme. *Lactobacillus acidophilus*, a commonly used LAB in dairy products due to its probiotic effects, is one of the most well-defined *Lactobacillus* phylogenetic subgroups. *Lactobacillus acidophilus* was first isolated from infant feces and designated as *Bacillus acidophilus* in 1900. This bacterium was renamed to *L. acidophilus* by Holland in 1920 (Winslow et al. 1920). From these early descriptions of *L. acidophilus* until around 1970, numerous *Lactobacillus* isolates from the human oral, intestinal, and vaginal tracts, were collectively identified as *L. acidophilus* on the basis of their phenotypic characteristics (Bull et al. 2013).

Table 2.1 Classification of the genus *Lactobacillus*

Characteristics	Group I	Group II	Group III
Pentose fermentation	-	+	-
CO_2 from glucose	-	-	+
CO_2 from gluconate	-	+	+
FBA[a] present	+	+	-
XFP[b] present	-	+[c]	+
Representative species	*L. acidophilus* *L. amylovorus* *L. delbruecckii* *L. farciminis* *L. gasseri* *L. helveticus* *L. johnsonii* *L. kefiranofaciens* *L. ruminis* *L. salivarius*	*L. acetotolerans* *L. alimentarius* *L. bifermentans* *L. casei* *L. curvatus* *L. pentosus* *L. plantarum* *L. rhamnosus* *L. sakei*	*L. brevis* *L. buchneri* *L. collinoides* *L. fermentum* *L. fructivorans* *L. hilgardii* *L. kefir* *L. panis* *L. pontis* *L. reuteri* *L. sanfranciscensis* *L. vaginalis*

a. Fructose 1,6-diphosphate aldolase. b. Xylulose 5-phosphate phosphoketolase. c. Inducible by pentose. Source: Adapted from Stiles and Holzapfel (1997).

The advent of the genomics era now offers a definitive way to identify *Lactobacillus* species (Claesson et al. 2007; Claesson et al. 2008), and the *L. acidophilus* group is sub-classified into approximately 20 different species with the aid of sequence analyses of genes (e.g., 16S rRNA and *gro*EL), DNA fingerprinting methods, and pulsed-field gel electrophoresis (Bull et al. 2013).

Group I lactobacilli exclusively ferment hexose sugars (e.g., glucose) to lactate via the Embden-Meyerhof (EM) pathway; i.e., fructose 1,6-diphosphate aldolase (FBA) plays a key role in homolactic fermentation. However, they do not metabolize pentose sugars or gluconate. Group I lactobacilli have been used as starter cultures for Swiss- and Italian-type cheeses and for fermentation of sourdough bread and kefir grain (Stiles and Holzapfel 1997). Group II lactobacilli

ferment hexoses to lactate via the EM pathway and produce carbon dioxide from gluconate. Another key feature in this group is an inducible phosphoketolase that enables the fermentation of pentose sugars to lactate and acetate. Group II lactobacilli, including *L. casei*, *L. plantarum*, and *L. sakei*, have important associations with fermented meat and vegetable products (Stiles and Holzapfel 1997). Group III lactobacilli exclusively ferment hexoses to lactate, ethanol and/or acetate via the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway; i.e., xylulose 5-phosphate phosphoketolase (XFP) is a critical enzyme for heterolactic fermentation. Carbon dioxide production during the metabolism of hexose is a distinct feature of the group III lactobacilli. A few group III lactobacilli are involved in sourdough fermentation; however, most bacteria in this group are commonly associated with food spoilage (Stiles and Holzapfel 1997). Among group III lactobacilli, *L. reuteri* has gained special interest due to its ability to produce reuterin (3-hydroxypropionaldehyde, 3-HPA), a metabolite with broad-spectrum antimicrobial activity (Bauer et al. 2010) .

2.2 Applications of Lactobacilli through Metabolic and Genetic Engineering

Traditionally, lactobacilli have been widely used in fermented food, especially dairy products, and considerable research efforts focusing on these bacteria have been conducted with aims to improve flavor, texture, and nutritional values. Advances in genetic techniques in the 1980s accelerated the manipulation of lactobacillus strains and contributed to a significant body of accumulated knowledge about their physiology, metabolism, and genetics. In addition, the combination of a rapidly evolving understanding of cellular pathways in combination with increasingly-sophisticated genetic tools led to a gradual shift in the paradigm of lactobacilli's utilization from that of traditional starter cultures to cell factories for the production of food ingredients, value-added metabolites, and commodity chemicals.

2.2.1 Food Flavors

Acetoin and diacetyl are key flavor compounds responsible for the butter aroma of dairy products. The production of these compounds is associated with citrate metabolism by a few lactobacilli, and α-acetolactate synthesized from two pyruvate molecules is converted to acetoin and diacetyl by α-acetolactate decarboxylase and non-enzymatic oxidative decarboxylation, respectively (Hugenholtz 1993). Thus, early metabolic engineering strategies focused on the

production of these flavor compounds from lactose or glucose, rather than from citrate that is in low abundance in raw dairy materials. Recently, Nadal et al. (2009) reported the production of acetyl/diacetyl compounds (1.4 g/L) from lactose in whey permeate, a waste product from cheese industry, by using an engineered *Lactobacillus brevis* strain in which lactate dehydrogenase (*ldh*) and pyruvate dehydrogenase E2 subunit (*pdhC*) genes were deleted and the acetohydroxy acid synthase (*ilvBN*) gene from *Lactococcus lactis* was over-expressed.

2.2.2 Ethanol

Ethanol is a major biofuel widely used as an additive in gasoline, and the recent increased demand for this chemical includes the utilization of lactobacilli that can ferment various sugars (e.g., glucose and xylose) in biomass hydrolysates and have higher ethanol tolerance (up to 18%) (Gaspar et al. 2013). Liu et al. (2006) expressed the pyruvate decarboxylase (*pdc*) gene of *Sarcina ventriculi* in a LDH-deficient strain of *Lactobacillus plantarum*. This LDH-deficient engineered strain produced 7-fold higher ethanol (130 mM) than the negative control strain; however, ethanol production was unexpectedly accompanied by high lactate production (170 mM), which might be induced by other dehydrogenases activated by antibiotic stress (Liu et al. 2006).

2.2.3 Lactate

Traditionally, lactate has been used as a flavor additive and preservative in food industry. However, this organic acid has also seen recent use in pharmaceutical, leather, and textile industries due to the feature of its carboxylic and hydroxyl groups which can be modified to produce useful chemicals, including pyruvic acid, acrylic acid, 1,2-propanediol, and lactate ester (Gao et al. 2011). Lactate can be produced by chemical synthesis as well as by microbial fermentation. Most lactate production is achieved by the latter method due to relatively low substrate cost, production temperature, and energy consumption (Gao et al. 2011). Lactobacilli are leading candidates for industrial lactate production and, from them, *Lactobacillus rhamnosus* and *Lactobacillus delbrueckii* have been widely used for L- and D-form lactate production, respectively (Okano et al. 2010a). These lactobacilli theoretically produce lactate from hexose fermentation via the EM pathway; thus, the high cost of fermentable substrates, including glucose, sucrose, or maltose, remains a drawback for biotechnological lactate production. Various studies have focused on lactate production utilizing cheap and renewable raw materials such as corn

stover (Cui et al. 2011), wheat bran (Li et al. 2010), and cassava powder (Wang et al. 2010). However, these raw materials must be pretreated by physicochemical or enzymatic methods since most lactobacilli cannot directly use them (Okano et al. 2010a). Alternatively, metabolic engineering strategies have been applied to lactobacilli candidates for the direct utilization of cellulosic compounds. According to Okano et al. (2010b), an engineered *L. plantarum* strain can produce 1.47g/L lactate from a medium containing 0.2% (w/v) β-glucan as the sole carbon source.

2.2.4 Succinate

Succinate has been widely used in industrial applications, including food and pharmaceutical products, biodegradable plastics and ingredients to trigger animal and plant growth, surfactants, and green solvents (Cheng et al. 2012; da Silva et al. 2009). After the first evidence of succinate production by *L. reuteri* strains (Kaneuchi et al. 1988), the succinate metabolic pathway has been studied in a few lactobacilli (Cselovszky et al. 1992; Torino et al. 2005; Tsuji et al. 2013). These strains can produce succinate from citrate via the reductive route using a part of the tricarboxylic acid (TCA) cycle under anaerobic conditions. Tsuji et al. (2013) recently reported the production of succinate from glucose in the absence of citrate by an engineered *L. plantarum* strain. For this work, the pyruvate carboxylase *(pycA)* and phosphoenolpyruvate carboxykinase (*pckA*) genes of *L. plantarum* were introduced into a LDH-deficient strain of *L. plantarum*, and this engineered strain produced a 22-fold higher amount of succinate (56 mM) than did the wild-type strain (Tsuji et al. 2013).

2.2.5 Reuterin

Reuterin, a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-HPA, is of industrial interest due to its potential use as a food preservative and sanitizing agent (Talarico and Dobrogosz 1989). In addition, 3-HPA is used as a precursor for acrolein, acrylic acid, and 1,3-propanediol (1,3-PDO), and the production of 3-HPA is achieved both through traditional chemistry and bacterial fermentation (Vollenweider and Lacroix 2004). A few *Lactobacillus* species, including *L. reuteri* (Bauer et al. 2010), *L. hilgardii* (Pasteris and Strasser de Saad 2009), *L. collinoides* (Sauvageot et al. 2000), and *L. coryniformes* (Martin et al. 2005), can convert glycerol to 3-HPA by the activity of glycerol dehydratase. Normally this chemical is further reduced to 1,3-propanediol (1,3-PDO) by 1,3-PDO dehydrogenase, thereby negating the

accumulation of 3-HPA. The accumulation of 3-HPA has been well studied in *L. reuteri* and *L. collinoides*. In these studies, the accumulation of 3-HPA by *L. reuteri* was strongly favored at pH 6.0 and molar ratio of glucose to glycerol less than 0.33 (Bauer et al. 2010; Lüthi-Peng et al. 2002). Also, this glucose to glycerol molar ratio was a critical determinant in the accumulation of 3-HPA by *L. collinoides* along with temperature (Garai-Ibabe et al. 2008; Sauvageot et al. 2000). Thus, this feature in glycerol metabolism suggests that the conversion of glycerol to 3-HPA by lactobacilli can be achieved by a similar mechanism.

2.3 Glycerol: an Abundant and Cheap C3 Carbon Source

Glycerol, a major component of triglycerides in animal fat, vegetable oil, and crude oil, is mainly derived from soap or biodiesel production. Three hydrophilic hydroxyl groups in the glycerol molecule are responsible for its soluble (in water) and hygroscopic properties, which enables glycerol to be a versatile substance in many applications, along with its non-toxicity to human and environment (Ayoub and Abdullah 2012; Quispe et al. 2013). This chemical is categorized into three major types; crude glycerol, refined glycerol, and commercially-synthesized glycerol, according to their purity (Table 2.2).

Table 2.2 Quality parameters of different categories of glycerol

Parameter	Crude glycerol	Refined glycerol	Commercial glycerol
Glycerol content (%)	60-80	99.1-99.8	99.2-99.8
Moisture content (%)	1.5-6.5	0.11-0.8	0.14-0.29
Ash (%)	1.5-2.5	0.054	< 0.002
Soap (%)	3.0-5.0	0.56	N.D.
Acidity (pH)	0.7-1.3	0.10-0.16	0.04-0.07
Chloride (ppm)	N.D.	1.0	0.6-9.5

N.D. not detected. Adapted from Ayoub and Abdullah (2012)

Concerns about high energy prices, petroleum supplies, and environmental consequences by fossil fuels have caused interest in developing alternative sources of renewable energy. Accordingly, the concept of bio-based resources has received much attention, and the demand for biofuels has been on the increase. Crude glycerol is a significant by-product of biodiesel and bioethenol production processes; thus, the major source of glycerol was shifted from the fatty acids industry to the

biodiesel industry over the past 10 years (Ayoub and Abdullah 2012). Crude glycerol has a low value due to its impurities and low glycerol level; thus, it requires refinement to be suitable for more valuable applications. A behemoth in biodiesel production can purify their crude glycerol and distribute the refined glycerol to the food, pharmaceutical, and cosmetic industries (Quispe et al. 2013). However, this refining process involves considerable costs for small- and medium-sized biodiesel producers, and typically, they dispose of crude glycerol or store it until a better market opportunity arises, thereby negatively-affecting the biodiesel economy (Ayoub and Abdullah 2012; Quispe et al. 2013).

2.3.1 Glycerol Market

From the 1970s until 2004, the price of high-purity glycerol had remained relatively stable at 1,200 and 1,800 U.S. $/ton. This value is considerably more expensive than that of glucose (e.g., 220 U.S. $/ton vs. greater than 1,100 U.S. $/ton) (Ayoub and Abdullah 2012; Cameron et al. 1998). However, the advent of biodiesel production has dramatically impacted the stable glycerol market. Currently, biodiesel is produced by the transesterification of triglycerides along with significant amounts of crude glycerol (about 10% w/w) (Quispe et al. 2013). Thus, the dramatic increase in biodiesel for transportation fuels in the United States and the European Union resulted in tremendous glycerol surplus, leading to a collapse in glycerol prices. As seen in Table 2.2, differences found between refined and commercial glycerol preparations are minor, and, therefore, conventional glycerol preparation has totally lost its competitiveness in the glycerol market since 2006. The price of refined glycerol dropped from approximately 1,200 U.S. $/ton in 2003 to 600 U.S. $/ton in 2006. Significantly, the price of crude glycerol was quoted between 0 and 70 U.S. $/ton in 2006, whereas in 2011, this price slightly increased by 40 to 110 U.S. $/ton in the United States (Ayoub and Abdullah 2012; Quispe et al. 2013). It is very difficult to predict glycerol pricing within the volatile glycerol market, which is dependent on the global demand and supply of biodiesel. However, low-price trends are expected to be sustainable as long as renewable-fuels policies (e.g., mandates, tax incentives, and subsidies) in top biodiesel-producing countries (e.g., European Union, United States, and Brazil) continue to be implemented. It was estimated that biodiesel production would reach 8.2 billion gallons by 2020, thereby contributing 5.9 billion pounds of crude glycerol that was previously less than 1 billion pounds prior to 2006 (Glycerin Market Analysis 2007).

2.3.2 Glycerol Applications

Traditionally, purified glycerol was considered to be a high-value and commercial chemical with more than 1500 uses. Glycerol is an ideal ingredient in many personal and oral care products, food, and pharmaceuticals as humectants and lubricants (Ayoub and Abdullah 2012; Quispe et al. 2013). Thus, new and economic applications of crude glycerol have emerged in recent years to absorb the glycerol glut (Clomburg and Gonzalez 2013; Yang et al. 2012; Yazdani and Gonzalez 2007). The microbial bioconversion of low-price glycerol to value-added chemicals would be a promising application; accordingly, glycerol could be substituted for traditional carbohydrates, such sucrose, glucose and starch, and enable the production of reduced chemicals, including propionate, dihydroxyacetone, polyhydroxyalcanoate, as well as amino acids, at higher yields than those obtained using sugars. Several attempts have been made to convert glycerol into more valuable chemicals, as described below.

2.3.2.1 Propionate

Propionate plays a key role in numerous industrial applications, such as cellulose-based plastics, herbicides, flavors and perfumes, arthritis and dermatosis drugs, antifungal agents in food and feed (Boyaval and Corre 1995). Two propionibacteria, *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp. *shermanii*, have been widely studied for the production propionate; both propionibacteria produced 12 g/L and 9 g/L propionate, respectively, from 20 g/L glycerol as the sole carbon and energy source (Himmi et al. 2000). The authors concluded that glycerol showed similar or superior conversion yield and productivity to that of glucose, and *P. acidipropionici* showed higher efficiency in glycerol conversion to propionate than that of *P. freudenreichii* ssp. *shermanii*.

2.3.2.2 Dihydroxyacetone

Dihydroxyacetone (DHA) is a multi-purpose building block utilized for the organic synthesis of fine chemicals, and for the cosmetic industry, the chemical synthesis of DHA is relatively expensive compared with microbial synthesis processes due to meticulous safety requirements (Hekmat et al. 2003). *Gluconobacter oxydans* has been widely used in the conventional industrial microbial DHA production, showing constant 1.6 g/L/h rates of DHA production in a repeated fed-batch fermentation maintained over a period of several months with

no decrease in productivity (Hekmat et al. 2003). Gätgens et al. (2007) over-expressed the glycerol dehydrogenase enzyme in *G. oxydans*, after which the recombinant strain produced up to 350 mM of final DHA concentration compared to 200 to 280 mM in control strains in culture medium supplemented with 50 g/L of glycerol.

2.3.2.3 Polyhydroxyalcanoate

Polyhydroxyalcanoate (PHA) can replace petroleum-derived polymers due to its biodegradable and biocompatible characteristics; thus, it is anticipated that PHA will see wide application in medicine, agriculture and horticulture, the fiber industry (Solaiman et al. 2006). Many species of *Pseudomonas* can accumulate PHA polymers when grown under nutrient limited conditions. *Pseudomonas oleovorans* NRRL B-14682 and *P. corrugata* 388 synthesized PHA and polyhydroxybutyrate (PHB) blends from up to 5% glycerol with varying blend ratios (Ashby et al. 2005). In an effort to minimize PHA production costs, Koller et al. (2005) utilized hydrolyzed whey permeate (HWP) and glycerol liquid phase (GLP) from biodiesel production as cheap carbon sources and reported 5.5 g/L and 16.2 g/L of PHA from the HWP and GLP (including 70% glycerol), respectively, by using an osmophilic *Escherichia coli* strain.

2.3.2.4 Amino Acids

Wild-type *Corynebacterium glutamicum* ATCC 13032 cannot utilize glycerol as the sole carbon source; however, a recombinant *C. glutamicum* strain, in which the glycerol facilitator, glycerol kinase, and glycerol 3-phosphate dehydrogenase genes from *E. coli* were introduced, could grow and produce 15 mM of glutamate with a product yield of 0.11 g/g, showing a similar glutamate yield on glucose (0.2 g/g) in minimal medium supplemented with 20 g/L glycerol (Rittmann et al. 2008). When these three genes were introduced into a lysine-producing *C. glutamicum* strain, the recombinant strain could used up available glycerol (20 g/L) and accumulated 26 mM of lysine in the culture medium. The product yield of lysine (0.19 g/g) was comparable to the lysine yield on glucose (Rittmann et al. 2008).

2.4 Glycerol Metabolism and Regulation of Glycerol Genes in Relevant Species

In bacteria, glycerol is transported via passive diffusion like other small uncharged molecules (Voegele et al. 1993). Glycerol uptake is also facilitated by an integral membrane

protein, the glycerol facilitator (GlpF), which is a highly-selective channel (Voegele et al. 1993). Thus, glycerol influx by GlpF is 100- to 1000-fold greater than by passive transport alone and is non-saturatable over 200 mM glycerol (Fu et al. 2000). Intracellular glycerol is metabolized by a few microorganisms, including *Citrobactor freundii*, *Klebsiella pneumoniae, Clostridium pasteurianum, Clostridium butyricum, Enterobacter agglomerans,* and *Enterobacter aerogenes*, as a sole carbon and energy source under anaerobic and/or aerobic conditions (da Silva et al. 2009). These organisms possess three pathways for glycerol metabolism. In the first pathway (an oxidative glycerol route), glycerol enters into cells via GlpF and is then converted to glycerol 3-phosphate (G3P) by glycerol kinase (GlpK). Subsequently, G3P is used to synthesize either dihydroxyacetone phosphate (DHAP) by glycerol 3-phosphate dehydrogenase (GlpD) or lipids through glycerophospholipid. The converted DHAP is transformed into glyceraldehyde 3-phosphate (GAP) by triosephosphate isomerase (TPI) in glycolysis, or can serve as a substrate for the synthesis of other metabolites. This pathway is responsible for the aerobic degradation of glycerol in *K. pneumoniae* and *Clostridium acetobutylicum* (da Silva et al. 2009; Gonzalez-Pajuelo et al. 2005; Raynaud et al. 2003; Seo et al. 2009). Another type of oxidative glycerol pathway is responsible for the anaerobic degradation of glycerol. In this route, the NAD^+ dependent glycerol dehydrogenase (DhaD) catalyzes the conversion of glycerol to dihydroxyacetone (DHA). Then dihydroxyacetone kinase (DhaK) phosphorylates DHA to produce DHAP which then enters glycolysis to produce energy for growth and reducing equivalents for redox balance. This pathway is found in most bacterial genera, such as *Klebsiella, Citrobacter, Clostidium* and *Enterobacter* (Biebl et al. 1999; Celinska 2010; da Silva et al. 2009; Saxena et al. 2009). The third pathway, a glycerol reductive route, is catalyzed by the coenzyme B_{12}-dependent glycerol dehydratase (DhaB), converting glycerol to 3-HPA. The 3-HPA is then reduced by the NADH-dependent enzyme 1,3-propanediol dehydrogenase (DhaT) to 1,3-PDO, thereby regenerating NAD^+. This reductive pathway is a distinct feature of bacteria producing 1,3-PDO from glycerol, such as *Klebsiella, Citrobacter, Clostridium, Enterobacter,* and *Lactobacillus* (Biebl et al. 1999; Celinska 2010; da Silva et al. 2009; Saxena et al. 2009).

2.4.1 Glycerol Metabolism in *Escherichia coli*

The *glp* and *dha* regulons reportedly contribute to the conversion of glycerol to DHAP in *E. coli*. The *glp* regulon is composed of five operons located at three different chromosomal regions

of *E. coli* (Schweizer et al. 1985; Weissenborn et al. 1992; Zeng et al. 1996; Zhao et al. 1994) (Figure 2.1), and this regulon is responsible for the transcription and regulation of the GlpK and GlpD pathway for the first oxidative glycerol metabolism. These genes are: *glpQ*, glycerophosphodiester phosphodiesterase; *glpT*, sn-glycerol 3-phosphate permease; *glpABC*, anaerobic sn-glycerol 3-phosphate dehydrogenase; *glpF*, glycerol uptake facilitator; *glpK*, glycerol kinase; *glpD*, aerobic glycerol 3-phosphate dehydrogenase; and *glpR*, transcriptional repressor. The GlpE gene has been reported to be a prototype sulfurtransferase (Spallarossa et al. 2001), whereas the GlpG gene currently has an unknown function. Transcription of the *glp* regulon is negatively regulated by the *glp* repressor, a tetrameric protein encoded by the *glpR* gene (Zhao et al. 1994).

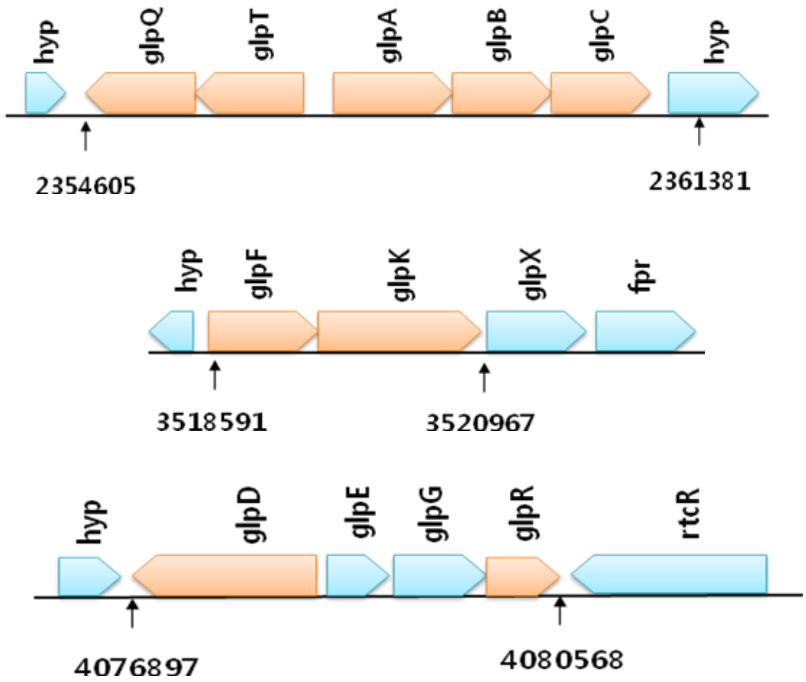

Figure 2.1 Position of the *glp* regulon in the *E. coli* W3100 chromosome. All annotated data are adapted from the DOE Joint Genome Institute database (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=findGenes). GlpQ, glycerophosphodiester phosphodiesterase; GlpT, glycerol 3-phosphate permease; GlpABC, anaerobic glycerol 3-phosphate dehydrogenase; GlpF, glycerol uptake facilitator; GlpK, glycerol kinase; GlpX, fructose 1,6-bisphosphatase II; FPR, ferredoxin-NADP reductase; GlpD, aerobic glycerol 3-phosphate dehydrogenase; GlpE, thiosulfate sulfurtransferase; GlpG, intramembrane serine protease; GlpR, transcriptional repressor; RtcR, sigma 54-dependent transcriptional regulator of RtcBA expression; and HYP, hypothetical protein.

Glycerol 3-phosphate, which acts as the true inducer of the *glp* regulon, inhibits binding of the repressor to the operators (Zhao et al. 1994). The control regions of *glpACB*, *glpD*, and *glpFK*

possess tandemly-repeated repressor-binding sites, and a repressor-mediated DNA looping mechanism controls the transcription of the divergent *glpTQ-glpACB* operons (Zhao et al. 1994).

The *dha* regulon is composed of two operons, and four genes have been identified in the *dha* regulon of *E. coli* (Figure 2.2), providing the second glycerol metabolic pathway (DhaD and DhaK pathway). These genes are: *dhaMLK* (DHA kinase) and *dha*R (transcriptional regulator). Transcription of the *dhaKLM* operon is up-regulated by DhaR that also auto-represses *dhaR* transcription (Bächler et al. 2005). DHA kinase of *E. coli* utilizes the bacterial phospoenolpyruvate carbohydrate phosphotransferase system (PTS) as a phosphate donor, and consists of three subunits; DhaK, DhaL, and DhaM. DhaK contains the DHA binding site, DhaL includes adenosine diphosphate (ADP) as cofactor for shifting of phosphate from DhaM to DHA, and DhaM offers a phospho-histidine relay between the PTS and DhaL-ADP (Bächler et al. 2005).

Escherichia coli uses two pathways to metabolize glycerol under aerobic or anaerobic conditions. The GlpKD pathway is expressed under aerobic conditions; whereas, the DhaDK pathway is activated under anaerobic conditions (Durnin et al. 2009). These two pathways enable *E. coli* to use glycerol as carbon source and energy source. However, *E. coli* cannot use glycerol as an external electron acceptor source, unlike *Klebsiella and Lactobacillus*, due to the absence of the glycerol dehydratase and 1,3-PDO dehydrogenase genes in its *dha* regulon.

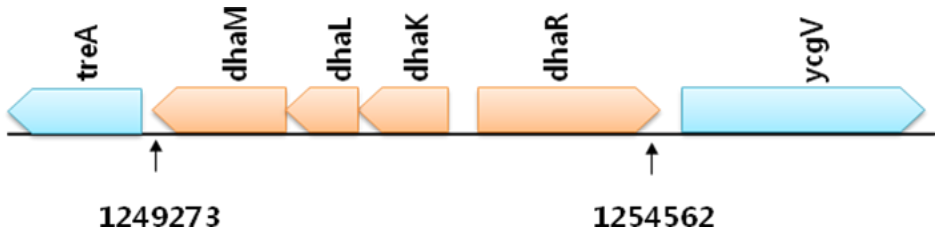

Figure 2.2 Position of the *dha* regulon in the *E. coli* W3100 chromosome. All annotated data are adapted from the DOE Joint Genome Institute database (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=findGenes). TreA, periplasmic trehalase; DhaMLK, PTS-dependent dihydroxyacetone kinase; DhaR, transcriptional regulator; and YcgV, predicted adhesin.

2.4.2 Glycerol Metabolism in *Bacillus subtilis*

The *glp* regulon of *Bacillus subtilis* enables growth on glycerol or glycerol 3-phosphate as the sole carbon source (Beijer and Rutberg 1992; Reizer et al. 1984). So far, six *glp* genes have been identified in the *glp* regulon of *B. subtilis* (Beijer et al. 1993; Nilsson et al. 1994). These

genes are: *glpP*, anti-terminator; *glpF*, glycerol uptake facilitator; *glpK*, glycerol kinase; *glpD*, NAD-independent glycerol 3-phosphate dehydrogenase; *glpT*, glycerol 3-phosphate permease; and *glpQ*, glycerophosphodiester phosphodiesterase. These six genes are organized into four operons: *glpP*, *glpKF*, *glpD*, and *glpTQ* (Figure 2.3). Glycerol is transported into *B. subtilis* by facilitated diffusion involving the GlpF protein. Intracellular glycerol is converted by the activity of GlpK to glycerol 3-phosphate, which is not a substrate for GlpF; thus, it remains entrapped in the cell and is further metabolized (Darbon et al. 2002). Glycerol 3-phosphate can also be taken up by GlpT where it functions as a true inducer of the genes and operons involved in the metabolism of glycerol (Darbon et al. 2002). In *B. subtilis*, expression of the GlpD gene was regulated by GlpP and it bound to the ρ-independent terminator located at the beginning of the *glpD* mRNA transcript (Darbon et al. 2002). The *glpFK* and *glpTQ* operons showing the inverted repeats similar to the *glpD* transcript in *B. subtilis* were thought to be controlled by GlpP (Darbon et al. 2002).

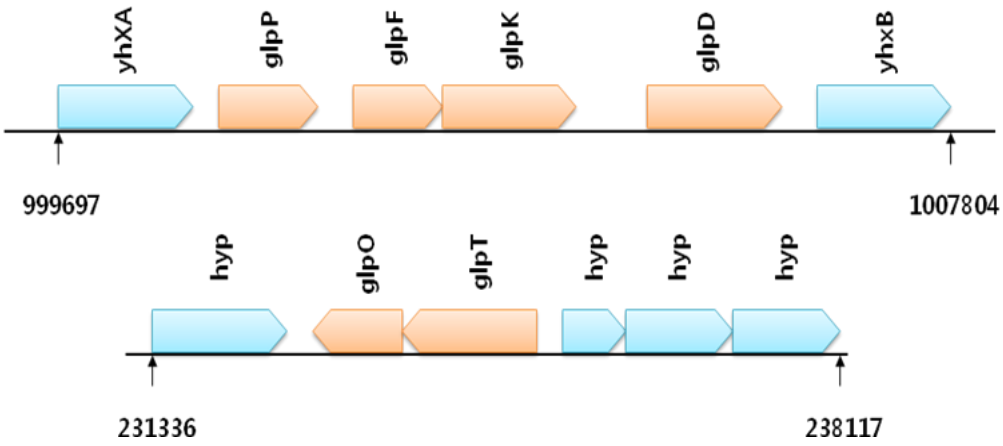

Figure 2.3 Position of the *glp* regulon in the *B. subtilis* chromosome. All annotated data are adapted from the DOE Joint Genome Institute database (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=findGenes). YhxA, adenosylmethionine 8-amino-7-oxononanoate transminase; GlpP, regulatory protein; GlpF, glycerol uptake facilitator; GlpK, glycerol kinase; GlpD, NAD-independent glycerol 3-phosphate dehydrogenase; YhxB, alpha-phosphoglucomutase; GlpT, glycerol 3-phosphate permease; GlpQ, glycerophosphodiester phosphodiesterase; and HYP, hypothetical protein.

As mentioned above, glycerol and glycerol 3-phosphate uptake in *B. subtilis* is not involved in the PTS. However, studies with mutants have revealed that glycerol uptake in *B. subtilis* defective in the general components of the PTS, Enzyme I (EI) or Hpr, was decreased and resulted in a failure to grow on glycerol. In contrast, these mutants could grow on glycerol 3-

phosphate as sole source of carbon and energy (Beijer and Rutberg 1992). Thus, it was suggested that the PTS differently influences glycerol and glycerol 3-phosphate catabolism in *B. subtilis*. Whereas, in Gram-positive bacteria, such as *Enterococcus faecalis* or *Enterococcus casseliflavus*, GlpK is phosphorylated by P~His-HPr at a conserved histidyl residue, leading to a more than 10-fold stimulation of its activity (Charrier et al. 1997). *Bacillus subtilis* mutants in the general components of the PTS showed the complete absence of P~GlpK and lacked growth on glycerol media as the sole carbon source (Beijer and Rutberg 1992; Henkin et al. 1991). Some revertants of *B. subtilis* Hpr mutants which could grow on glycerol possessed a PTS-independent GlpK, in which a phosphorylatable His-230 was replaced with an arginine. Further, the PTS-independent GlpK provided an enzyme exhibiting elevated activity similar to P~GlpK in *E. casseliflavus* (Charrier et al. 1997).

2.4.3 Glycerol Metabolism in *Klebsiella pneumoniae*

In silico analysis of the genome sequence of *K. pneumoniae* strain MGS78578 using the DOE Joint Genome Institute database (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=findGenes) revealed that the genes related to glycerol metabolism are mainly clustered in four chromosomal loci (Figures 2.4 and 2.5). The *glp* regulon consists of 11 genes which are organized into five operons: *glpQT*, *glpABC*, *glpFK*, *glpR*, and *glpD*. These genes are: *glpQ*, glycerophosphodiester phosphodiesterase; *glpT*, sn-glycerol 3-phosphate permease; *glpABC*, anaerobic glycerol 3-phosphate dehydrogenase; *glpF*, glycerol uptake facilitator; *glpK*, glycerol kinase; *glpR*, transcriptional repressor; and *glpD*, aerobic glycerol 3-phosphate dehydrogenase. Two of the 11 genes (*glpE* and *glpG*) have been reported as having unknown function (Figure 2.4). The *dha* regulon consisted of 9 genes which are organized into four operons: *dhaB*, *dhaT*, *dhaRD*, and *dhaKLM*. These genes are: *dhaB123*, glycerol dehydratase; *dhaT*, 1,3-PDO dehydrogenase; *dhaR*, transcriptional regulator; *dhaD*, glycerol dehydrogenase; and *dhaKLM*, DHA kinase. The genome sequence of *K. pneumoniae* strain MGS78578 revealed that two putative genes encoding a second glycerol facilitator (*glpF*) and a second DHA kinase (*dhaK*) are present in two operons of the *dha* regulon (Figure 2.5).

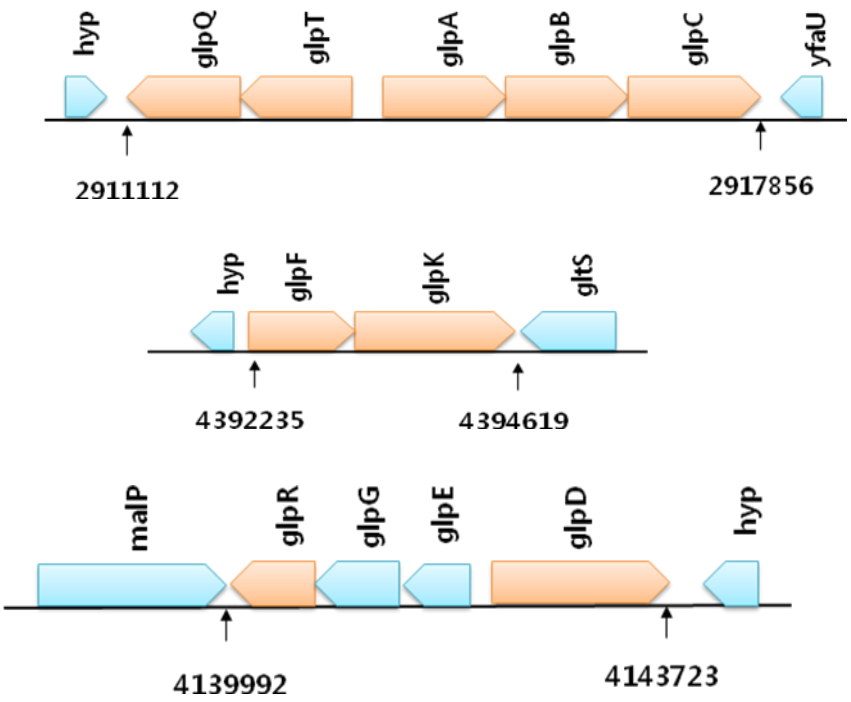

Figure 2.4 Position of the *glp* regulon in the *K. pneumoniae* chromosome. All annotated data are adapted from the DOE Joint Genome Institute database (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=findGenes). GlpQ, glycerophosphodiester phosphodiesterase; GlpT, glycerol 3-phosphate permease; GlpABC, anaerobic glycerol 3-phosphate dehydrogenase; YfaU, 2,4-dihydroxyhept-2-ene-1,7-dioic acid aldolase; GlpF, glycerol uptake facilitator; GlpK, glycerol kinase; G*ltS*, glutamate transport; MalP, maltodextrin phosphorylase; GlpR, transcriptional repressor; GlpG, intramembrane serine protease; GlpE, thiosulfate sulfurtransferase; GlpD, aerobic glycerol 3-phosphate dehydrogenase; and HYP, hypothetical protein.

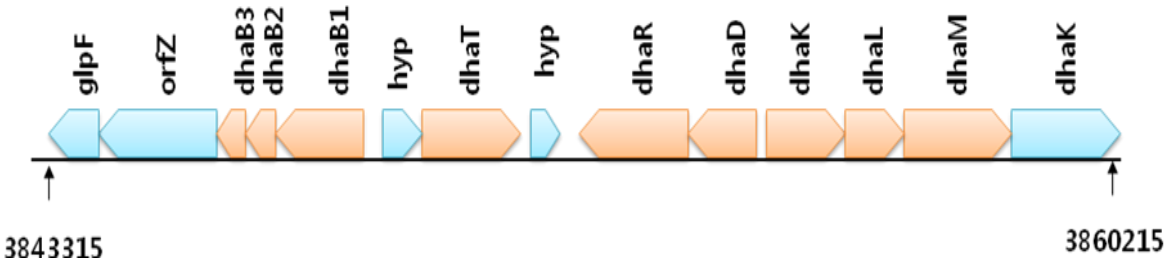

Figure 2.5 Position of the *dha* regulon in the *K. pneumoniae* chromosome. All annotated data are adapted from the DOE Joint Genome Institute database (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=findGenes). GlpF, putative glycerol uptake facilitator; ORFZ, glycerol dehyrdratase activator; DhaB123, glycerol dehydratase; DhaT, 1,3-propanediol dehydrogenase; DhaR; transcriptional regulator; DhaD, glycerol dehydrogenase; DhaKLM, dihydroxyacetone kinase; and HYP, hypothetical protein .

Klebsiella pneumoniae is the most notable strain capable of producing 1,3-PDO from glycerol, having a well-studied metabolic pathway for 1,3-PDO production. Wild-type *K. pneumoniae* relies on two different metabolic pathways for growth on glycerol (Forage and Lin 1982). Under aerobic conditions, the *glp* regulon is expressed. Glycerol is converted to glycerol 3-phosphate by GlpK, which is then dehydrogenated to DHAP by GlpD. On the other hand, the *dha* regulon is expressed under anaerobic conditions. Because the production of 1,3-PDO from glycerol is generally conducted under anaerobic conditions, the *dha* regulon has been the focus of more attention than the *glp* regulon in *K. pneumoniae*. The *dha* regulon largely consists of reductive and oxidative pathways (Seo et al. 2010; Seo et al. 2009). In the reductive pathway, glycerol is converted to 1,3-PDO by the activities of the DhaB and DhaT enzymes. In a coupled oxidative pathway, glycerol is oxidized to DHA by DhaD and then DHA is phosphorylated to DHAP by DhaKLM, thereafter entering into the glycolysis pathway.

2.4.4 Glycerol Metabolism in Lactobacilli

Glycerol metabolism in lactobacilli is species-specific, depending upon the presence or absence of genes encoding specific enzymes as well as the conditions for their expression. *Lactobacillus rhamnosus* ATCC 7496 can use glycerol as the sole carbon source using GlpK activity under aerobic conditions. However, it cannot produce 1,3-PDO (Alvarez Mde et al. 2004); whereas, *Lactobacillus* species able to produce 1,3-PDO, including *L. reuteri* (Lüthi-Peng et al. 2002), *L. brevis* and *L. buchneri* (Veiga da Cunha and Foster 1992b), and *Lactobacillus hilgardii* (Pasteris and Strasser de Saad 2009), only possess the glycerol reductive pathway and cannot grow on glycerol. The sequences of the key genes in *dha* regulon (glycerol dehydrogenase, dihydroxyacetone kinase, glycerol dehydratase, and 1,3-PDO dehydrogenase) and *glp* regulon (glycerol uptake facilitator, glycerol kinase, and glycerol 3-phosphate dehydrogenase) were searched against 19 other lactobacillus strains released by the DOE Joint Genome Institute (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=findGenes) (Table 2.3). This *in silico* analysis revealed that no lactobacilli possess complete glycerol operons and genes for the oxidative and reductive pathways as seen in the case of *K. pneumoniae*. Both glycerol oxidative routes, GlpKD and DhaDKLM, were found in few lactobacilli of groups I and II (*L. salivarius* CECT 5713, *L. casei* ATCC334, and *L. rhamnosus* GG); however, previous studies

Table 2.3 Identification of the key genes of *glp* and *dha* operons in 19 lactobacilli strains

Group	Species and strains	Genes ID[a]						
		DhaD	**DhaKLM**	**DhaB**	**DhaT**	**GlpF**	**GlpK**	**GlpD**
I	*L. acidophilus* 30SC	N.F.	N.F.	N.F.	N.F.	650747275	650747596	650746299
	L. amylovorus GRL 1112	N.F.	N.F.	N.F.	N.F.	650724866 650726093	650726360	650725063
	L. crispatus ST1	N.F.	N.F.	N.F.	N.F.	646763141	646763494	646762261
	L. delbrueckii bulgaricus 2038	N.F.	N.F.	N.F.	N.F.	650390022 650391693	650391796	650390507 650391680
	L. gasseri ATCC 33323	N.F.	N.F.	N.F.	N.F.	639672338	N.F.	639673159
	L. helveticus DPC 4571	N.F.	N.F.	N.F.	N.F.		N.F.	641311351
	L. johnsonii DPC 6026	N.F.	N.F.	N.F.	N.F.	651137482	N.F.	651138262
	L. kefiranofaciens ZW3	N.F.	650903633 650903634 650903635 650905436 650905437	N.F.	N.F.	650905439	650904875	650904434
	L. ruminis ATCC 27782	2521929483	2521930803 2521930804 2521930805	N.F.	N.F.	2521930527 2521930806	N.F.	2521929748
	L. salivarius CECT 5713	648249771	648251113 648251114 648251115	N.F.	N.F.	648249841 648250178 648251112	648249839	648250052

Table 2.3 Identification of the key genes of *glp* and *dha* operons in 19 lactobacilli strains (cont'd)

Group	Species and strains	Genes ID[a]						
		DhaD	**DhaKLM**	**DhaB**	**DhaT**	**GlpF**	**GlpK**	**GlpD**
II	*L. casei* ATCC 334	639665798	639663396 639663397 639664554	N.F.	N.F.	639663622 639663760	639663624 639665567	639663623 639663904
	L. plantarum WCFS1	N.F.	N.F.	N.F.	637390007	637387512 637387564 637387740 637388425 637390301 637390319	637387738 637388146	637387739 637388081
	L. rhamnosus GG	645015149	645012727 645012729 645012730 645013882	N.F.	N.F.	645012863 645013005	645012865	645013082 645013140
	L. sakei sakei 23K	N.F.		N.F.	637794658	637795235	637795233	637795098 637795234
III	*L. brevis* ATCC 367	639654320	N.F.	N.F.	639654345	639653788 639654017 639654153	639652433 639653079	639652831
	L. buchneri NRRL B-30929	2505384017	N.F.	N.F.	2505382405	N.F.	2505382268 2505382855	2505383133
	L. fermentum CECT5716	N.F.	N.F.	N.F.	648249105	N.F.	648249327	648248848
	L. reuteri JCM 1112	642585505	N.F.	640591592	642583731	642585421	640590904	640590176
	L. sanfranciscensis TMW 1.1304	N.F.	N.F.	N.F.	N.F.	N.F.	2511696617 2511697062	2511696879

a. Each gene (ID) was searched in the DOE Joint Genome Institute (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindGenes&page=findGenes).
N.F. not found.

reported that *L. salivarius* CECT 5713 and *L. rhamnosus* GG could not ferment glycerol when provided as the sole carbon source (Martin et al. 2006; Saxelin 1997). In addition, although *L. reuteri* JCM 1112 possessed the glycerol oxidative (GlpKD) and reductive (DhaBT) routes, glycerol was only utilized as an electron acceptor in this strain when co-fermentated with glucose, producing 1,3-PDO (Santos et al. 2011). Thus, most of the annotated genes in lactobacilli may not function in actual glycerol metabolism, suggesting that lactobacilli cannot use glycerol as both energy and external electron acceptor sources at the same time. While massive amounts of sequence information of lactobacilli has been released to the public database, functional studies on this sequence information of glycerol metabolism in lactobacilli have not been investigated in detail to date.

2.5 New Candidate Strain, *Lactobacillus panis* PM1, for Glycerol Utilization

Generally, lactobacilli are common inhabitants of the human intestine and well-known for use as probiotics, and are considered as GRAS microorganisms. *Lactobacillus panis* PM1 is a natural 1,3-PDO-producing microorganism originally isolated from thin stillage (TS) (Khan et al. 2013). TS is a major by-product (approximately 85% of total volume) that remains after bioethanol production from various carbon-rich substrates (e.g., corn, barley, and wheat) by yeast fermentation and contains carbohydrates, organic compounds, protein, and various minerals. In addition, glycerol (2.4 to 7.9 g/L) is a major component in wheat-based TS (Ratanapariyanuch et al. 2011) from which *L. panis* PM1 was isolated. In a stored wheat-based TS sample, 1,3-PDO was spontaneously produced with the concurrent depletion of glycerol (Khan et al. 2013). Bacteria were isolated from TS and the isolated pure strains were inoculated into sterilized TS. After 1-week incubation, the TS was tested using ^{1}H-NMR for the conversion of glycerol to 1,3-PDO (Reaney et al. 2011). Subsequently, a non-pathogenic *Lactobacillus panis* strain (designated as PM1) capable of converting glycerol to 1,3-PDO was isolated (Khan et al. 2013). This strain would, therefore, appear to be a good bacterial candidate for a cell factory with potential for converting low-price glycerol to platform chemicals (e.g., 1,3-PDO).

2.5.1 1,3-PDO: a Platform Chemical from Low-price Glycerol

The compound 1,3-PDO is a platform chemical, and is widely utilized in industrial applications, including laminates, adhesives, resins, films, solvents, detergents, cosmetics, engine

coolants, fibers, and carpets (Kaur et al. 2012; Saxena et al. 2009). This chemical is receiving more attention due to its use as a monomer in manufacturing polytrimethylene terephthalate (PTT), a biodegradable polymer. The 1,3-PDO market was previously small; however, in 1995, the commercialization of a new PTT-based polyester by Shell Chemical Company significantly changed the market size (Saxena et al. 2009). Currently, a single manufacture (DuPont Tate & Lyle) dominates the 1,3-PDO market; however, other manufacturers (e.g., Metabolic Explorer and Huamei Biomaterial) are expected to enter in this market soon. These new entries will create new market opportunities for 1,3-PDO. The global 1,3-PDO market has been estimated to be U.S. $157 million in 2012 and is expected to grow by 15.7% annually until 2014 (MarketsandMarkets 2012). Conventionally, 1,3-PDO is produced by catalytic chemical reactions, such as hydration of acrolein (by Degussa) or hydroformylation of ethylene oxide (by Shell) to 3-HPA, followed by hydrogenation to 1,3-PDO, showing 40% and 80% yield, respectively (Cameron et al. 1998; Saxena et al. 2009). However, these conventional processes require high pressure, temperature, expensive catalysts, along with the release of toxic intermediates. Industry trends in 1,3-PDO production have moved from petroleum-based feedstock to renewable resources (e.g., glucose from corn and glycerol from biodiesel). Dupont Tate & Lyle currently produces 1,3-PDO from corn sugar by fermentation, which imposes expensive sugar usage. A lower cost renewable resource is broadly considered to be more appropriate for 1,3-PDO production. Thus, production of 1,3-PDO through microbial fermentation using low-price glycerol from biodiesel industries has been received attention as an alternative. This provides a promising option for utilization of the glycerol surplus mentioned in section 2.3.1. Bacteria able to convert glycerol to 1,3-PDO have been identified to include the genera *Klebsiella* (*K. pneumoniae* and *K. oxytoca*), *Enterobacter* (*E. agglomerans*), *Citrobacter* (*C. freundii*), *Clostridium* (*C. butyricum and C. pasteurianum*), and *Lactobacillus* (*L. brevis*, *L. reuteri*, and *L. buchneri*) (Kaur et al. 2012; Saxena et al. 2009). Recent studies on the bioconversion of crude glycerol to 1,3-PDO have mostly focused on two species, *K. pneumoniae* and *C. butyricum*. Mu et al. (2006) reported that fed-batch fermentations by *K. pneumoniae* produced 53 g/L of 1,3-PDD from crude glycerol obtained from soybean oil, and during fed-batch fermentation under non-sterile conditions; whereas, *C. butyricum* VPI strain 1718 could convert crude glycerol to 1,3-PDO at the concentration of 67.9 g/L and yield of 0.55 g/g (Chatzifragkou et al. 2011).

2.5.2 1,3-PDO Fermentation by *Lactobacillus*

A few lactobacilli, including *L. brevis*, *L. reuteri*, and *L. buchneri*, have been reported as natural 1,3-PDO producers. These bacteria can convert glycerol to 1,3-PDO via their glycerol reductive pathway during cofermentation of glucose and glycerol. In the 1990s, 1,3-PDO production by *Lactobacillus* was mostly studied as a part of understanding of the glycerol reductive pathway; i.e., characteristics of enzymes comprising the reductive pathway (Veiga da Cunha and Foster 1992a), regulatory mechanism and role of the reductive pathway (Veiga da Cunha and Foster 1992b), and factors affecting the reductive pathway (De Valdez et al. 1997). In the 2000s, this reductive pathway was found and characterized in few more *Lactobacillus*, including *L. hilgardii* (Pasteris and Strasser de Saad 2009), *L. collinoides* (Claisse and Lonvaud-Funel 2000; Garai-Ibabe et al. 2008; Sauvageot et al. 2000), and *L. coryniformes* (Martin et al. 2005); however, a major focus still was the production of 3-HPA and not 1,3-PDO. These bacteria were commonly isolated from spoiled alcoholic beverages (e.g., an unpleasant bitter taste); thus, most of the studies dealt with mechanism of formation and factors affecting the accumulation of 3-HPA, a precursor of acrolein that causes actual bitter tastes. Recently, 1,3-PDO production by *Lactobacillus* species has received more interest due to the market trends of crude glycerol and 1,3-PDO, as mention in sections 2.3.1 and 2.5.1. A high amount of 1,3-PDO production (84.5 g/L) was achieved in a fed-batch fermentation by *Lactobacillus diolivorans*, suggesting the feasibility of the industrial utilization of this strain (Pflügl et al. 2012). In a further study, the authors attempted to use crude glycerol from biodiesel production from palm oil and lignocellulosic hydrolysate, instead of purified glycerol and glucose, to reduce the material costs. Fed-batch fermentation by *L. diolivorans* successfully achieved a high amount of 1,3-PDO production (75 g/L) under more economic conditions than before, suggesting the feasibility of 1,3-PDO production by this strain under actual industrial conditions (Pflügl et al. 2014).

2.6 Connection to the Studies in This Thesis

As discussed above, recent trends in the biofuel sector have provided impetus for the development of new applications of crude glycerol, and the glycerol reductive pathway of *Lactobacillus panis* PM1 would be a good target for potential applications of low-price glycerol. In this regard, an improved physiological knowledge of *L. panis* PM1, including its metabolism (e.g., pathway elucidation and redox balance), genetics, and fermentation conditions, would

clearly aid in the utilization of this strain as a host for industrial conversion of low-price glycerol to platform chemicals (e.g., 3-HPA and 1,3-PDO). To achieve these goals, this thesis was designed with three major foci: (i) elucidation of central metabolic pathway details operative in *L. panis* PM1, including how they are regulated (Chapters 3 and 4), (ii) understanding the types of auxiliary metabolic pathways that function in *L. panis*, and how they enable or support central metabolism during adaptation of PM1 to various environmental conditions (Chapters 5, 6, 7, and 8), and (iii) metabolic engineering of *L. panis* PM1 with the goal of improving the understanding of metabolic pathways during production of value-added chemicals from glycerol in thin stillage (Chapters 9 and 10).

3. CHARACTERIZATION OF NOVEL 1,3-PROPANEDIOL-PRODUCING BACTERIUM, *LACTOBACILLUS PANIS* PM1[i]

3.1 Abstract

This study was conducted to understand metabolic characterisistics of *Lactobacillus panis* PM1, a new 1,3-propanediol (1,3-PDO) producing strain isolated from thin stillage (TS), a major by-product after bioethanol production. Strain PM1 showed the same 16S rRNA sequence and carbohydrate fermentation pattern as *L. panis* strain DAF 335 which is classified into the group III heterofermentative lactobacilli. Glucose fermentation by the PM1 strain yielded acetate, lactate, and ethanol as major end-products, which is the typical heterolactic fermentation via the 6-phosphogluconate/phosphoketolase pathway in group III lactobacilli. *Lactobacillus panis* PM1 produced 1,3-PDO from glycerol in co-fermentation of glucose; however, this strain could not ferment glycerol and its intermediates on the glycerol oxidative pathway (glycerol to dihydroxyacetone phosphate) when supplied as the sole carbon source, suggesting that the glycerol reductive pathway (glycerol to 1,3-PDO) was the only route for glycerol metabolism. The presence of glycerol led to a decrease in ethanol and an increase in acetate along with 1,3-PDO production. These metabolic characteristics strongly suggested that NADH recycling for glucose fermentation shifted from ethanol production to 1,3-PDO production, which contributed to additional ATP generation. All of the experiments contained in this chapter were performed by Tae Sun Kang.

[i] A version of this chapter has been published: Nurul H. Khan, Tae Sun Kang, Douglas A. S. Grahame, Monique C. Haakensen, Kornsulee Ratanapariyanuch, Martin J. Reaney, Darren R. Korber, Takuji Tanaka. 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. This chapter is reproduced in this thesis with the permission of Springer.

3.2 Introduction

Thin stillage (TS) is a major fermentation residue from bioethanol production by yeast fermentation and contains a variety of complex nutrients, including various carbohydrates, minerals, and amino acids. Regardless of feedstock (i.e., sugar cane or corn), significant amounts of glycerol (up to 2%) are a key feature of TS (Clomburg and Gonzalez 2013). A patent by Reaney et al. (2011) reported that a stored bioethanol TS sample spontaneously produced 1,3-propanediol (1,3-PDO) with the concurrent depletion of glycerol. Subsequently, a non-pathogenic *Lactobacillus panis* strain (designated as PM1) capable of converting glycerol to 1,3-PDO was isolated by Khan et al. (2013).

The biological production of 1,3-PDO from glycerol is well documented in various genera, including *Klebsiella*, *Citrobacter*, *Clostridium*, *Enterobacter*, and *Lactobacillus* (Saxena et al. 2009). Among these bacteria, *Clostridium butyricum* and *Klebsiella pneumoniae* are reported as the best natural 1,3-PDO producers (Gonzalez-Pajuelo et al. 2005; Saxena et al. 2009; Zhang et al. 2006). In these bacteria, glycerol is mainly metabolized by three pathways (Figure 3.1). A glycerol oxidative pathway converts glycerol into dihydroxyacetone phosphate (DHAP) using glycerol dehydrogenase (DhaD) and dihydroxyacetone kinase (DhaK) under anaerobic conditions; whereas aerobic conditions activate another oxidative route in which glycerol is phosphorylated with glycerol kinase (GlpK) into glycerol 3-phosphate (G3P), and G3P dehydrogenase (GlpD) converts it into DHAP. The DHAP produced by the glycerol oxidative routes is a key intermediate of glycolysis for energy metabolism, redox balance, and metabolite synthesis. On the other hand, the third pathway, a reductive route, mostly serves as an electron sink under fermentative conditions (Clomburg and Gonzalez 2013). In the reductive route, glycerol is converted to 3-hydroxypropionaldehyde (3-HPA) by coenzyme B_{12}-dependent glycerol dehydratase (DhaB). The compound 3-HPA is further reduced by the NADH-dependent enzyme, 1,3-PDO dehydrogenase (DhaT), to 1,3-PDO, thereby regenerating NAD^+.

The glycerol reductive pathway is reported in a few lactobacilli, including *L. brevis*, *L. reuteri*, and *L. buchneri*, and are capable of producing 1,3-PDO (Lüthi-Peng et al. 2002; Veiga da Cunha and Foster 1992a). These bacteria belong to the group III heterofermentative lactobacilli that metabolize glucose via the 6-phosphogluconate/phosphoketolase (6-PG/PK)

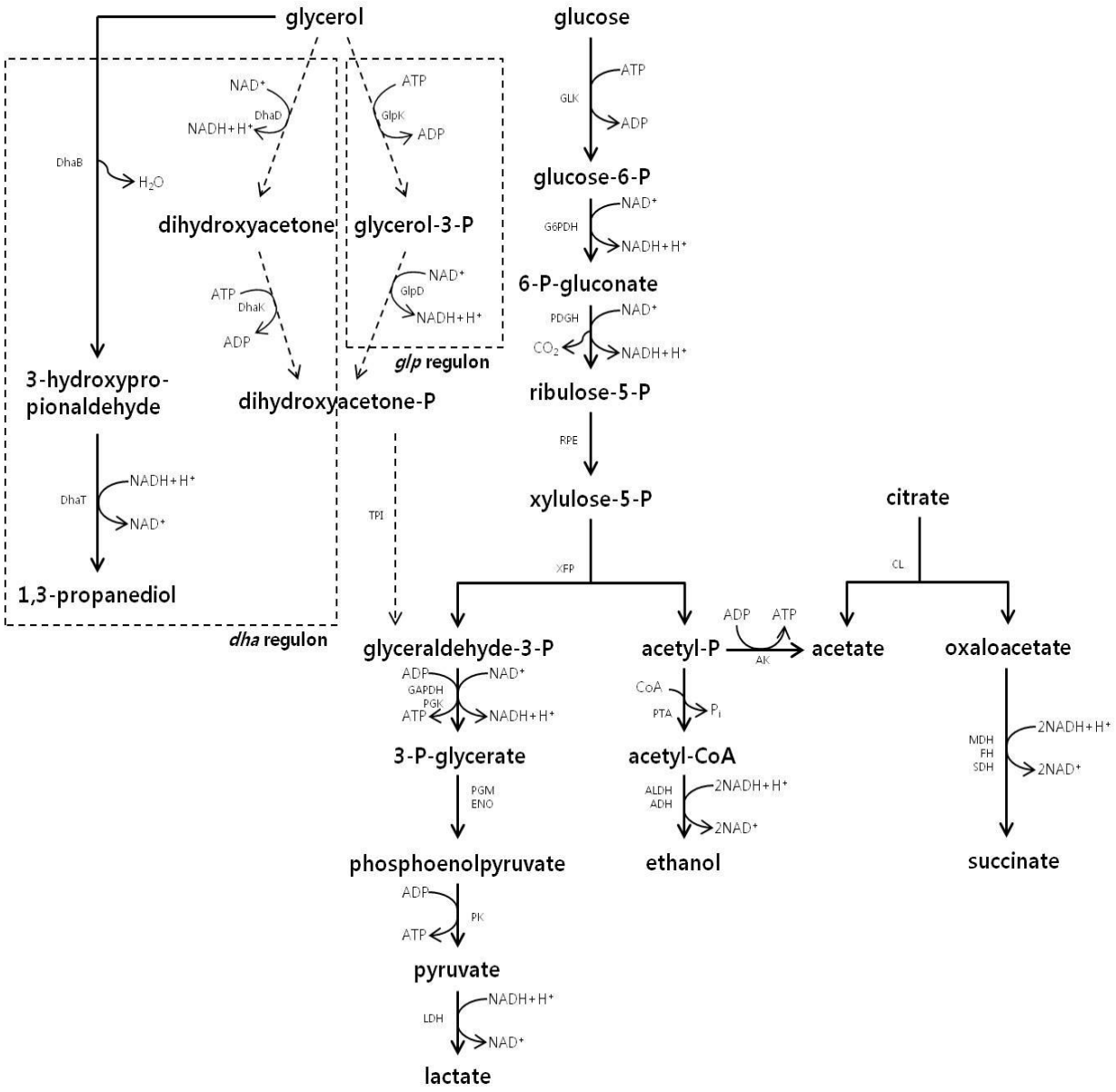

Figure 3.1 Schematic diagram of the glycerol and glucose metabolic pathways in *L. panis* PM1. Dashed lines, missing pathway elements in *L. panis* PM1. Abbreviation, ADH, alcohol dehydrogenase; AK, acetate kinase; ALDH, acetaldehyde dehydrogenase; CL, citrate lyase; CoA, coenzyme A; DhaB, glycerol dehydratase; DhaD, glycerol dehydrogenase; DhaK, dihydroxyacetone kinase; DhaT, 1,3-propanediol dehydrogenase; ENO, enolase; FH, fumarate hydratase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLK, glucokinase; GlpD, glycerol 3-phosphate dehydrogenase; GlpK, glycerol kinase; G6PDH, glucose 6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PGDH, 6-phosphogluconate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglyceromutase; PK, pyruvate kinase; PTA, phosphotransacetylase; RPE, ribulose 5-phosphate 3-epimerase; SDH, succinate dehydrogenase; TPI, triosephosphate isomerase; and XFP, xylulose 5-phosphate phosphoketolase.

pathway, theoretically producing 1 mol each of lactate, ethanol, and CO_2 and 1 mol ATP per mol glucose consumed. In group III bacteria, glycerol cannot be used as the sole carbon source; however, the presence of glycerol can reoxidize NADH in conjunction with the ethanol production pathway, and the phosphoketolase/glycerol reductive pathway can generate one or two extra ATP per glucose (Figure 3.1) (Zaunmuller et al. 2006).

In this study, physiological characteristics of this newly-isolated PM1 strain were investigated and its 1,3-PDO-producing ability (from glycerol) was confirmed. For an improved understanding of glucose and glycerol metabolism, end-product analysis methods were developed, and the primary carbohydrate and glycerol reductive pathways were elucidated based on metabolite profiles.

3.3 Materials and Methods

3.3.1 Bacterial Strains and Growth Conditions

Lactobacillus panis PM1 strain was isolated from bioethanol thin stillage (Khan et al. 2013) and has been deposited under accession number 180310-01 at the International Depository Authority of Canada, National Microbiology Laboratory, Public Health Agency of Canada (IDAC, NML-HCCC; Winnipeg, MB, Canada) under the provision of the Budapest Treaty. Strain PM1 was pre-cultured at 37 ℃ under microaerobic conditions until late log phase using commercial MRS medium (BD, Franklin Lakes, NJ, USA), at which time 1% (v/v) of this pre-culture was transferred into fresh modified MRS (mMRS) medium. The mMRS medium consisted of 5 g yeast extract, 10 g peptone, 10 g meat extract, 2 g K_2HPO_4, 2 g ammonium citrate, 5 g sodium acetate, 100 mg $MgSO_4 \cdot 7H_2O$, 50 mg $MnSO_4$, and a defined concentration of carbon sources (sugars, glycerol, or glycerol pathway intermediates) per liter. The cultures were incubated at 37 ℃ under microaerobic conditions, unless otherwise stated. Air-tight 15-mL tubes, filled to the two-thirds level, were incubated under static conditions to establish microaerobic conditions. For qualitative analyses, *L. panis* PM1 and *L. reuteri* DSM20016 (as a positive control) were cultured by the two-step fermentation method (Lüthi-Peng et al. 2002). Briefly, in the first step, both bacteria were cultured in MRS medium for 24 h. Cells were harvested and then washed with phosphate buffer (pH 7.0) twice. The washed cells were then resuspended in mMRS medium containing either glycerol (220 mM) or glucose (10 mM) and glycerol (220

mM), while the resuspension was kept under microaerobic conditions at 37℃ for 4 h. For conversion kinetics of glycerol, *L. panis* PM1was cultured mMRS media supplemented with 55 mM glucose and 41, 81, 163, and 326 mM glycerol. To evaluate the effect of carbon source on PM1 growth, citrate, fructose, galactose, glucose, lactose, raffinose, or sucrose was added at 20 mM to the mMRS broth. As a negative control, mMRS broth was used without any additional carbohydrate. The ability to use glycerol or its metabolites (glycerol 3-phosphate, dihydroxyacetone and dihydroxyacetone phosphate) was examined using mMRS supplemented with 2% (w/v) of one of these carbon compounds as the sole carbon source.

3.3.2 Qualitative and Quantitative Analyses of Metabolites in Culture Media

The culture optical density at 600 nm (OD_{600}) was measured as an index of growth with a DU 800 spectrophotometer (Beckman Coulter, Mississauga, ON, Canada). After centrifugation, the supernatant was filtered through a 0.22-µm-pore-size filter and stored at -20 ℃ for ^{1}H NMR and HPLC analyses. The qualitative analysis of end-products in the culture broth was conducted by double-pulsed field gradient spin echo nuclear magnetic resonance (DPFGSE NMR) using a 500-MHz ^{1}H NMR (Model AMX; NMR Bruker, Mississauga, Canada) at the Saskatoon Structural Sciences Center (University of Saskatchewan), according to the method of Ratanapariyanuch et al. (2011). A 500-µL aliquot of the filtered supernatant was mixed with 50 µL of deuterium oxide (D_2O). The DPFGSE was utilized to mitigate the strong singlet peaks of water. The DPFGSE 1H NMR (1D) spectra were recorded on the 500-MHz unit for metabolite analysis. Concentrations of citrate, glucose, fructose, erythritol, succinate, lactate, glycerol, 3-HPA, acetate, 1,3-PDO, and ethanol were quantitatively determined using high-performance liquid chromatography (HPLC) using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector. Filtered culture medium (40 µL) was loaded on an Aminex HPX-78H analytical column (Bio-Rad, Hercules, CA, USA), and eluted with 5 mM sulfuric acid at a flow rate of 0.6 mL/min at 55 ℃; the elution was observed for 30 min. Identification of the potential metabolites was determined by comparing retention times with ACS-grade reagents.

3.4 Results and Discussion

3.4.1 Carbohydrate Fermentation by *L. panis* PM1

Previously, three different strains (DAF1, DAG76, and DAF 355) of *L. panis* have been reported by Pederson et al. (2004), and their 16S rRNA sequences were identical to the PM1 strain. However, differences in fermentation of carbohydrates were observed among these *L. panis* strains (Khan et al. 2013; Pedersen et al. 2004). *Lactobacillus panis* PM1 showed the same sugar fermentation pattern to that of strain DAF 355 (no growth on fructose or sucrose); whereas the other two strains could grow on both sugars, suggesting that the PM1 strain was most closely related to the DAF 335 (Figure 3.2). In addition, carbon sources affected the degree of growth of the PM1 strain. The cell densities of *L. panis* PM1 cultures grown on glucose, galactose, and raffinose were similar (OD_{600} of 0.7 to 0.9 at 48 h); whereas the cell density of cultures grown on lactose was almost double at 48 h (1.8 OD_{600}) compared with the other sugars (Figure 3.2). However, glycerol and its direct metabolites, glycerol 3-phosphate, dihydroxyacetone and dihydroxyacetone phosphate, could not support the growth of the PM1 strain (Khan et al. 2013).

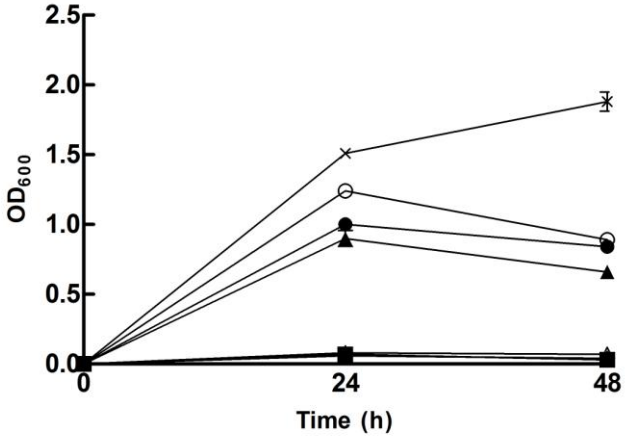

Figure 3.2 Growth of *L. panis* PM1 in mMRS media containing various carbohydrate sources at 37°C under microaerobic conditions. Media (mMRS) supplemented with carbohydrates (20 mM): no addition (plus symbols), citrate (white squares), fructose (black squares), galactose (black triangles), glucose (white circles), lactose (times symbols), raffinose (black circles), or sucrose (white triangles).

3.4.2 Confirmation of *L. panis* PM1 as a 1,3-PDO-Producing Bacterium

The ability of *L. panis* PM1 to produce 1,3-PDO was determined by ^{1}H-NMR. ^{1}H-NMR spectra of two-step fermentation samples taken from *L. panis* PM1 and *L. reuteri* DSM20016 cultures showed increased peak areas with chemical shifts of ethanol ($\underline{CH_3}$-CH_2-OH, 1.00 to 1.04 ppm), lactate ($\underline{CH_3}$-CHOH-COOH, 1.18 to 1.22 ppm), 1,3-PDO (OH-CH_2-$\underline{CH_2}$-CH_2-OH, 1.60 to 1.66 ppm), and acetate ($\underline{CH_3}$-COOH, 1.86 to 1.90 ppm), when compared with the spectrum of the time-zero sample (Figure 3.3A and B). However, in mMRS media containing either glycerol or glucose as the sole carbon source, the peaks corresponding for 1,3-PDO were not detected (Figure 3.3C and D). Glucose fermentation by the PM1 strain produced peaks for ethanol, lactate, and acetate as major end-products, which is the typical pattern of group III lactobacilli, including *L. reuteri*, *L. buchneri*, and *L. brevis* (Veiga da Cunha and Foster 1992a). Thus, glycerol was only converted to 1,3-PDO in the presence of glucose, suggesting the absence of the glycerol oxidative pathway in *L. panis* PM1.

3.4.3 Optimal Conditions for Quantitative Analysis

Quantitative analysis of glucose and/or glycerol fermentation by *L. panis* PM1 was performed by an HPLC method. Under optimized condition, good separation and selectivity for a total of 11 standard chemicals, spiked into mMRS without carbon sources (e.g., glucose and glycerol), were obtained. The retention times of citrate, glucose, fructose, erythritol, succinate, lactate, glycerol, 3-HPA, acetate, 1,3-PDO, and ethanol were 8.5, 9.2, 10.1, 11.8 12.3, 13.1, 13.5, 14.5, 15.5, 17.5, and 21.7 min, respectively. Precision and accuracy of all standards were within 0.5% to 8.8% and 80.4% to 125.5%, respectively, over a concentration range of 0.5 to 5.0 mg/mL, indicating that the validation of this method was excellent. Recovery was examined by analysis of three test samples that had been prepared by adding 200 µL of mixed standard solution containing 0.5, 1.0 and 2.0 mg/mL of each compound to 800 µL of a culture sample. Recovery rates of all standards was between 92.7% and 119.5% except for glucose (123.8% in 1.0 mg/mL), which is within acceptable limits. Relationships between peak area and concentration for the standards were examined under the optimized condition, and linear regression equations, correlation coefficients (R^2), and detection limits are summarized in Table 3.1.

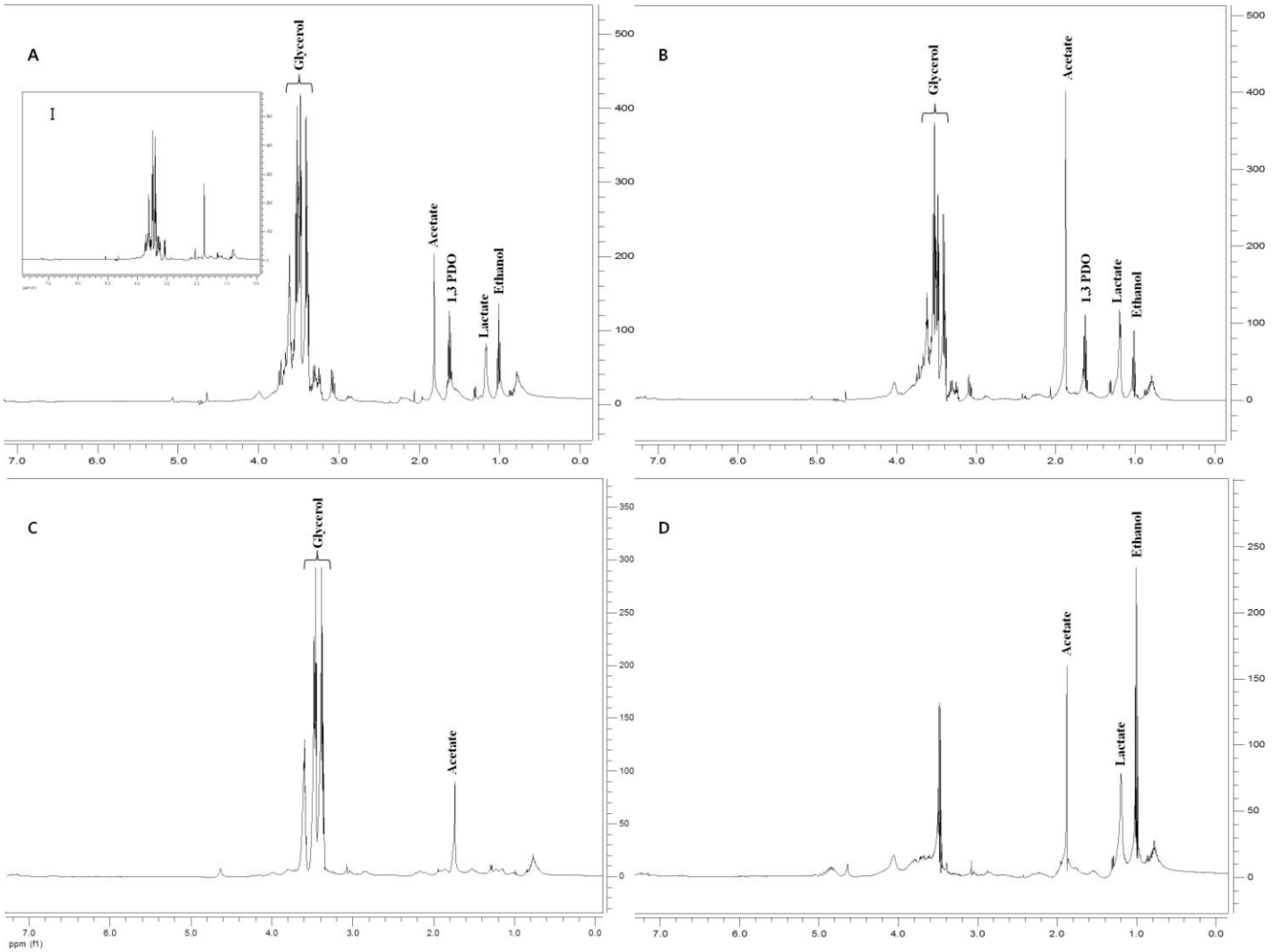

Figure 3.3 ^{1}H-NMR spectra of two-step fermentation samples (4 h) obtained from *L. panis* PM1 (A) or *L. reuteri* DSM20016 (B) cultured in media containing glucose and glycerol. The insert "I" corresponds to the ^{1}H-NMR spectrum of the time-zero sample. C and D represent ^{1}H-NMR spectra of two-step fermentation samples obtained from *L. panis* PM1 cultured in media containing either glycerol or glucose as the sole carbon source. 1.00 to 1.04 ppm for ethanol (CH_3), 1.18 to 1.22 ppm for lactate (CH_3), 1.60 to 1.66 ppm for 1,3-PDO (CH_2), and 1.86 to 1.90 ppm for acetate (CH_3).

Table 3.1 The results of regression equations, correlation coefficients, and detection limits for 11 standards

Compounds	Regression Equation	Correlation Coefficient (R^2)	Linear Range (mg/mL)	Retention Time (min)
Citrate	y = 520323x - 3543	1	0.2-20	8.5
Glucose	y = 585569x + 24642	0.9999	0.2-20	9.2
Fructose	y = 510115x + 37181	0.9964	0.2-20	10.1
Erythritol	y = 544348x + 5718	0.9992	0.2-20	11.8
Succinate	y = 421818x + 14308	0.9999	0.2-20	12.3
Lactate	y = 365744x - 10880	1	0.2-20	13.1
Glycerol	y = 461104x + 3978	1	0.2-20	13.5
3-HPA	y = 22465x + 35037	0.9971	0.47-4.59	14.5
Acetate	y = 270161x - 12145	1	0.2-20	15.5
1,3-PDO	y = 411972x + 2195	1	0.2-20	17.5
Ethanol	y = 228282x - 17732	0.9999	0.2-20	21.7

3.4.4 Conversion Kinetics of Glucose and/or Glycerol by *L. panis* PM1

Under microaerobic conditions, initially-available glucose (55 mM) was converted into almost the same amount of ethanol (55 mM) and lactate (53 mM) by *L. panis* PM1. After available glucose depletion (48 h), lactate was reduced from 53 to 33 mM; whereas acetate still increased to 84 mM (Figure 3.4A). Another key feature of glucose fermentation by *L. panis* PM1 was succinate production. Succinate was converted from citrate, a MRS medium component from ammonium citrate as described in section 3.3.1, which was not observed in the most closely-related strain, *L. reuteri* DSM 20016 (Khan et al. 2013). The production of 1,3-PDO by *L. panis* PM1 was accompanied by the consumption of glycerol, as well as glucose, which resulted in the production of more acetate (84 to 99 mM) and less ethanol (55 to 29 mM) compared with cells grown in glucose medium (Figure 3.4B).

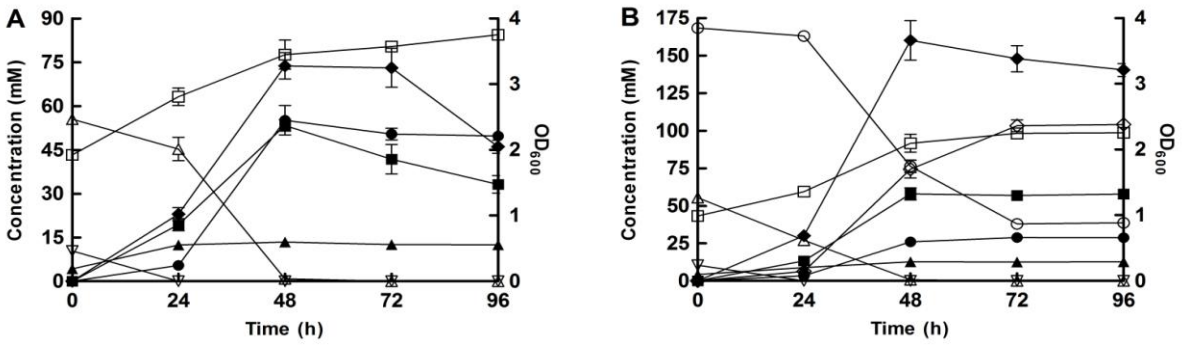

Figure 3.4 Concentration change of metabolites during *L. panis* PM1 culture. *Lactobacillus panis* PM1 was inoculated to mMRS medium amended with either 55 mM glucose (A) or 55 mM glucose and 168 mM glycerol (B). The concentrations of glycerol (white circles), glucose (white up-pointing triangles), ethanol (black circles), acetate (white squares), lactate (black squares), succinate (black up-pointing triangles), 1,3-PDO (white diamonds), and citrate (white down-pointing triangles) are provided on the left-hand *y*-axis, and the cell density (black diamonds) is provided on the right-hand *y*-axis.

However, the production of the other metabolites, lactate and succinate, was comparable under both conditions. Glycerol concentrations equal to 81 mM were a limiting factor for 1,3-PDO conversion under 55 mM glucose conditions; whereas production of 1,3-PDO was dependent of glucose concentration at glycerol concentrations above 163 mM (Table 3.2).

Table 3.2 Glycerol consumption and 1,3-PDO production by *L. panis* PM1 at 72 h

	Initial glycerol concentration (mM)			
	326	**163**	**81**	**41**
Final glycerol concentration (mM)	178.41±2.23	35.50±0.16	N.D.	N.D.
Final 1,3-PDO concentration (mM)	96.86±0.68	104.22±0.48	69.44±1.98	42.93±0.38

N.D., not detected.

Lactobacillus panis PM1 was isolated from TS and reported to be a natural 1,3-PDO-producer (Khan et al. 2013). In this study, physiological characteristics of this strain were investigated with an emphasis on interpreting possible metabolic pathways associated with 1,3-PDO production. The growth pattern observed in Figure 3.2 strongly suggested that the new isolate, PM1, belonged to *L. panis* and was most similar to the DAF 335 strain. The maximum OD_{600} of the PM1 strain in mMRS containing lactose (20 mM) was twice as high as in the media containing 20 mM glucose, galactose, or raffinose after 48 h, which can be explained by the composition of the carbon source. Lactose is a disaccharide formed from galactose and glucose, and raffinose is a trisaccharide composed of galactose and sucrose. Since sucrose is a carbon

compound that strain PM1 cannot efficiently utilize, only the galactose moiety of raffinose could be used for growth; whereas both sugar moieties of lactose are metabolizable. As a result, lactose has twice the carbon source available for utilization by the PM1 strain.

The HPLC method developed in this study was useful for the simultaneous determination and quantification of citrate, glucose, fructose, erythritol, succinate, lactate, glycerol, 3-HPA, acetate, 1,3-PDO, and ethanol in fermentation samples. End-product patterns from glucose and/or glycerol fermentation by *L. panis* PM1 were analyzed using this method. When *L. panis* PM1 was cultured in mMRS containing glucose, the major fermentation metabolites consisted of acetate, lactate, and ethanol along with the production of carbon dioxide. The analysis of metabolites indicated that *L. panis* PM1 could produce 1,3-PDO from glycerol during co-fermentation with glucose. The conversion of glycerol to 1,3-PDO was dependent on glucose concentration (Table 3.2) and affected the production of acetate and ethanol (Figure 3.4B). These metabolic characteristics of *L. panis* PM1 were similar to previously-reported heterofermentative lactobacilli strains, including *L. reuteri, L. brevis*, and *L. buchneri* (Lüthi-Peng et al. 2002; Veiga da Cunha and Foster 1992a). *Lactobacillus panis* PM1 could not utilize substances on the glycerol oxidative pathway (i.e., glycerol 3-phosphate, dihydroxyacetone, and dihydroxyacetone phosphate). This result suggests that missing pathways (GlpKD and DhaDK) were the reason that *L. panis* PM1 could not use glycerol as the sole carbon source (Figure 3.1). Combined with the sugar utilization results, it is concluded that strain PM1 belongs to the group III heterofermentative lactobacilli that utilize the 6-PG/PK pathway for central sugar metabolism (producing acetate, lactate, and ethanol), and where glycerol is used as an electron acceptor (producing 1,3-PDO).

Like some group III lactobacilli, strain PM1 can produce 1,3-PDO via the glycerol reductive pathway under anaerobic conditions. This strain was able to only utilize glycerol with the aid of a fermentable sugar like glucose that provided NADH via the 6-PG/PK pathway. While glucose was observed to become depleted within 48 h, 1,3-PDO increased until 72 h. Little additional production of 1,3-PDO was observed after 72 h. This suggests that NADH accumulation by glucose metabolism is a key factor in 1,3-PDO production, since glucose consumption yields reducing power (in the form of excess NADH). These observations explain why 1,3-PDO conversion under higher glycerol levels than glucose is ultimately dependent on glucose concentration (Table 3.2).

Primary metabolism of group III lactobacilli produces 3 NADH when a glucose molecule is used to produce ATP (Figure 3.1). These NADH are generally processed via ethanol and lactate production in the absence of external electron acceptors. The production of 1,3-PDO is utilized to regenerate NAD^+ in the presence of glycerol, producing more acetate (Figure 3.4B). This suggested an advantage of glycerol utilization, since acetate formation is associated with ATP production (Figure 3.1). Instead of using acetyl phosphate for ethanol production, the presence of glycerol may allow the scavenging of extra energy along with simultaneous oxidation of NADH. In addition, *L. panis* PM1 produced succinate from citrate, and this conversion has been reported as a main NAD^+ regeneration route in a few lactobacilli (Kaneuchi et al. 1988; McFeeters and Chen 1986). Thus, this data suggests the presence of another NAD^+ regeneration route in *L. panis* PM1.

3.5 Conclusions

Lactobacillus panis PM1 belongs to group III heterofermentative lactobacilli. The 6-PG/PK pathway is a main metabolic route for glucose fermentation, and glycerol is metabolized via only the glycerol reductive pathway. Thus, *L. panis* PM1 is not able to utilize glycerol as the sole carbon source for growth and survival; however, the production of 1,3-PDO contributes to NAD^+ regeneration and additional ATP generation.

3.6 Connection to the Next Study

This initial investigation of *L. panis* PM1 demonstrated that the 6-PG/PK pathway is the primary metabolism route enabling this strain to ferment various sugar as carbon sources; however, this strain cannot ferment fructose, a sugar widely utilized in microbial fermentation, in contrast to typical group III lactobacilli. This inability of fructose fermentation suggests the central metabolism of strain PM1 (the 6-PG/PK pathway) is different from typical group III lactobacilli. Thus, the following Chapter (Chapter 4) investigated differences in metabolic pathways and end-products that were formed during fructose fermentation by strain PM1. The intent of these analyses was to elucidate unique aspects of PM1 metabolism from other group III lactobacilli, while providing clues for further engineering work to utilize this strain for glycerol conversion.

4. REGULATION OF DUAL GLYCOLYTIC PATHWAYS FOR FRUCTOSE METABOLISM IN HETEROFERMENTATIVE *LACTOBACILLUS PANIS* PM1[ii]

4.1 Abstract

Lactobacillus panis PM1 belongs to the group III heterofermentative lactobacilli that use the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway as their central metabolic pathway and is reportedly unable to grow on fructose as a sole carbon source. In this study, following extended culture of the PM1 strain, three growth patterns were observed on fructose medium: full growth, half growth and little growth. The cells showing these growth patterns were identified as the same PM1 strain based on sequences of genes necessary for central metabolism and 16*S* RNA. Accordingly, PM1 strain growth on fructose medium was further characterized to delineate how fructose was metabolized. The end-product pattern was different from what is expected in typical group III lactobacilli using the 6-PG/PK pathway (i.e., strain PM1 produced more lactate, less acetate, and no mannitol). In addition, *in silico* analysis revealed the presence of genes encoding most of critical enzymes in the Embden-Meyerhof (EM) pathway. Fructose metabolism in the PM1 strain was influenced by the activities of two enzymes, triosephosphate isomerase (TPI) and glucose 6-phosphate isomerase (PGI). A lack of TPI resulted in the intracellular accumulation of dihydroxyacetone phosphate (DHAP) in PM1, the toxicity of which caused early growth cessation during fructose fermentation. The activity of PGI was enhanced by the presence of glyceraldehyde 3-phosphate (GAP), which allowed additional fructose to enter into the 6-PG/PK pathway to avoid toxicity caused by DHAP. Exogenous TPI gene expression shifted fructose metabolism from that of a heterolactic to homolactic fermentation, indicating that TPI enabled the PM1 strain to mainly use the EM pathway for fructose fermentation. These findings clearly demonstrated that the balance in the accumulation of GAP and DHAP determines the fate of fructose metabolism and the activity of TPI plays a critical role during fructose fermentation via the EM pathway in *L. panis* PM1.

[ii] Tae Sun Kang, Darren R. Korber, and Takuji Tanaka. 2013. Regulation of dual glycolytic pathways for fructose metabolism in heterofermentative *Lactobacillus panis* PM1. Applied and Environmental Microbiology 79(24):7818-7826. This chapter is reproduced in this thesis with the permission of American Society for Microbiology.

4.2 Introduction

Lactobacilli generate metabolic energy (i.e., ATP) mainly by substrate-level phosphorylation using relatively simple routes: the Embden-Meyerhof (EM) pathway and/or the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway. The members of genus *Lactobacillus* are subdivided into three groups according to their fermentative characteristics on the basis of the presence of these pathways (Kandler 1983; Stiles and Holzapfel 1997). Group I lactobacilli are obligate homofermentative lactobacilli that exclusively ferment hexose sugars (e.g., glucose) to lactate via the EM pathway; however, they do not metabolize pentose sugars or gluconate. Group II lactobacilli are the facultative heterofermentative lactobacilli that ferment hexoses to lactate via the EM pathway. They can also ferment pentose sugars using an inducible phosphoketolase, producing lactate and acetate, and they can yield carbon dioxide from gluconate but not from glucose. Group III lactobacilli are the obligate heterofermentative lactobacilli that ferment hexoses to lactate, ethanol and/or acetate, and carbon dioxide, which is a distinct feature of the group III lactobacilli. Group III lactobacilli exclusively use the 6-PG/PK pathway to achieve this metabolism.

Fructose, an abundant hexose sugar found in many plants, is one of the main monosaccharides for bacteria growth in most ecosystems associated with plants and in lactobacilli is typically fermented by the two metabolic pathways described above (Saha and Racine 2011; Wisselink et al. 2002). During homofermentative fructose metabolism in group I and II lactobacilli, fructose is converted by the phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase system (PTS) and 6-phosphofructokinase (PFK) to fructose 1,6-diphosphate (FDP) and is then split by a fructose 1,6-diphosphate aldolase (FBA) into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). Triosephosphate isomerase (TPI) isomerizes DHAP into GAP, which is then further metabolized into lactate with the production of ATP. These enzymes comprise the EM pathway. In typical fructose fermentation by group III lactobacilli, fructose is taken up by fructose permease, and fructokinase (FK) and glucose 6-phosphate isomerase (PGI) convert the assimilated fructose to glucose 6-phosphate (G6P), which is further metabolized via the 6-PG/PK pathway, producing two intermediates: GAP and acetyl phosphate. GAP further follows the same metabolism as it does in the EM pathway and produces ATP, while acetyl phosphate is metabolized into either acetate or ethanol. Theoretically, fructose

metabolism via the 6-PG/PK pathway has a low energy yield (1 ATP molecule per fructose molecule) compared to that of the EM pathway (2 ATP molecules per fructose molecule) (Heinrich et al. 1997). However, many group III lactobacilli can compensate for this disadvantage by using fructose not only as a carbon source but also as an electron acceptor, producing mannitol by a NAD^+ mannitol dehydrogenase (Poolman 1993). This mannitol pathway allows NAD^+ regeneration as an alternative to ethanol production. As a result, a part of the acetyl phosphate is redirected to acetate production, along with yielding one extra ATP.

Lactobacillus panis PM1 has been described as a group III lactobacillus that uses the 6-PG/PK and various external electron acceptor pathways (e.g., glycerol, citrate, and oxygen) for its central metabolism and NADH recycling system (Kang et al. 2013a; Kang et al. 2013b; Kang et al. 2013c), and its inability to grow on fructose medium has been reported in Chapter 3. In this study, growth of the PM1 strain on fructose as the sole carbon source was sporadically observed. This growth on fructose revealed characteristics distinct from those of typical group III lactobacilli: sporadic growth, no mannitol but erythritol, less acetate, and more lactate production. Possible fructose metabolism and its regulation were studied using *in silico* and metabolic profile analyses, enzyme assays, and the introduction of an *Escherichia coli* TPI gene into *L. panis* PM1.

4.3 Materials and Methods

4.3.1 Bacterial Strains and Growth Conditions

Lactobacillus panis PM1 was cultured in modified MRS (mMRS) medium at 37 ℃ under microaerobic conditions as described in section 3.3.1, unless otherwise stated. *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, CA, USA) cells were used for cloning and preparation of the target plasmid, and cultured in LB medium at 37 ℃ with vigorous shaking. Concentrations of erythromycin (Em) for selective plating were 10 μg/mL and 300 μg/mL for *L. panis* PM1 and *E. coli*, respectively.

4.3.2 Preparation of Crude Cell Extracts

Lactobacillus panis PM1 cells grown in mMRS containing different carbon sources (fructose and/or glucose) were harvested by centrifugation, washed with 100 mM phosphate buffer (pH 7.0), and the cells in pellets then disrupted using sonication: three times for 1 min with a 3 min rest interval at output level 2 using a Sonifier 450 (Branson, CT, USA) using the

same buffer. Crude cell extract was obtained by centrifugation for 10 min at 16,160 × g, and protein concentration was determined using the Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as a standard.

4.3.3 Enzyme Assays

All enzyme assays were spectrophotometrically evaluated at 37 ℃ by measuring the changes in absorbance at 340 nm (ε^{NADH} = 6,220 M^{-1} cm^{-1}). One unit of activity corresponded to the generation or consumption of 1 μmol of NADH per min, and specific activity was expressed as units per milligram of protein. For PGI (EC 5.3.1.9; fructose 6-phosphate [F6P] to G6P) activity, the reaction mixture was composed of 100 mM potassium phosphate pH 7.0, 1.5 mM NAD^+, 1.0 mM ATP, 1.0 unit/mL of hexokinase, 1.0 unit/mL of glucose 6-phosphate dehydrogenase, 2 mM D-fructose, 0 to 20 mM GAP (for tests of the inhibition or activation of PGI activity), and 0.05 to 0.1 mg of protein. Mannitol dehydrogenase (ManDH; EC 1.1.1.67) activity was measured in 90 mM Tris-HCl, pH 9.0, 3.0 mM NAD^+, 1.5 mM D-mannitol, and 0.05 mg of protein. TPI (EC 5.3.1.1; GAP to DHAP) was assayed in 100 mM potassium phosphate (pH 7.4) containing 1.0 unit/mL of glycerol 3-phosphate dehydrogenase, 0.3 mM NADH, 0.4 mM GAP, and 0.05 to 0.1 mg of protein.

4.3.4 Determination of Hexoses (Glucose and Fructose) and End-products

Optical density and concentration of metabolites (glucose, fructose, organic acids, and ethanol) were analyzed by a spectrophotometer and the optimized HPLC method, respectively, as described in section 3.3.2. The mannitol concentration in the culture broth was measured in accordance with the instructions in the D-Mannitol/L-Arabitol Kit (Megazyme, Bray, Co. Wicklow, Ireland).

4.3.5 Quantification of Intracellular DHAP Level

For the quantification of intracellular DHAP levels, *L. panis* PM1 was cultured in mMRS medium containing either 60 mM fructose or glucose. After 24-h culture, the cells were harvested and the crude extract was prepared as described above. A standard curve for the DHAP concentration was evaluated spectrophotometrically (at 340 nm, ε^{NADH} = 6,220 M^{-1} cm^{-1}) in 100 mM potassium phosphate (pH 7.4) containing 1.0 unit/mL of glycerol 3-phosphate dehydrogenate, 0.2 mM NADH, and external DHAP standards (0, 10, 20, 40, and 80 μM). The

DHAP concentrations in the crude extract were calculated from the standard curve. Intracellular DHAP concentrations were calculated on the basis of the dry cell mass conversion factor (0.255g/liter/OD_{600} unit) and water content (3.53 µL/mg [dry weight]) of *L. panis* PM1.

4.3.6 RNA Preparation

RNA was extracted from *L. panis* PM1 cells by the acid-hot phenol method, as described by Oh and So (2003) with minor modifications. Briefly, 10 mL of exponentially-growing bacteria from liquid media were added to a tube containing 1.25 mL of ice-cold ethanol/phenol stop solution (5% water-saturated phenol, pH < 4.5, in 95% ethanol), and harvested by centrifugation for 5 min at 16,160 × g. The cell pellets were resuspended in 600 µL of diethylpyrocarbonate (DEPC)-treated water. Glass beads (0.8 g, 452-600 µm in diameter; Sigma, St. Louis, MO, USA) and 600 µL of pre-warmed acid-hot phenol (pH < 4.5):chloroform:isoamylacohol (PCI, 25:24:1, v/v) were added to the cell suspensions, and the mixture was incubated at 65 ℃ for 10 min with vigorous vortexing for 30 s duration every 30 s. The samples were centrifuged for 10 min at 16,160 × g and then the supernatants (500 µL) were transferred to fresh 1.5-mL micro-tubes containing 500 µL of the pre-warmed PCI and incubated at 65 ℃ for 5 min with vortexing every 30 s. Samples were then centrifuged for 10 min at 16,160 × g. The aqueous layer (400 µL) was transferred to new 1.5-mL microtubes and mixed with 95% ethanol (800 µL) and 3 M sodium acetate (40 µL). The mixtures were kept at -80 ℃ for 30 min and centrifuged at 16,160 × g. The RNA pellet was washed with 70% ethanol and resuspended in 50 µL of RNase-free water. RNA was treated with DNase and purified using the RNeasy kit (Qiagen, Toronto, ON, Canada). The quantity of RNA was determined by measuring the absorbance at 260 nm (optical density (OD) 1 at A_{260} = 40 $\mu g \cdot ml^{-1}$ RNA) using a DU 800 spectrophotometer (Beckman Coulter, Mississauga, ON, Canada), and its purity was determined by measuring the A_{260}/A_{280} ratio.

4.3.7 Reverse Transcription

The primers used in this study were specifically designed by Primer3 (http://frodo.wi.mit.edu/primer3/) for real time RT-PCR applications of *L. panis* PM1 from the nucleotide sequence of the annotation data (Table 4.1). The RT reaction mixture contained 0.5 µg of total RNA and 0.25 µM of reverse primers of the selected genes.

Table 4.1 The plasmids and primers used in this study

Plasmids	Relevant features	source or reference
pEGFP-N1	Source of EGFP[a] gene	Clontech[b]
pSIP411	Source of P*orfX* promoter	Sörvig et al. 2005
pCER	*E. coli-L. panis* PM1 shuttle vector, Em^r Cm^r, 5.0 kb	Kang et al. 2013a
pCER-EGFP	pCER derivative expressing the EGFP gene under control of the P*orfX* promoter, Em^r Cm^r, 6.0 kb	This study
pCER-TPI	pCER-EGFP derivative, in which the EGFP gene is replaced with the TPI gene, Em^r Cm^r, 6.0 kb	This study

Primer	Restriction enzymes	T_m (℃)[c]	Nucleotide sequence (5' →3')[d]	Target gene	Function
f16S		58	tggcccaactgatatgac	16S rRNA	16S ribosomal RNA
r16S		58	ctctcatgcacgttcttctt		
fFBA		60	tagtggggaactggcttcag	*fba*	Fructose 1,6-diphosphate aldolase
rFBA		60	atcccttgtggtccttgtca		
fXFP		60	catggaacacaaggacatgc	*xfp*	Xylulose 5-phosphate phosphoketolase
rXFP		60	aagccacggaatgtatctgg		
fTPI	*NotI*	69	ataagaatgcggccgcatgcgacatcctttagtgatgggt	*tpi*	Triosephosphate isomerase
rTPI	*SalI*	65	acgcgtcgacttaagcctgtttagccgcttc		

a. EGFP, enhanced green fluorescent protein. b. Clontech (Mountain View, CA, USA). c. T_m, melting temperature. d. Underlined primer sequences represent restriction enzyme sites.

The RT reaction was performed using qScript cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. The reaction mixture was incubated at 25 ℃ for 5 min and at 42 ℃ for 30 min, and the reaction was terminated by incubation at 85 ℃ for 5 min with a Techne thermal cycler (*FTGENE-5D*, *Techgene*, Burlington, NJ, USA).

4.3.8 Quantitative Real-time PCR

Real-time PCR amplification was performed in a CFX96 real-time detection system (Bio-Rad) using SsoFast EvaGreen Supermix (Bio-Rad). The total volume of the PCR master mixture was 20 µL, to which cDNA template equivalent to 25 ng RNA starting material and 0.5 µM of each primer (Table 4.1) were added. PCR amplification was initiated at 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 10 s. Amplification was followed by a melt-curve analysis between 65 ℃ and 95 ℃ using a 0.5 ℃ increment. All sample and primer combinations were assessed in three biological replicates with two technical replicates per biological replicate. A no-template control was used for the negative control PCR, and PCR specificity and product detection were verified by examining the temperature-dependent melting curves of the PCR products as well as by ethidium bromide staining on 1% agarose gels. For relative gene expression, the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method, using the 16S rRNA gene for normalization, was performed as described by Livak and Schmittgen (2001). The steps for calculating the expression ratio were as follows:

$\Delta C_{T(test)} = C_{T(\textit{fba} \text{ and } \textit{xfp}, \text{ test})} - C_{T(16S \text{ rRNA, test})}$

$\Delta C_{T\ (control)} = C_{T(\textit{fba} \text{ and } \textit{xfp}, \text{ control})} - C_{T(16S \text{ rRNA, control})}$

$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(control)}$

Normalized expression ratio of $\textit{fba}_{(test)}$ and $\textit{xfp}_{(test)} = 2^{-\Delta\Delta CT}$.

The Real-Time PCR data were processed using CFX Manager Software (Bio-Rad).

4.3.9 General DNA Techniques, Plasmid Construction, and Bacterial Transformation

DNA work was carried out according to standard protocols described in *Molecular Cloning* (Sambrook et al. 2001). Restriction endonucleases were purchased from Fermentas (Waltham, MA, USA) and digestions were performed as recommended by the manufacturer. A Bio-Basic gel extraction kit (Bio-Basic Inc., Markham, ON, Canada) was used to isolate specific

restriction and PCR fragments from agarose gels. DNA ligations and subsequent transformations into competent *E. coli* TOP 10 cells (Invitrogen) were carried out according to standard protocols (Sambrook et al. 2001). *Escherichia coli* JM109 genomic DNA was used as a template for TPI gene amplification. For PCR amplification, a 50-µL PCR mixture was prepared using 0.5 µM (each) fTPI and rTPI primers (Table 4.1), 1.25 U *Pfu* DNA Polymerase (Fermentas), 1× *Pfu* buffer with $MgCl_2$, and 0.2 mM of deoxynucleoside triphosphates (dNTPs). The DNA template was denatured for 3 min at 95 ℃, and the PCR continued with 30 cycles of 30 s at 95℃, 30 s at 55℃, and 1 min at 72℃. After the final cycle, the mixtures were further incubated for 5 min at 72 ℃ using a thermal cycler (*FTGENE-5D*). The amplified TPI gene was digested with *NotI* and *SalI* restriction endonucleases and cloned into the expression vector pCER-EGFP (Table 4.1). The resultant plasmids were transformed into *L. panis* PM1 as described by Mason et al. (2005). Briefly, *L. panis* PM1 was statically-cultured until early-log phase (OD_{600} 0.5) in MRS medium at 37 ℃ and glycine was added in the culture at final concentration of 0.27 M and the culture was then incubated for another 90 min at 37℃. The cells were collected, washed twice with 10% glycerol and resuspended in 1/100 of the original culture volume of 10% glycerol. The concentrated cell (100 µL) mixture was combined with 1 µg plasmid DNA and electroporated using a MicroPulser electroporator (Bio-Rad) in a cuvette with a 0.2-cm electrode gap (Bio-Rad). A pre-programmed electroporation setting (StA, 1.8 kV) was used. After electroporation, the cells were diluted immediately into 1 mL of MRS broth and incubated at 37 ℃ for 3 h before plating on MRS agar media containing antibiotics.

4.3.10 Statistical Analysis

For determinations of end-product concentrations and enzyme activities, data were presented as mean values ± standard errors of the means calculated from at least three independent experiments. Differences in end-products, specific enzyme activities, and transcriptional expression were analyzed by the two-way analysis of variance with Bonferroni post-tests for three groups or the unpaired *t* test for two groups using GraphPad Prism, version 5.0, software (GraphPad Software, Inc., San Diego, CA, USA). A *P* value of < 0.05 was considered significant.

4.4. Results and Discussion

4.4.1 Sporadic Growth on Fructose Medium

The inability of *L. panis* PM1 to grow solely on fructose was previously reported in Chapter 3. However, a culture left in the incubator for over a 1-month period revealed some growth of the PM1 strain on fructose did occur. Subsequent extended growth tests were conducted in multiple independent cultures using this fructose-cultured strain. This strain showed three types of fructose consumption patterns (Figure 4.1): (I) full growth, consuming all available fructose (60 mM), (II) arrested cell growth at the mid- to late- log growth phase, consuming one third of the available fructose, and (III) early cell growth cessation, leaving most of the available fructose unconsumed. In this thesis, these three growth patterns were, together, defined as sporadic growth.

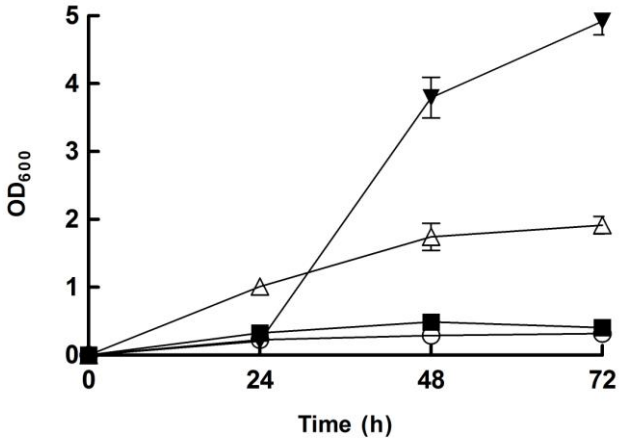

Figure 4.1 Growth pattern of *L. panis* PM1 on fructose medium. The PM1 strain was cultured in mMRS medium without a carbon source (circles) or containing 60 mM fructose. Three types of patterns were observed: full growth (down-pointing triangles), arrested cell growth at mid- to late log phase (up-pointing triangles), and early cell growth cessation (squares).

In the present study, sequences of 16S rRNA, a possible sugar permease, and genes necessary for the EM and 6-PG/PK pathways were compared among the PM1 cells showing the three growth patterns on fructose in order to identify reasons for variable growth responses. The results revealed the sequences of these genes were identical. Subsequent culture of the PM1 strain showing the type II pattern retained the same three patterns on fructose. However, once the PM1 strain fully grew on fructose medium (type I growth), successive subcultures in fructose medium showed a growth pattern similar to that observed in glucose medium. In this case, the lag phase

of the PM1 grown using fructose medium was longer than that observed when it was grown on glucose (24 h vs. 12 h). Interestingly, once the strain showing the type I growth became dormant, i.e., after it was frozen for storage, the cell cultures reacquired the same inability of the parent *L. panis* PM1 on fructose (Figure 4.1). Therefore, it was apparent that the sporadic growth was the result of naturally-occurring phenotypic differences by *L. panis* PM1 on fructose. The type I pattern cells were subsequently used to examine the growth on fructose throughout the rest of this chapter, and is simply referred to as *L. panis* PM1.

4.4.2 Metabolic Profiles of Fructose Fermentation

When *L. panis* PM1 was cultured in mMRS medium with different carbon sources (fructose and/or glucose), each culture showed different end-product production patterns. As shown in Figure 4.2, fructose fermentation by *L. panis* PM1 significantly increased lactate production (25% in 60 mM glucose and fructose medium and 51% in 120 mM fructose medium), along with decreases in acetate (28% in 60 mM glucose and fructose medium and 50% in 120 mM fructose medium) ($P < 0.01$).

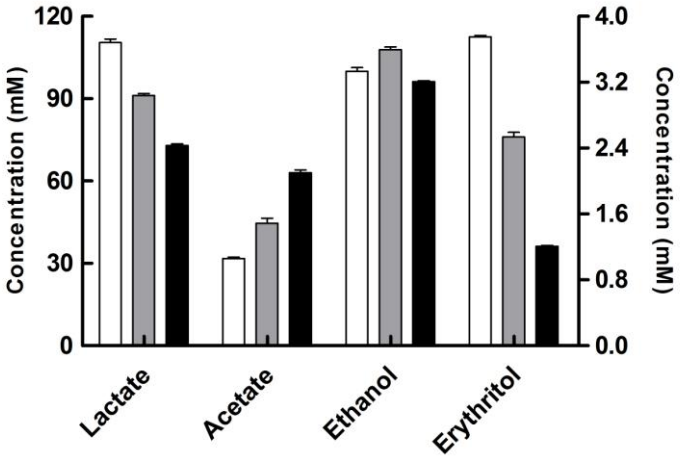

Figure 4.2 Main end-products yielded from fructose and/or glucose fermentation by *L. panis* PM1. *Lactobacillus panis* PM1 was cultured in mMRS media containing 120 mM fructose (white bars), 60 mM (each) fructose and glucose (gray bars), or 120 mM glucose (black bars) for 48 hours. The concentrations of lactate, acetate, and ethanol are provided on the left-hand *y*-axis, and the concentration of erythritol is provided on the right-hand *y*-axis.

In contrast, ethanol concentrations were comparable under all conditions ($P > 0.05$). Interestingly, mannitol, which is a typical main end-product of fructose fermentation in group III lactobacilli, was not detected in any fermentation broths, and the activity of ManDH was not observed from

cells grown on either glucose or fructose, indicating that fructose is used only as a carbon source in *L. panis* PM1. Instead of mannitol, erythritol was detected in glucose and fructose fermentation by the fructose-utilizing *L. panis* PM1, and the presence of fructose significantly increased the final yield of this end-product (by 102% in 60 mM glucose and fructose medium and 210% in 120 mM fructose medium compared with that in 120 mM glucose medium) ($P < 0.01$). These changes in metabolites indicated that the PM1 strain used different pathways, in addition to the 6-PK/PG pathway, to metabolize fructose, suggesting the possibility of the simultaneous use of the 6-PG/PK and EM pathway for fructose metabolism.

4.4.3 *In silico* Analysis of the EM Pathway and Quantitative Real-time PCR

To identify genes encoding enzymes involved in the EM pathway, annotation data from the draft genome of *L. panis* PM1 (M.C. Haakensen, V. Pittet, D.A.S. Grahame, D.R. Korber, and T. Tanaka, unpublished data) were analyzed at the UniProt database (http://www.uniprot.org; default parameters were applied), identifying similarities with other proteins from related bacteria. This *in silico* analysis revealed the presence of the genes encoding the most critical enzymes of the EM pathway: FK (EC 2.7.1.4, GenBank accession No. KF312453), PGI (EC 5.3.1.9, GenBank accession No. KF312454), FBA (EC 4.1.2.13, GenBank accession No. KF312455), and TPI (EC 5.3.1.1, GenBank accession No. KF384506), but not PFK (EC 2.7.1.11). These genes were mainly clustered in two chromosomal loci. PGI and FK genes were organized in a five-gene operon structure coding for glyoxalase (GenBank accession No. KF384502)**,** sorbitol dehydrogenase (GenBank accession No. KF384503), and a small hypothetical protein. A gene annotated as a member of the major facilitator superfamily (GenBank accession No. KF384501) with a suggestive sugar transporter function was located in the upstream (1,446 bp) region of the PGI gene (Saier 2000). Two other genes for the EM pathway were grouped in an operon structure coding for phosphoenolpyruvate synthase (GenBank accession No. KF384504), a hypothetical protein (GenBank accession No. KF384505), the TPI gene, the FBA gene, and phosphoglycerate mutase (GenBank accession No. KF384507). The sequences of the key five genes (those encoding FK, PFK, PGI, FBA, and TPI) were searched against and compared to those of 19 other lactobacillus strains released by the DOE Joint Genome Institute (http://www.jgi.doe.gov/) (Table 4.2). Overall, the genes of the

Table 4.2 Identification of the five genes in 19 lactobacilli strains and sequence conservation similarity

Group	Strains	Genes ID and percent similarity with the PM1 strain[a]				
		FK	**PFK**	**PGI**	**FBA**	**TPI**
I	*L. acidophilus* 30SC	650745597, 44.97%	650746564	650746372, 69.25%	650747281, 71.80%	650746322, 40.31%
	L. amylovorus GRL 1112	650724351, 8.22% 650724347, 44.97%	650725350	650725157, 69.25%	650726109, 71.80%	650725087, 40.31%
	L. crispatus ST1	646761531, 41.95%	646762585	646762330, 68.14%	646763201, 70.49%	646762284, 41.47%
	L. delbrueckii bulgaricus 2038	N.F.	650390714 650391575	650390580, 67.55%	650391374, 69.18%	650390527, 40.31%
	L. gasseri ATCC 33323	639671876, 44.82%	639672737	639673092, 67.77%	639672325, 66.56%	639673139, 42.02%
	L. helveticus DPC 4571	641310735, 37.20%	641311598	641311408, 67.48%	641312090, 71.15%	N.F.
	L. johnsonii DPC 6026	651136969, 44.15%	651138043	651138189, 67.77%	651137472, 66.23%	651138237, 42.41%
	L. kefiranofaciens ZW3	650904929, 43.29%	650904173	650904375, 68.14%	650903657, 71.80	650904413, 39.92%
	L. ruminis ATCC 27782	2521929235, 46.62%	2521929885	2521930531, 71.02%	2521930559, 66.32%	2521929706, 39.30%
	L. salivarius CECT 5713	648250961, 48.31%	648250462	648250176, 70.58%	648250105, 66.21%	648250709, 38.91%
II	*L. casei* ATCC 334	N.F.	639664304	639664077, 67.70%	639663308, 27.52% 639663408, 55.07% 639665611, 29.29%	639663926, 39.69% 639665610, 15.44%
	L. plantarum WCFS1	637387577, 20.55% 637390475, 28.82%	637389046	637389578, 72.79%	637387703, 59.31%	637388111, 43.19%
	L. rhamnosus GG	645012628, 41.84%	645013595	645013306, 67.92%	645012414, 28.38% 645012634, 27.72% 645012635, 30.97% 645012745, 54.73%	645013156, 40.86%
	L. sakei sakei 23K	637796403, 60.80%	637795615	637795762, 69.45%	637796116, 59.31%	637795189, 39.69%
III	*L. brevis* ATCC 367	639654318, 73.63%	N.F.	639653487, 73.67%	N.F.	639652851, 40.47%
	L. buchneri NRRL B-30929	2505384060, 75.69%	N.F.	2505384064, 75.66%	N.F.	2520958147, 43.97%
	L. fermentum CECT 5716	648249646, 81.29%	N.F.	648249647, 77.88% 648248900, 77.66%	N.F.	648248902, 51.94%
	L. reuteri JCM 1112	642584601, 28.23% 642584602, 29.93%	N.F.	640590226, 88.27%	640590034, 82.64%	642583870, 62.26% 642583938, 64.37% 642584103, 41.63%
	L. sanfranciscensis TMW 1.1304	2511696944, 56.42%	N.F.	2511696945, 73.07%	N.F.	2511696895, 40.47%

a. Each gene (gene ID) was searched in DOE Joint Genome Institute (http://www.jgi.doe.gov/) and similarity with the PM1 strain was analyzed at the UniProt database (http://www.uniprot.org). N.F. not found.

PM1 strain showed higher similarities with those of group III lactobacilli (64% to 88%) than those of groups I and II (42% to 73%), and the sequence of the *L. reuteri* JCM 1112 strain showed the highest similarity (64%, 83%, and 88% in the TPI, FBA, and PGI genes, respectively) with that of the PM1 strain for all genes except the FK gene (28% and 30% to the two FK genes of the *L. reuteri* JCM 1112 strain). Previously, phylogenetic work showed higher identity (96%) in 16S rRNA sequences to the16S rRNA sequence of *L. reuteri* (Khan et al. 2013). Combined with the present results, this indicates that *L. panis* PM1 and *L. reuteri* are close relatives. The PFK gene in the annotation data of *L. panis* PM1I was not found; however, this gene was found only in group I and II lactobacilli in this comparison. The PM1 strain possessed a gene annotated as ribokinase (RBSK, EC 2.7.1.15, GenBank accession No. KF312456), showing 82% similarity to a *pfkB* orthologous gene (GenBank accession No. EF547651) in *L. reuteri* (Å rsköld et al. 2008). The similarity of the FK gene of group I and II lactobacilli with that of strain PM1 was relatively low (61% to 29%). In addition, the TPI gene showed 39% to 64% similarity across all groups. In contrast, the PGI gene had two well-conserved sugar isomerase domains and showed higher similarity than other genes among groups I, II, and III (67% to 88%). Table 4.2 shows that the FBA gene, containing a specific tetrameric class II aldolase, was found only in *L. reuteri* among group III lactobacilli, and the FBA gene of strain PM1 had a higher similarity with the FBA gene of group I lactobacilli (66% to 72%) than with the FBA gene of group II lactobacilli (55% to 59%).

The activities of the EM and 6-PK/PG pathways were evaluated using qRT-PCR with RNA prepared from a variety of cultures. Two representative genes, the FBA and the xylulose 5-phosphate phosphoketolase gene (XFP, GenBank accession No. KF384508), were evaluated for their use of the EM and 6-PG/PK pathways, respectively, including analysis of (i) early- and mid-log-phase cells grown on fructose medium and (ii) mid-log-phase cells grown on different carbon sources (glucose and/or fructose) (Table 4.3). During growth on fructose medium, the expression of the XFP gene was 2.9-fold higher in the early log phase than in the mid-log phase; whereas the expression level of the FBA gene was induced (4.1-fold) in the mid-log phase ($P < 0.01$). The difference in carbon source up-regulated the expression of both genes (1.5-fold in 30 mM fructose medium and 3.7-fold in 60 mM fructose medium relative to 60 mM glucose medium) ($P < 0.01$).

Table 4.3 Growth phase- and carbon source-dependent expression of the FBA and XFP genes determined using quantitative real-time PCR

Growth phase or Carbon sources[a]	Relative gene expression level	
	FBA gene	XFP gene
early log phase[b] (OD_{600}, 0.65)	1.00±0.37	1.00±0.31
mid-log phase (OD_{600},1.86)	4.09±0.38	0.35±0.04
Glc[b]	1.00±0.16	1.00±0.12
Glc and Frc	1.49±0.31	1.52±0.32
Frc	3.65±0.33	3.63±0.39

a. Strain PM1 was cultured in 60 mM glucose (Glc), 30 mM glucose and fructose (Glc/Frc), or 60 mM fructose (Frc) medium for 24 h. b. Control condition for qRT-PCR.

4.4.4. Enzyme Activities of the EM Pathway

The activity of PGI is essential to convert F6P to G6P for channeling fructose fermentation to the 6-PG/PK pathway. PGI enzyme activity was monitored for 42 h in cultures grown on medium with different carbon sources (glucose and/or fructose). The activity of PGI was induced by the presence of glucose, and the activities observed in medium with 30 mM (each) of fructose and glucose were 10 times higher than those observed in 60 mM fructose medium ($P < 0.0001$) (Figure 4.3).

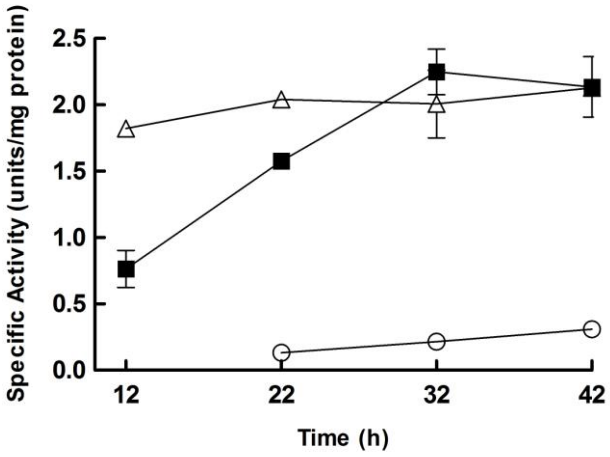

Figure 4.3 Changes in glucose 6-phosphate isomerase activity on different carbon sources. *Lactobacillus panis* PM1 was cultured in mMRS medium containing 60 mM fructose (circles), 60 mM glucose (squares), or 30 mM (each) fructose and glucose (triangles). At the 12-h time point, PGI activity could not be determined in 60 mM fructose medium due to insufficient growth.

Furthermore, the PGI activity on 60 mM glucose medium gradually increased from 0.76 to 2.25 units/mg protein until glucose depletion at the 32-h time point.

In group I and II lactobacilli, TPI is a key enzyme necessary to metabolize fructose via the EM pathway without carbon loss. In the absence of TPI, one-half of the fructose mass is theoretically converted to a three-carbon intermediate (DHAP), reducing the yield of end-product (e.g., lactate). To detect this enzyme activity, *L. panis* PM1 was cultured in MRS medium and was successively subcultured in mMRS medium containing either 60 mM glucose or fructose for 24 h. Cell growth on glucose medium followed a typical bacterial growth pattern; whereas cell growth on fructose as the sole carbon source ceased at the early log phase ($OD_{600,}$ 0.48). The specific activity of the TPI enzyme was not detected in PM1 cells grown on glucose medium; whereas TPI activity was observed in cells grown on fructose medium. However, the specific activity was not very high and in fact was 65 times lower than that of the crude extracts of *E. coli* culture ($P < 0.0001$), which was performed in LB medium for 12 h and served as the positive control (Table 4.4).

Table 4.4 The specific activity of TPI enzyme observed in *L. panis* PM1 and *E. coli* cultures

	E. coli	**PM1_Glc**	**PM1_Frc**
TPI activity (units/mg protein)	59.17±0.17	N.D.	0.91±0.02

PM1 strain was cultured in 60 mM glucose (Glc) or 60 mM fructose (Frc) medium for 24 h, and final cell density (OD_{600}) was 1.58 and 0.48, respectively. One unit is defined as the amount an enzyme able to generate 1 µmol of DHAP per min. N.D., not detected.

4.4.5 Repressor and Inducer of Fructose Metabolism

The enzyme assay data prompted me to focus on the two three-carbon intermediates, DHAP and GAP, which are substrates of TPI reactions. To investigate the roles of GAP on fructose metabolism, the PGI activity in the crude extracts of PM1 cells cultured in glucose medium until mid-log phase was measured. PGI enzyme assays were conducted in the presence of 0, 5, 10, and 20 mM GAP. Figure 4.4 shows the increased activity of PGI in the presence of GAP. The enzyme reaction was accelerated by 34 times in the presence of 10 mM GAP. However, 20 mM GAP lost much of this stimulatory effect, with the activity of PGI being only 6 times that in the absence of GAP. This result clearly suggests a significant role of GAP on the activation of PGI, which can stimulate channeling of fructose to the 6-PK/PG pathway.

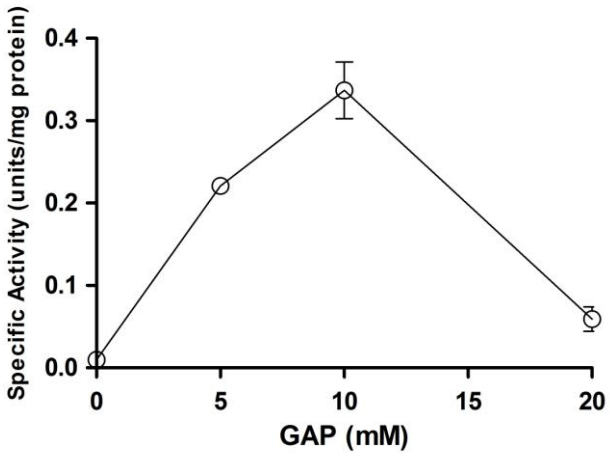

Figure 4.4 Relation of glucose 6-phosphate isomerase activity to the availability of glyceraldehyde 3-phosphate. Specific activities of glucose 6-phosphate isomerase were measured from crude extracts of *L. panis* PM1 cultured in the presence of glyceraldehyde 3-phosphate (GAP).

A significant level of intracellular DHAP (5.03 mM), along with the early cessation of growth, was detected in cells grown on fructose medium; whereas the presence of this chemical was not observed in cells grown on glucose medium, on which they exhibited a normal growth pattern (Table 4.5).

Table 4.5 Quantification of intracellular DHAP concentration

Carbon sources	Glucose	Fructose
Final cell density (OD_{600})	1.88±0.08	0.48±0.03
DHAP (mM)	N.D.	5.03±0.49

N.D., not detected.

The negative effect of dihydroxyacetone (DHA; a precursor and nonphosphorylated form of DHAP) on the cell growth was observed in media containing 0, 5.6, 11.2, 28, and 56 mM DHA. The growth of *L. panis* PM1 cells was completely inhibited by 28 mM DHA (Figure 4.5). Generally, DHAP is formed by the EM or glycerol oxidative (glycerol-to-DHAP) pathway; however, *L. panis* PM1 does not possess the glycerol oxidative routes (Kang et al. 2013b; Khan et al. 2013). Thus, the accumulation of DHAP suggested the utilization of the EM pathway and provided a direct reason for growth cessation with fructose but not with glucose.

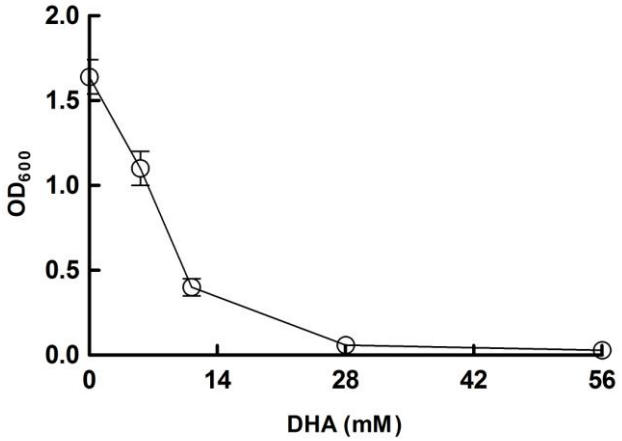

Figure 4.5 Growth inhibition by the presence of DHA in *L. panis* PM1 culture. *Lactobacillus panis* PM1 was cultured in MRS medium containing 0, 5.6, 11.2, 28, and 56 mM DHA, and cell density was determined after 24-h of culture.

4.4.6 The Role of TPI Activity on Fructose Metabolism

Although TPI genes were annotated from the genome data of *L. panis* PM1, functional activity was absent or very low in *L. panis* PM1, leading me to speculate that the accumulation of intracellular DHAP was occurring during fructose fermentation via the EM pathway. To circumvent DHAP accumulation, a TPI gene of *E. coli* was expressed in a wild-type *L. panis* PM1. The TPI gene was cloned into the expression vector pCER-EGFP and was under the control of P*orfX* promoter from plasmid pSIP144 (Sörvig et al. 2005) (Table 4.1), and the resulting plasmid, pCER-TPI, was introduced into a wild-type PM1 strain (i.e., a strain unable to readily utilize fructose). The resultant strain, PM1-TPI, was cultured in fructose medium, and cell growth and end-product profiles were monitored. Interestingly, the introduction of the TPI gene enabled *L. panis* PM1 to grow on fructose medium without undergoing sporadic growth (Figure 4.6) and was accompanied by a shift in fructose fermentation pattern. Accordingly, at the 24-h culture time point, the recombinant strain mainly produced lactate (51.89 mM) and ethanol (10.82 mM) from the fructose consumed (30 mM) but did not produce acetate, representative of a typical homolactic fermentation pattern.

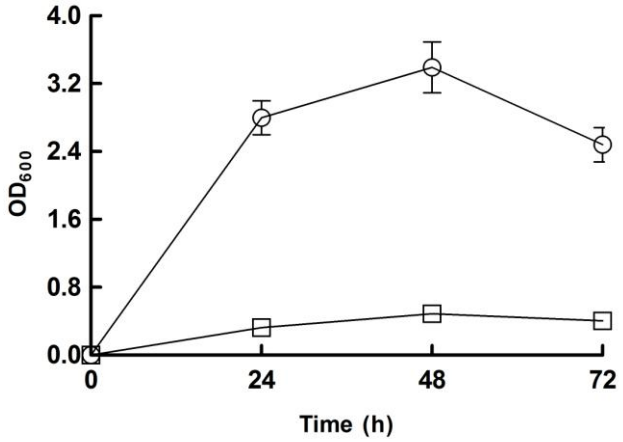

Figure 4.6 Effect of triosephosphate isomerase expression in *L. panis* PM1 growth on fructose-containing mMRS medium. PM1(pCER) (squares) and PM1(pCER-TPI) (circles) strains were cultured in mMRS medium containing 60 mM fructose, and samples taken from a 24-h culture were used for end-product analysis.

Group III lactobacilli generally tend to lack PTS activity, and the reaction involving PTS is the first reaction in routing glucose into the EM pathway in group I and II lactobacilli. As a result, group III lactobacilli do not use the EM pathway, but rather, use the 6-PK/PG pathway to utilize glucose. However, the presence of fructose-specific PTS has been reported in the group III lactobacilli, *L. brevis* and *L. reuteri* CRL 1098 (Saier et al. 1996; Taranto et al. 1999). In addition, the utilization of both the EM and 6-PK/PG pathways has been evidenced in *L. brevis* and *L. reuteri* ATCC 55730 during their fructose fermentation (Å rsköld et al. 2008; Saier et al. 1996). In the present study, I also observed evidence of the utilization of dual pathways for fructose metabolism in *L. panis* PM1 and that its fructose-metabolizing patterns were unique compared with those of known fructose-utilizing lactobacilli. Further study elucidated the detailed mechanism of fructose fermentation, the regulation of this mechanism, and the role of the TPI enzyme in the shift between homolactic and heterolactic fermentation patterns.

Fructose fermentation by *L. panis* PM1 did not produce mannitol due to the absence of ManDH activity, indicating that fructose is only used as a carbon source. This characteristic of *L. panis* PM1 offer fewer benefits in terms of energy metabolism since the PM1 strain cannot regenerate NAD^+ using fructose as an electron acceptor. Thus, the amount of NADH produced during rapid growth on fructose could exceed the capacities of NADH-consuming enzymes (lactate dehydrogenase, acetaldehyde dehydrogenase, and alcohol dehydrogenase) under the

circumstances described above without external electron acceptors (Kang et al. 2013b). Instead of mannitol, *L. panis* PM1 produced erythritol, and its final yield was increased by the availability of fructose (Figure 4.2). The activity of phosphoketolase cleaves fructose 6-phosphate into acetyl phosphate and erythrose 4-phosphate, which are used for the disposal of 1 mol (or 3 mol, if acetyl phosphate is converted to ethanol) of NADH per mol of glucose consumed in various heterofermentative lactobacilli (Richter et al. 2001; Stolz et al. 1995; Veiga da Cunha and Foster 1992a). Thus, erythritol production by *L. panis* PM1 suggests the role of this pathway as an alternate route for NAD^+ regeneration. However, the contribution of this pathway to NAD^+ regeneration is limited when the low yield of erythritol (3.75 mM) relative to that of lactate (110.47 mM) is considered.

If fructose is solely fermented by the 6-PG/PK pathway, the end-product pattern should be the same as that for glucose metabolism. However, fructose fermentation by strain PM1 did not follow this model and produced more lactate and less acetate than the amounts seen during glucose fermentation (Figure 4.2). If utilization of the EM pathway is assumed, fructose would be processed only to lactate and would contribute to the increased lactate observed in Figure 4.2. These observations strongly suggest the utilization of both the EM and 6-PK/PG pathways during fructose fermentation by *L. panis* PM1.

A possible fructose metabolic pathway was examined on the basis of *in silico* and metabolite analyses (Figure 4.7). Draft genomic information of *L. panis* PM1 revealed the presence of genes encoding most of the critical enzymes of the EM pathway (FK, PGI, FBA, and TPI); the exception was PFK. The absence of this gene has also been reported in the genome of group III lactobacilli released in the GenBank database (Å rsköld et al. 2008). However, Å rsköld et al. (2008) found that *L. reuteri* with orthologs to *pfkB*, which encodes a minor PFK in *E. coli*, possesses PFK activity. Since relatively high similarities of the key EM pathway genes were observed among *L. panis* PM1 and *L. reuteri* strains and the *rbsK* gene showed a similarly high degree of similarity to the *pfkB* gene, it is suggested that *rbsK* also functions as an ortholog of PFK. The lower similarity of the FK gene in groups I and II indicated the presence of a fructose-importing system different from group III; i.e., the PTS system of group I and II strains take up fructose in the form of fructose 6-phosphate, followed by intracellular phosphorylation by PFK, and thus require little FK activity during the conversion of fructose to fructose 6-phosphate. The similarity of the TPI gene was relatively low among the three groups of lactobacilli, and the TPI

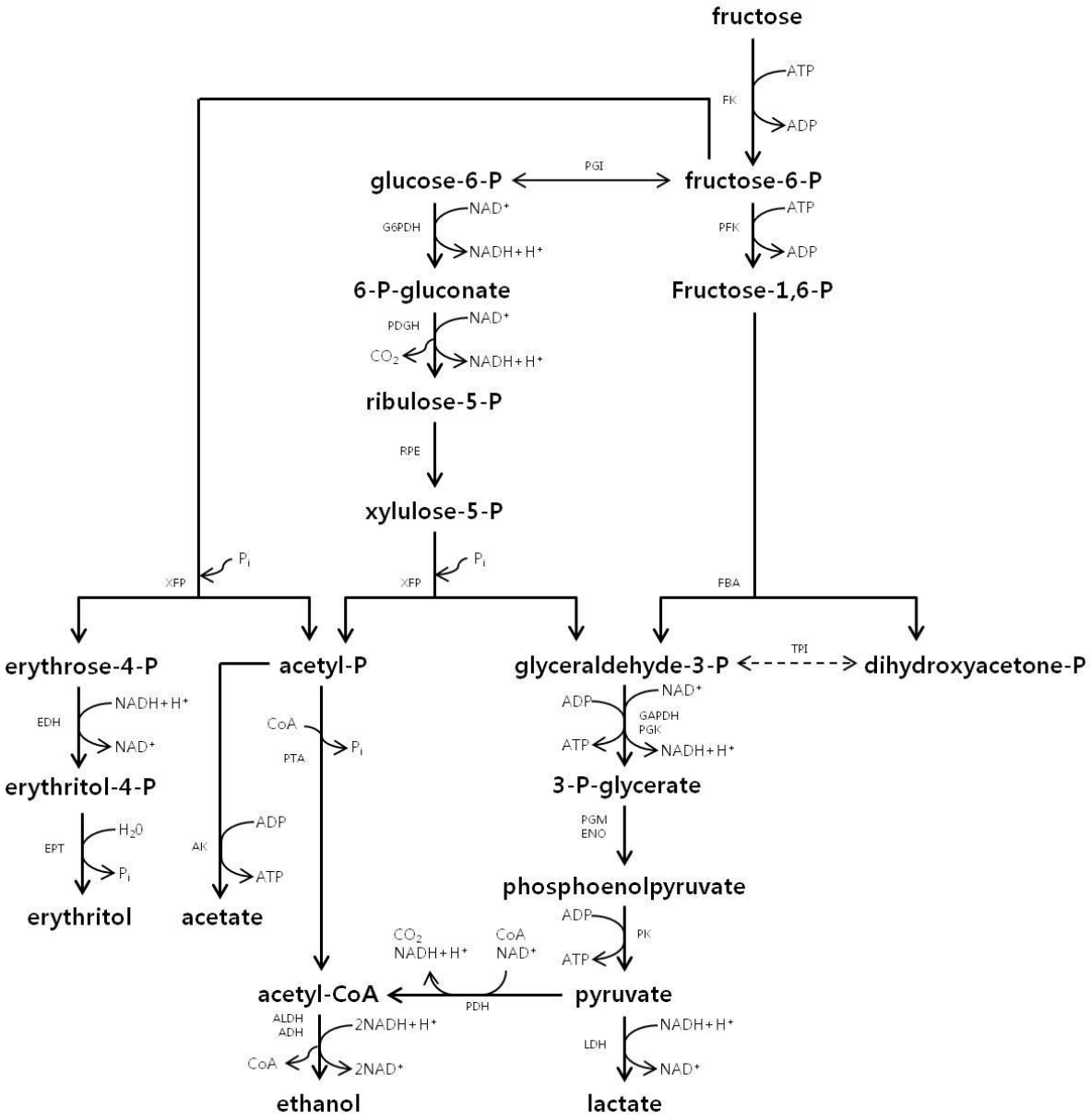

Figure 4.7 Schematic diagram of the fructose metabolic pathway in *L. panis* PM1. Dashed line, the absence of enzyme activity in *L. panis* PM1. Abbreviation, ADH, alcohol dehydrogenase; AK, acetate kinase; ALDH, acetaldehyde dehydrogenase; CoA, coenzyme A; EDH, erythritol dehydrogenase; ENO, enolase; EPT, erythrose 4-phosphate phosphotransferase; FBA, fructose 1,6-diphosphate aldolase; FK, fructokinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G6PDH, glucose 6-phosphate dehydrogenase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PFK, 6-phosphofructokinase; PGDH, 6-phosphogluconate dehydrogenase; PGI, glucose 6-phosphate isomerase; PGK, phosphoglycerate kinase; PGM, phosphoglyceromutase; PK, pyruvate kinase; PTA, phosphotransacetylase; RPE, ribulose 5-phosphate 3-epimerase; TPI, triosephosphate isomerase; and XFP, xylulose 5-phosphate phosphoketolase.

gene of *L. panis* PM1 showed 33% similarity to the TPI gene of *E. coli*, explaining the low or absent TPI activity, as observed in Table 4.4; whereas the higher similarities of the PGI and FBA genes among the three groups suggest that these annotated genes function in the upper EM pathway.

FBA and XFP were chosen as representative enzymes of the EM and 6-PK/PG pathways, respectively, due to their critical roles in the fate of carbon (i.e., lactate, acetate, or ethanol). The FBA and XFP genes were up-regulated by the availability of fructose, and both genes were differently activated according to growth phase on fructose medium (Table 4.3). This observation suggests two important aspects of fructose utilization: (i) the presence of dual pathways for fructose metabolism and (ii) the fructose-directed regulation of those pathways. However, a question still remained: why does *L. panis* PM1 sporadically grow on fructose medium, even though it possesses these two pathways?

To address this question, it was determined that the activities of two enzymes, PGI and TPI, are critical to the functioning of these two pathways. These enzymes are critical because PGI channels fructose to the 6-PK/PG pathway and TPI governs the fate of the two split products of FBA activity to prevent the over-accumulation of DHAP, i.e., loss of carbon. The activity of PGI was induced by glucose, and 10 times less activity was observed in cells grown on medium containing fructose as the sole carbon source than on medium containing glucose as the sole carbon source (Figure 4.3), suggesting that fructose fermentation through the 6-PK/PG pathway did not occur at a high rate and that glucose could accelerate the channeling of fructose to the 6-PG/PK pathway. In addition, glucose concentration significantly affected the activity of this enzyme. At the 12-h time point, PGI activity in cells grown on medium containing 30 mM glucose was two times higher than that in cells grown on 60 mM glucose. PGI enzyme activity on 60 mM glucose increased with glucose consumption and peaked at the time of glucose depletion (32 h). Therefore, this enzyme assay data indicate that intermediates from glucose metabolism indeed induce the activity of PGI enzyme.

While PGI enables the utilization of fructose, cell growth on fructose medium halted at an early log phase, suggesting that initial fructose fermentation can repress growth. If fructose is fully introduced into the 6-PK/PG pathway, there should not be any repression of growth, as it would essentially follow the same reaction flow that it does during glucose fermentation. This observation further suggested that the PM1 strain uses the EM pathway like group I and II

lactobacilli do and that the cessation of growth on fructose medium could be related to the accumulation of intermediates through utilization of the EM pathway. The significant accumulation of DHAP was observed only from cells grown on fructose medium. In this case, the low TPI activity was insufficient to process the DHAP produced by FBA; thus, fructose utilization through the EM pathway resulted in DHAP accumulation. The toxicity of DHAP was confirmed by a growth inhibition test with the nonphosphorylated form of this chemical (DHA) (Figure 4.5 and Table 4.5). Combining these observations, the accumulation of DHAP by the EM pathway caused early growth cessation during fructose metabolism. Interestingly, the other three-carbon metabolite, GAP, made from FDP by FBA, enhanced the activity of PGI at 5 and 10 mM; however, a higher GAP concentration (20 mM) inhibited the activity of this enzyme. These findings clearly demonstrate that the balance in the accumulation of the three-carbon intermediates (GAP and DHAP) functions to control fructose metabolism patterns in *L. panis* PM1, as follows. Initially, fructose is fermented via the EM pathway, producing GAP and DHAP. GAP increases the activity of PGI and is further metabolized to lactate. This enhanced PGI activity then allows F6P to be processed through the 6-PG/PK pathway for further fructose fermentation, producing lactate, ethanol and/or acetate. It should be noted that once PGI is activated, the 6-PK/PG pathway supplies GAP, keeping fructose channeled to the 6-PK/PG pathway. When this scheme is compared with glucose fermentation solely through the 6-PK/PG pathway, the production of acetate is reduced by the amount of reactants via the EM pathway, where no acetate is produced. When the majority of carbon is fructose (as in the culture on 120 mM fructose medium, for which the results are shown in Figure 4.2), the initial EM pathway fermentation provided a certain concentration of DHAP, and due to low or absent TPI activity, this DHAP accumulated, resulting in growth cessation at an early stage. Thus, this regulation nicely explains the sporadic growth and the concentrations of the different end-products (more lactate and less acetate) observed during fructose fermentation (figure 4.2).

Various NAD^+ regeneration routes (e.g., mannitol production) enable group III lactobacilli to compensate for the reduced ATP yield that occurs via the 6-PG/PK pathway by shifting acetyl phosphate for ATP production, producing acetate instead of ethanol (Condon 1987; Hugenholtz 1993; Wisselink et al. 2002; Zaunmuller et al. 2006). However, the absence of ManDH activity in *L. panis* PM1 made fructose metabolism less energy efficient than that in group III lactobacilli, which can produce mannitol. The absence of TPI activity was a key

bottleneck which hampered potential yield of energy from fructose metabolism via the EM pathway; i.e., DHAP accumulation keeps one-half of the carbon from generating ATP, resulting in a zero-sum ATP balance. To overcome this reduced efficiency and the sporadic growth on fructose medium, the TPI gene of *E.coli* was cloned into and expressed in *L. panis* PM1. The introduction of this gene eliminated the early growth cessation seen on fructose medium (Figure 4.6) and altered fructose metabolism from heterolactic to homolactic fermentation, wherein most fructose was processed to lactate. Also, citrate (initial concentration, 16.03 mM) was not converted to succinate, which is an alternate NAD^+ regeneration route for the 6-PG/PK pathway in the presence of citrate (Kang et al. 2013a), suggesting no NADH regeneration provision from this pathway. Therefore, this change in fermentation pattern indicated that in this recombinant PM1 strain, fructose was mostly fermented via the EM pathway, generating 2 ATP molecules per fructose molecule.

Under certain conditions (e.g., low pH or glucose limitation) a shift from homolactic to heterolactic fermentation has been observed in homofermentative lactobacilli, producing end-products such as acetate, ethanol, diacetyl, acetoin, and 2,3-butanediol (Borch et al. 1991; Torino et al. 2001). The PM1-TPI strain also produced approximately 11 mM ethanol from fructose fermentation. This ethanol may have been produced via two possible routes: (i) the pyruvate-to-acetyl coenzyme A pathway from the activity of pyruvate dehydrogenase (Kang et al. 2013a) or (ii) the 6-PG/PK pathway via the activity of PGI (Figure 4.7). Considering the low level of PGI activity in *L. panis* PM1 grown on fructose medium (Figure 4.3), part of the available pyruvate could have been directed to ethanol via the former route to meet the redox balance needs under conditions of rapid fructose metabolism.

4.5 Conclusions

Overall, these findings clearly indicate that *L. panis* PM1 can ferment fructose via both the EM and 6-PG/PK pathways and that the TPI enzyme plays a critical role in the shift between the 6-PK/PG and EM pathways, leading to an altered fermentation pattern. Despite the improved energetic efficiencies of the EM pathway, the reason why *L. panis* PM1 loses its TPI activity requires further study.

4.6 Connection to the Next Study

In this chapter, the utilization of dual glycolytic pathways for central metabolism was evidenced in *L. panis* PM1 and their regulation was elucidated. These central metabolic pathways are critical system to supply basic-life essentials, i.e., energy molecules and precursors for further metabolism to produce lipids, proteins, and so on. Along with this critical system, the PM1 strain has developed auxiliary pathways (e.g., glycerol-to-1,3-PDO and citrate-to-succinate) as described in Chapter 3. These auxiliary routes enable *L. panis* PM1 to adapt to various environmental and nutritive conditions. Oxygen is one of the most common electron acceptors on the Earth, and most bacteria's metabolism, such as redox potential maintenance through NADH-NAD^+, is affected by the presence of oxygen. Thus, in the following Chapter (Chapter 5), how molecular oxygen affects NADH recycling system and end-product formation in *L. panis* PM1 was studied in depth. This investigation would indicate the role of oxygen in *L. panis* PM1's metabolism and information about culture conditions for glycerol conversion by this strain.

5. INFLUENCE OF OXYGEN ON NADH RECYCLING AND OXIDATIVE STRESS RESISTANCE SYSTEMS IN *LACTOBACILLUS PANIS* PM1[iii]

5.1 Abstract

Lactobacillus panis strain PM1 is an obligatory heterofermentative microorganism that also produces 1,3-propanediol from glycerol. This study investigated the metabolic responses of *L. panis* PM1 to oxidative stress under aerobic conditions. Growth in aerobic culture triggered an early entrance of *L. panis* PM1 into the stationary phase, along with marked changes in end-product profiles. A ten-fold higher concentration of hydrogen peroxide was accumulated during aerobic culture compared to microaerobic culture. This H_2O_2 level was sufficient for the complete inhibition of *L. panis* PM1 cell growth, along with a significant reduction in end-products typically produced during anaerobic growth. *In silico* analysis revealed that *L. panis* PM1 possessed two genes for NADH oxidase and NADH peroxidase, but their expression levels were not significantly affected by the presence of oxygen. Specific activities for these two enzymes were observed in crude extracts from *L. panis* PM1. Enzyme assays demonstrated that the majority of the H_2O_2 in the culture media was the product of NADH:H_2O_2 oxidase which was constitutively-active under both aerobic and microaerobic conditions; whereas NADH peroxidase was positively-activated by the presence of oxygen and had a long induction time in contrast to NADH oxidase. These observations indicated that a coupled NADH oxidase-NADH peroxidase system was the main oxidative stress resistance mechanism in *L. panis* PM1, and was regulated by oxygen availability. Under aerobic conditions, NADH was mainly reoxidized by the NADH oxidase-peroxidase system rather than through the production of ethanol (or 1,3-propanediol or succinate production if glycerol or citrate is available). This system helped *L. panis* PM1 to directly use oxygen in its energy metabolism by producing extra ATP in contrast to homofermentative lactobacilli.

[iii] Tae Sun Kang, Darren R. Korber, and Takuji Tanaka. 2013. Influence of oxygen on NADH recycling and oxidative stress resistance systems in *Lactobacillus panis* PM1. AMB-Express 3:10. doi:10.1186/2191-0855-3-10. This chapter is reproduced in this thesis with the permission of Springer.

5.2 Introduction

Lactobacillus panis PM1 is an obligatory heterofermentative microorganism isolated from bioethanol thin stillage, and has been the focus of attention due to its ability to produce 1,3-propanediol (1,3-PDO) during the fermentation of glycerol under anaerobic conditions (Khan et al. 2013). *Lactobacillus panis* PM1 belongs to the group III heterofermentative lactobacilli, which include *L. brevis*, *L. buchneri* and *L. reuteri*, where the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway is the primary carbohydrate fermentation pathway (Khan et al. 2013; Lüthi-Peng et al. 2002; Pedersen et al. 2004; Veiga da Cunha and Foster 1992a). In theory, when one glucose molecule is consumed, three NADH and one ATP molecules are generated. Subsequently, one pyruvate and one acetyl phosphate molecules accept protons from one and two NADH molecules, respectively, and regenerate NAD^+. End-products of this metabolism are lactate and ethanol, respectively. Overall heterolactic fermentation of glucose through the 6-PG/PK pathway results in 1 mol each of lactate, ethanol, CO_2, and ATP per mol glucose consumed (Kandler 1983).

For heterofermentative lactic acid bacteria (LAB), external electron acceptors can be used as alternate routes to regenerate NAD^+. The presence or absence of electron acceptors determine whether ethanol (and no more ATP) or acetate (and 1 additional ATP) is produced from a glucose molecule (Chen and McFeeters 1986; Condon 1987; McFeeters and Chen 1986; Talarico et al. 1990; Veiga da Cunha and Foster 1992a). For example, when glycerol is available, the regeneration of NAD^+ for glucose metabolism can be achieved through the conversion of glycerol to 1,3-PDO using glycerol as an electron receptor (Saxena et al. 2009; Veiga da Cunha and Foster 1992a). The presence of external electron acceptors, therefore, affects the energy metabolism and end-product profiles, as well as further fermentation applications of LAB.

Molecular oxygen can act as an external electron acceptor and can be advantageous to LAB during cell growth, and its presence during culture greatly influences the physiology of many LAB (An et al. 2010; Condon 1987; Higuchi et al. 2000; Marty-Teysset et al. 2000; Miyoshi et al. 2003). While oxygen itself is not toxic, reactive oxygen species [ROS; including the superoxide anion radical (O_2^-), the hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2)] which are produced during cellular processes can cause a variety of damage to the cell (Condon 1987; Higuchi et al. 2000; Miyoshi et al. 2003). Unlike aerobes and facultative anaerobes, such

as *Escherichia coli* and *Salmonella typhimurium*, that have evolved efficient mechanisms for protection against ROS (Farr and Kogoma 1991), LAB lack catalases and functional cytochrome oxidases required for energy-linked oxygen metabolism (An et al. 2011; An et al. 2010; Anastassiadis et al. 2008; Jansch et al. 2011). Some LAB possess oxidases that utilize molecular oxygen to oxidize substrates such pyruvate or NADH (Condon 1987; Marty-Teysset et al. 2000; Sedewitz et al. 1984). Generally, NADH oxidase is the most common oxidative enzyme in LAB, and the systems are typically oxygen-inducible (Condon 1987; Higuchi et al. 2000; Komagata 1996; Miyoshi et al. 2003). However, the activity of NADH oxidase can produce hydrogen peroxide (H_2O_2), which can then directly oxidize protein cysteinyl residues and inactivate enzymes (Miyoshi et al. 2003). Hydrogen peroxide can also react with cations, such as Fe^{2+} and Cu^{2+}, giving rise to hydroxyl radicals via the Fenton reaction (Miyoshi et al. 2003).

Therefore, the presence of oxygen in the growth environment of LAB will induce oxidative stress to which bacteria have various responses mechanisms. A common oxidative stress resistance mechanism found in LAB is a coupled NADH oxidase-NADH peroxidase system (Miyoshi et al. 2003). In these coupled reactions, intracellular oxygen is first used to oxidize NADH into NAD^+ by NADH oxidase, thereby releasing H_2O_2. Subsequently, H_2O_2 is reduced to H_2O by NADH peroxidase (Condon 1987; Higuchi et al. 2000; Miyoshi et al. 2003). However, the activity of NADH peroxidase is generally low (10 to 30 times lower than that of NADH oxidase) in *L. lactis* and has not been detected in some *Latobacillus* strains. Thus, cellular H_2O_2 detoxification is inefficient in some LAB capable of producing H_2O_2 under aerobic conditions (Anders et al. 1970; Komagata 1996).

The presence of oxygen during the fermentation of glycerol by *L. panis* PM1 negatively affected cell growth, glucose consumption, and end-product production, including 1,3-PDO. Protective mechanisms targeting oxidative stress are key element to optimize *L. panis* PM1 for 1,3-PDO production in biofuel by-product applications. The NADH oxidase-NADH peroxidase system and conversion of glycerol to 1,3-PDO both use NADH as a key factor for their reactions. Therefore, the clarification of the control of oxidative stress by this strain can shed light on how it regulates 1,3-PDO production. In this study, the oxygen-dependent function of NADH oxidase and NADH peroxidase and its involvement in the NAD^+ regeneration system of *L. panis* PM1 was clearly demonstrated.

5.3 Materials and Methods

5.3.1 Bacterial Strains and Growth Conditions

Lactobacillus panis PM1 was cultured at 37 ℃ using commercial MRS medium (BD, Franklin Lakes, NJ, USA) until late log phase and was then transferred to modified MRS (mMRS) medium, as described in section 3.3.1. The cultures were incubated at 30 or 37 ℃ under aerobic or microaerobic conditions. Aerobic and microaerobic cultures were grown using the same medium and temperature. Continuous aeration was provided to aerobic cultures by agitation; whereas air-tight 15-mL tubes, filled to the two-thirds level, were incubated under static conditions to establish microaerobic conditions. It should be noted that there was little difference in the behavior of *L. panis* PM1 under anaerobic and microaerobic conditions (Khan et al. 2013), thus anaerobic culture was not included in this study.

5.3.2 Quantification of H_2O_2 Production

Lactobacillus panis PM1 cells were removed from the culture media using centrifugation (16,160 × g, 5 min). Hydrogen peroxide concentrations of the cell-free media were measured in accordance with the Pierce Quantitative Peroxide Assay Kit (Thermo Scientific, Rockford, IL, USA) based on oxidation of ferrous to ferric ion in the presence of xylenol orange.

5.3.3 RNA Extraction and Reverse Transcription

RNA was extracted by the acid-hot phenol method, as described in section 4.3.6. First-strand cDNA was synthesized using reverse primers of the selected genes (Table 5.1) according to section 4.3.7.

Table 5.1 The primers used in this study

Target gene	Function	Primer	T_m (℃)[a]	Nucleotide sequence (5' →3')
16S rRNA	16S ribosomal RNA	f16S	58	tggcccaactgatatgac
		r16S	58	ctctcatgcacgttcttctt
nox	NADH oxidase	fNOX	60	actggggctgagaagacaga
		rNOX	60	tgctcatcaaaggcagtgac
npx	NADH peroxidase	fNPX	60	tcatcaggtgtaacgccaaa
		rNPX	60	taacgcccatcttcaagtcc

a. T_m, melting temperature.

5.3.4 Quantitative Real-time PCR

Real-time PCR amplification was performed in a CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA) using SsoFast EvaGreen Supermix (Bio-Rad). The total volume of the PCR master mixture was 20 µL, to which cDNA template equivalent to 25 ng RNA starting material and 0.5 µM of each primer (Table 5.1) was added. PCR amplification and validation were conducted as described in section 4.3.8. For relative gene expression, the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method, using the 16S rRNA gene for normalization, was performed as described by Livak and Schmittgen (2001). The steps for calculating the expression ratio were as follows:

$\Delta C_{T\ (test)} = C_{T\ (nox \text{ and } npx, \text{ test})} - C_{T\ (16S\ rRNA, \text{ test})}$

$\Delta C_{T\ (control)} = C_{T\ (nox \text{ and } npx, \text{ control})} - C_{T\ (16S\ rRNA, \text{ control})}$

$\Delta\Delta C_T = \Delta C_{T\ (test)} - \Delta C_{T\ (control)}$

Normalized expression ratio of *nox* and *npx* (test) = $2^{-\Delta\Delta CT}$.

The Real-Time data were processed using CFX Manager Software (Bio-Rad).

5.3.5 Preparation of Crude Cell Extracts

Lactobacillus panis PM1 cells grown to mid-exponential phase under microaerobic or aerobic conditions were disrupted by sonication, and protein concentration in the crude extract was determined by the Bradford method, as described in section 4.3.2.

5.3.6 Enzyme Assays

NADH oxidase and NADH peroxidase activities were determined by measuring the H_2O_2 concentration generated and decomposed by the crude extracts, respectively. The assay mixture contained 200 µM of the reduced form of nicotinamide adenine dinucleotide (NADH) and 20 µM flavin adenine dinucleotide (FAD^+) in 50 mM phosphate buffer at pH 6.0. The assay was carried out at 30 °C under aerated or non-aerated conditions. For the NADH peroxidase assay, H_2O_2 was added into the above assay mixture to an initial concentration of 30 µM. The concentrations of H_2O_2 generated or decomposed were quantified, as described in section 5.3.2. In these determinations, one unit of activity corresponds to the generation (for NADH oxidase) and decomposition (for NADH peroxidase) of 1 µmol of H_2O_2 per min. Specific activity was expressed as units per milligram of protein.

5.3.7 Determination of End-products

Optical density and concentration of metabolites (glucose, glycerol, organic acids, 1,3-PDO, and ethanol) were analyzed by a spectrophotometer and the optimized HPLC method, respectively, as described in section 3.3.2.

5.3.8 Statistical Analysis

For growth experiments and determinations of H_2O_2 concentrations, data are presented as the mean values calculated from at least three independent experiments. For specific activities of NADH oxidase and NADH peroxidase, standard errors of the means from at least three independent experiments were also calculated and presented. Differences in culture and enzyme assay conditions with NADH oxidase or NADH peroxidase activity (units/mg protein) were analyzed by the *t* test (Mann–Whitney test) for two groups, or the one-way ANOVA test (Kruskal-Wallis test) for three groups, using GraphPad Prism, version 5.0, software (GraphPad Software, Inc., San Diego, CA, USA). A P value of < 0.05 was considered significant.

5.4 Results and Discussion

5.4.1 Influence of Oxygen on the Physiology of *L. panis* PM1

The rates of growth during the first 24 h of culture were similar between aerobic and microaerobic *L. panis* PM1; however, the aerobically cultured *L. panis* PM1 entered stationary phase earlier than the microaerobic culture (Figures 5.1A and B). This early entry into stationary phase was associated with a halt in production of end-products, but not with glucose depletion (as approximately 30 mM glucose remained after 24 h) (Figure 5.1A), indicating that glucose concentration was not a critical cause of the cellular growth interruption. Unlike aerobically cultured samples, the microaerobic cultures consumed all available glucose (55 mM) within 48 h and produced near-equimolar amounts of lactate and ethanol (Figure 5.1B), revealing a typical heterolactic fermentation of glucose via the 6-PG/PK pathway. Also, cells grown under microaerobic conditions were observed to consume lactate during stationary phase, reducing the concentration of lactate from 53 mM to 33 mM. In contrast, aerobic cultures did not utilize lactate after cessation of glucose consumption (Figure 5.1A). Furthermore, the ratio of ethanol production to glucose consumption was less during aerobic culture (11:26 mM) than during

microaerobic culture (55:55 mM). These results indicated that, under aerobic conditions, an alternate metabolic route re-oxidized NADH formed via the 6-PG/PK pathway, while not forming ethanol.

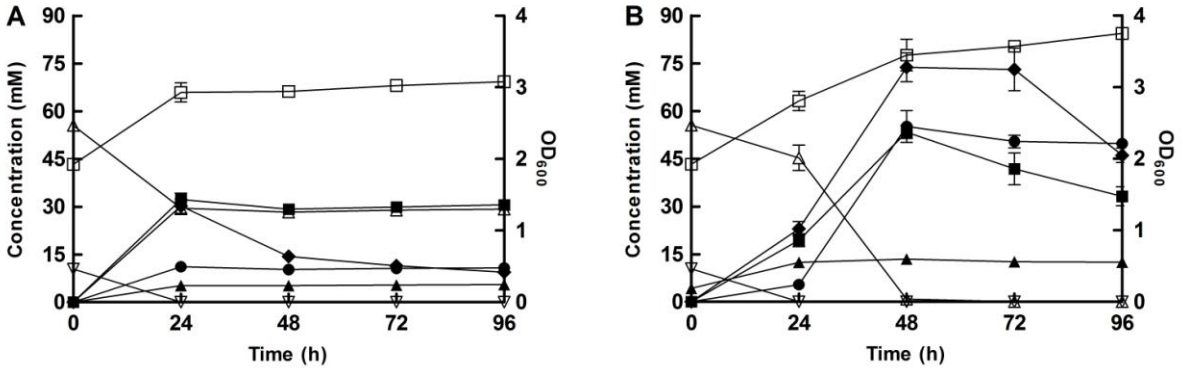

Figure 5.1 Effect of oxygen and time on growth and end-product formation in *L. panis* PM1. Growth response (OD_{600}) and end-product formation were monitored from *L. panis* PM1 cultures grown in mMRS under aerobic (A) or microaerobic (B) conditions. The concentrations of glucose (white up-pointing triangles), ethanol (black circles), acetate (white squares), lactate (black squares), succinate (black up-pointing triangles), and citrate (white down-pointing triangles) are given by the left-hand *y*-axis, and the cell density (black diamonds) is given by the right-hand *y*-axis.

5.4.2 Production of H_2O_2 by Aerobic Culture and Its Effects on Bacterial Growth

Aerobic culture resulted in the production of ten-fold higher concentrations of H_2O_2 than during microaerobic culture. Rapid accumulation of H_2O_2, reaching approximate 100 µM, was achieved in the first 24 h of aerobic culture (Figure 5.2A). The concentration of H_2O_2 necessary to completely inhibit the growth of *L. panis* PM1 was determined to be approximately 120 µM H_2O_2 (Figure 5.2B). Accumulation of H_2O_2 reached close to this inhibitory concentration level within 24 h of aerobic culture. Concurrently, a reduction of cell density was observed after 24 h aerobic culture (Figure 5.1A). This data, therefore, indicated a clear association between H_2O_2 produced under aerobic conditions and the early entrance of *L. panis* PM1 into stationary phase.

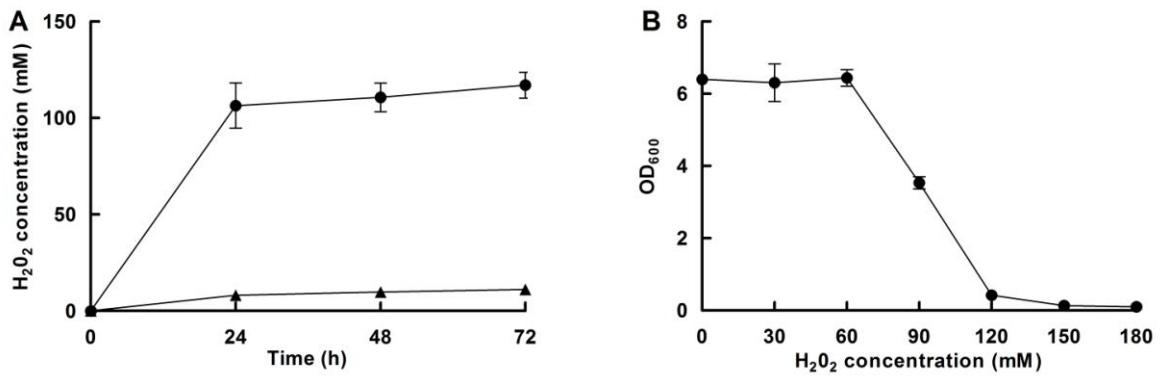

Figure 5.2 Effect of oxygen and time on the accumulation of hydrogen peroxide and growth by *L. panis* PM1. Growth response (OD_{600}) and H_2O_2 production by *L. panis* PM1 cultured in mMRS under aerobic (circles) or microaerobic (triangles) conditions after 24, 48, and 72 h (A). The inhibitory concentrations of H_2O_2 were determined from *L. panis* PM1 cultures grown in MRS broth containing H_2O_2 at concentration of 0, 30, 60, 120, 240, or 480 μM for 2 days under microaerobic conditions (B).

5.4.3 Specific Activities of NADH Oxidase and NADH Peroxidase

The draft genome data of *L. panis* PM1 (unpublished data) revealed only candidate genes for NADH oxidase and NADH peroxidase; whereas genes for other protective enzymes that might respond to the toxic effects caused by oxygen were not detected. The expression levels of these two genes were compared under aerobic and microaerobic conditions by qRT-PCR. It was determined that *nox* (NADH oxidase gene) and *npx* (NADH peroxidase gene) were expressed at similar levels under both culture conditions (Table 5.2) even though *L. panis* PM1 was shown to produce lethal levels of hydrogen peroxide under aerobic, but not microaerobic, conditions. The levels of activity of NADH oxidase and NADH peroxidase were measured from the cells grown under microaerobic and aerobic culture conditions. The specific activity of NADH oxidase was comparable ($P > 0.1$) under both aerobic and microaerobic cultures (Table 5.2). Interestingly, the activities of NADH oxidase were dependent on availability of oxygen in the respective assay reactions. When the specific activities of NADH oxidase were compared between aerated and non-aerated assay conditions, significant differences were observed ($P < 0.05$). Higher activities of NADH oxidase were observed in aerated assay than non-aerated assay for both aerobically- and microaerobically-grown cultures (158.8±7.6 vs. 92.5±2.2 and 144.0±2.0 vs. 103.1±5.6 units/mg, respectively). In contrast to NADH oxidase, NADH peroxidase activity was only detected in aerobic cultures. Enzyme assay conditions significantly affected the levels of activity of NADH peroxidase in the opposite direction of NADH oxidase ($P < 0.05$); i.e., higher enzyme

activity was observed under non-aerated assay conditions (148.3±9.7 vs. 197.3±1.7 units/mg) (Table 5.2).

Table 5.2 The specific activities of NADH oxidase and NADH peroxidase of *L. panis* PM1

Culture conditions (Enzyme sources) [a]	Assay conditions	Enzyme activity[b]		Relative gene expression level[j]	
		NADH oxidase	NADH peroxidase	NOX gene	NPX gene
Aerobic	Aerated	158.8±7.6[d]	148.3±9.7[h]	1.50±0.30	1.16±0.28
Microaerobic	Aerated	144.0±2.0[e]	N.D.[c]	1.00±0.22	1.00±0.10
Aerobic	Non-aerated	92.5±2.2[f]	197.3±1.7[i]		
Microaerobic	Non-aerated	103.1±5.6[g]	N.D.[c]		

a. *Lactobacillus panis* PM1 was cultured in mMRS for 24 h under aerobic or microaerobic conditions for enzyme assays. b. Units/mg protein, where 1 unit of activity was defined as 1 μmol of H_2O_2 generated (for NADH oxidase) or decomposed (for NADH peroxidase) per minute. c. Minus values, where H_2O_2-generating ability was higher than H_2O_2-decomposing ability, are defined as non-detectable (N.D.) in the NADH peroxidase assay. d. Significantly different to f, $P < 0.05$. e. Significantly different to g, $P < 0.05$. h. Significantly different to i, $P < 0.05$. j. Microaerobic culture was used for control sample for relative gene expression by qRT-PCR.

5.4.4. Role of Oxygen in Oxidative Stress

Oxygen availability in the culture media directly affected the coupled NADH oxidase-NADH peroxidase system of *L. panis* PM1, controlling the accumulation of H_2O_2 under aerobic conditions. When *L. panis* PM1 was cultured in 15-mL conical tubes containing 9-, 6-, and 3-mL mMRS under aerobic conditions (in order to incrementally-increase oxygen availability in the aerobic cultures), the H_2O_2 accumulation was greatest and most rapid in the 3-mL culture, reaching a maximal value by 12 h in all samples (Figure 5.3). The H_2O_2 concentration decreased after 12 h in all samples; however, the degree of H_2O_2 decomposition occurred in proportion to oxygen availability in the culture media (63% in the 3-mL culture, 33% in the 6-mL culture, and 13% in the 9-mL culture). The final amount of cell growth was in accordance with the amount of H_2O_2 accumulated in the culture media until 12 h (Figure 5.3 and Table 5.3). NADH oxidase activity was constitutively-expressed during the early stages of cell culture, and high NADH oxidase activities were determined in the all 6-h aerobic cultures (approximately 100 units/mg). The activity of this enzyme increased in a time-dependent manner until after 24 h of culture. The specific activities of NADH oxidase did not show significant differences in the three aerobic culture conditions ($P > 0.1$). In contrast to NADH oxidase, NADH peroxidase specific activity was only observed in the 3-mL 24-h culture (Table 5.3). This data clearly demonstrated that

NADH peroxidase activity was induced according to oxygen availability that also elevated the production of H_2O_2 by NADH oxidase.

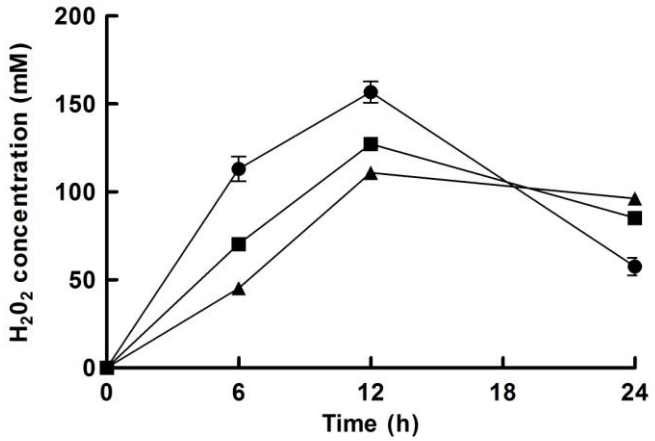

Figure 5.3 Effect of oxygen availability on accumulation of hydrogen peroxide in *L. panis* PM1 culture media. H_2O_2 production by *L. panis* PM1 cultured in 3 (circles)-, 6 (squares)-, or 9 (triangles)-mL mMRS in 15-mL conical tubes, respectively, under aerobic conditions.

Table 5.3 The result of cell growth and specific activities of NADH oxidase and NADH peroxidase according to oxygen availability

Culture Vol.	3 mL	6 mL	9 mL
Cell Growth[a] (OD_{600})	0.59	1.18	2.26
NADH oxidase activity[b]	177.6±3.0	193.2±2.5	192.8±5.7
NADH peroxidase activity[b]	120.1±12.51	N.D.	N.D.

a. *Lactobacillus panis* PM1 was cultured in mMRS under aerobic conditions, and samples for cell growth and enzyme assays were withdrawn from the cultures after 24 h. b. Units/mg protein, where 1 unit of activity was defined as 1 µmol of H_2O_2 generated (for NADH oxidase) or decomposed (for NADH peroxidase) per min. NADH oxidase and NADH peroxidase assays were carried out under aerated and non-aerated conditions, respectively.

5.4.5. The Change of NADH Flux by NADH Oxidase

Oxygen was a preferred electron acceptor for glycerol or citrate and changed the flux of NADH for reoxidation in *L. panis* PM1. Inhibitory levels of H_2O_2 were accumulated following 24 h culture of *L. panis* PM1 in mMRS containing either citrate (24 mM) or glycerol (160 mM) as electron acceptors under aerobic conditions (124 and 120 µM H_2O_2, respectively). The consumption of glucose (11 and 27 mM in citrate and glycerol media, respectively) and production of ethanol (4 and 6 mM in citrate and glycerol media, respectively) were suppressed similar to that observed in aerobic cultures lacking additional electron accepters (Figure 5.4A

and B). In addition, little citrate (7 mM) or glycerol (13 mM) was consumed. The amount of lactate produced correlated only with the amount of glucose utilized. Considering the amount of citrate or glycerol consumed and acetate produced (28 and 39 mM, respectively) in the culture media, it appeared that citrate and glycerol contributed only slightly to an increase in acetate production and utilization for NADH recycling.

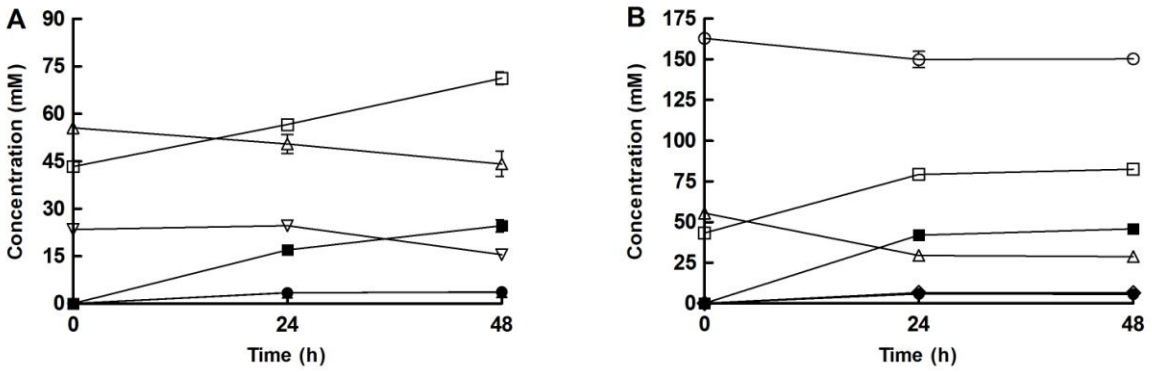

Figure 5.4 Effect of external electron acceptors on end-product formation by *L. panis* PM1. End-product formation by *L. panis* PM1 cultured in mMRS containing either 24 mM citrate (A) or 160 mM glycerol (B) as external electron acceptors under aerobic conditions. The concentrations of glycerol (white circles), glucose (white up-pointing triangles), ethanol (black circles), acetate (white squares), lactate (black squares), succinate (black up-pointing triangles), 1,3-PDO (white diamonds), and citrate (white down-pointing triangles) are provided on the left-hand *y*-axis.

The ability of *L. panis* PM1 to use glycerol as a means of NADH recycling in the absence of oxygen was previously discussed in Chapter 3. However, the presence of oxygen prevented 1,3-PDO formation and thus markedly-affected NADH recycling in this strain. In this study, the influence of oxygen on NADH recycling system and the oxidative stress resistance mechanism in its aerotolerance was investigated. Moreover, the metabolic profile was further investigated to understand how oxidative stress resistance mechanisms of *L. panis* PM1 influenced the profile of metabolic end-products.

During aerobic culture, *L. panis* PM1 prematurely entered into a stationary phase without depleting glucose (Figure 5.1A). This early entry into stationary phase was also associated with a ten-fold higher accumulation of H_2O_2 compared with microaerobic culture (Figure 5.2A). Therefore, the accumulation of H_2O_2 in aerobic culture was the apparent reason for the early cessation of growth. Anaerobic metabolism theoretically makes one ethanol per every glucose consumed, but the presence of oxygen altered this pattern to less than 1:1 ratio.

These observations suggested that H_2O_2 could be a major end-product of an alternate pathway for NADH recycling under aerobic conditions, and that this could compete with NAD^+ regeneration through ethanol production.

The production of H_2O_2 by LAB grown under aerobic conditions is commonly the result of flavoprotein oxidases, including NADH oxidase, pyruvate oxidase, α-glycerophosphate oxidase, and superoxide dismutase (Condon 1987). However, candidate genes for these enzymes were not found in the draft genome data of *L. panis* PM1, with the exception of NADH oxidase. Pyruvate oxidase has been documented in a few species of lactobacilli and is known to convert pyruvate to CO_2 and acetyl phosphate, along with the formation H_2O_2 (Condon 1987). Pyruvate oxidase has its highest activity during the early stationary phase of growth and is induced and repressed by oxygen and glucose, respectively, in *L. plantarum* (Saxena et al. 2009; Veiga da Cunha and Foster 1992a). However, the presence of pyruvate oxidase does not adequately explain the early entry into stationary phase observed during the aerobic culture of *L. panis* PM1. As seen in Figure 5.1A, most of pyruvate produced during glucose consumption was used to produce lactate in aerobic culture, indicating that pyruvate oxidase apparently removed little pyruvate from this pathway. NADH oxidase is the most common enzyme responsible for the production of H_2O_2 from oxygen and is highly-active in LAB (Condon 1987; Higuchi et al. 2000; Tachon et al. 2011). LAB are known to possess either a NADH:H_2O_2 or a NADH:H_2O oxidase, or sometimes both (Condon 1987; Higuchi et al. 2000). Final products of the reaction of NADH oxidase include either NAD^+ and H_2O_2, or NAD^+ and H_2O, depending on whether two- or four-electrons are transferred by NADH:H_2O_2 oxidase or NADH:H_2O oxidase (Condon 1987; Higuchi et al. 2000; Miyoshi et al. 2003). In this study, the crude extract from *L. panis* PM1 cultured under aerobic and microaerobic conditions could directly produce H_2O_2 using oxygen as a substrate, and the activity of the enzyme was found to increase with the addition of FAD^+ as well as the aeration of the assay mixture (approximately 1.5 fold). These results indicated that the NADH oxidase in *L. panis* PM1 was a NADH:H_2O_2 oxidase and a flavoprotein-like NADH oxidase, as seen in other gram-positive bacteria (Komagata 1996; Marty-Teysset et al. 2000; Tachon et al. 2011).

Most LAB can respond (and protect themselves) to high concentrations of H_2O_2 produced through their oxidase enzymes during sugar fermentation (Higuchi et al. 2000). In fact, most LAB possess NADH peroxidase or pseudocatalase, and superoxide dismutase exists in

some LAB (Condon 1987). These enzymes can enable LAB to overcome otherwise-lethal concentrations of hydrogen peroxide. The annotation data of the *L. panis* PM1 genome sequence and the results of the enzyme assays of NADH oxidase and NADH peroxidase suggest that these enzymes are main factors in oxidative stress resistance. The levels of accumulated H_2O_2 in the culture media could be accounted for by the differences in the activities of NADH peoxidase and NADH oxidase. qRT-PCR analyses showed that oxygen did not regulate *nox* and *npx* at the transcriptional-level, and mainly affected enzyme activities in *L. panis* PM1 (Table 5.2). While transcription levels were similar, activity assays revealed that NADH peroxidase was positively-activated by oxygen but required a long induction time to express its activity contrary to NADH oxidase. The oxygen-availability analyses indicated that higher oxygen availability in the 3-mL culture could provide higher amounts of substrate (i.e., oxygen) for NADH oxidase, resulting in greater accumulation of H_2O_2 in the first 12 h. In the subsequent 12 h, the accumulated H_2O_2 was decomposed by NADH peroxidase activity. The degree of degradation of H_2O_2 was dependent on NADH peroxidase activity, and the amount of activity was in proportion with oxygen availability (Figure 5.3 and Table 5.3). Therefore, it was concluded that a coupled NADH oxidase-NADH peroxidase system, regulated by oxygen availability, was a key oxidative stress resistance mechanism in *L. panis* PM1.

Accumulation of H_2O_2 by NADH oxidase has been reported in group I homofermentative lactobacilli, like *L. delbrueckii*, where approximate 97% of NADH was reoxidized by lactate dehydrogenase and NADH oxidase accounted for only 3% of NADH reoxidation (Marty-Teysset et al. 2000). Thus, NADH recycling in group I LAB depends on a pyruvate supply from glycolysis, rather than oxygen. Unlike homofermentative lactobacilli, the presence of electron acceptors, such as oxygen, citrate, or glycerol, directly influenced the flux of NADH reoxidation in *L. panis* PM1. In other studies, when *L. panis* PM1 was cultured in mMRS containing citrate (24 mM) and glycerol (150 mM) under microaerobic conditions, the major changes in end-product formation included a decrease in ethanol, an increase in acetate, and the production of succinate (19 mM) and 1,3-PDO (88 mM), respectively (Kang et al. 2013a; Kang et al. 2013b). The results of HPLC analyses in the present study showed that aerobic conditions negatively-affected the production of ethanol relative to glucose consumption, regardless of the presence of electron acceptors (Figures 5.1A and 5.4). Also, when *L. panis* PM1 was cultured under aerobic conditions in mMRS containing citrate and glycerol, oxygen was used as the

preferred electron acceptor, resulting in a shift of NADH flux along with a significant decrease of the production of succinate (4 mM) and 1,3-PDO (7 mM) (Figure 5.4). This data indicated that the activity of NADH oxidase was a key mechanism for the reoxidation of NADH during growth in aerobic culture.

In addition to oxidative stress responses, NADH oxidase can also help *L. panis* PM1 to use oxygen during energy metabolism, directly. That is, the shift of NADH recycling with molecular oxygen redirected acetyl phosphate, which normally would be used to produce ethanol, to the formation of acetate. This acetate production via acetate kinase can stoichiometrically-generate ATP (Condon 1987). Thus, O_2-directed NADH recycling should be advantageous with respect to energy metabolism. However, regeneration of NAD^+ via NADH oxidase in *L. panis* PM1 led to overproduction of H_2O_2, with subsequent negative effects on growth and end-product formation.

5.5 Conclusions

Findings in this chapter clearly indicate that varied oxygen availabilities of culture environments greatly affected energy metabolism as well as oxidative stress in *L. panis* PM1. Under aerobic conditions, the coupled NADH oxidase-NADH peroxidase system is the main oxidative stress resistance mechanism and NADH recycling system in *L. panis* PM1. Therefore, this result suggests that anaerobic or microaerobic conditions should be required for the production of commodity chemicals, latate, succinate, or 1,3-PDO by the PM1 strain.

5.6 Connection to the Next Study

It was found that oxygen was a significant factor affecting the physiology of *L. panis* PM1 strain among various environmental conditions evaluated in this chapter. Citrate is another factor that can affect the energy metabolism and end-product production of *L. panis* PM1, as described in Chapter 3. Thus, in the following Chapter (Chapter 6), citrate metabolism and its regulation were investigated in detail to elucidate how this pathway influences the NADH recycling systems and end-product formation in *L. panis* PM1. The results would show distinctive characteristics of citrate fermentation by the PM1 strain, and provide additional knowledge in the metabolism of this strain to base further metabolic engineering work.

6. CONTRIBUTIONS OF CITRATE IN REDOX POTENTIAL MAINTENANCE AND ATP PRODUCTION: METABOLIC PATHWAYS AND THEIR REGULATION IN *LACTOBACILLUS PANIS* PM1[iv]

6.1. Abstract

Lactobacillus panis PM1 belongs to the group III heterofermentative lactobacilli and can utilize various NADH-reoxidizing routes (e.g., citrate, glycerol, and oxygen) according to environmental conditions. In this study, the ability of *L. panis* PM1 to produce succinate, acetate, and lactate via citrate utilization was investigated. Possible pathways, as well as regulation, for citrate metabolism were examined on the basis of the genome sequence data and metabolic profiles of *L. panis* PM1. The presence of citrate led to the up-regulation, at the transcriptional level, of the genes encoding for citrate lyase, malate dehydrogenase, and malic enzyme of the citrate pathways by 10- to 120-fold. The transcriptional regulator (PocR) of the *dha* operon coding for glycerol dehydratase of *L. panis* PM1 repressed the expression of the citrate lyase gene by 10-fold. Metabolite analyses indicated that the transcriptional enhancement by citrate stimulated succinate yield. Citrate metabolism contributed to energy production by providing a major alternate pathway for NAD^+ regeneration and allowed acetyl phosphate to yield acetate/ATP instead of ethanol/NAD^+. Additionally, a branching pathway from oxaloacetate to pyruvate increased the pool of lactate, which was then used to produce ATP during stationary phase. However, the redirection of NADH-to-citrate utilization resulted in stress caused by end-products (i.e., succinate and acetate). This stress reduced succinate production by up to 50% but did not cause significant changes at the transcriptional level. Overall, citrate utilization was beneficial for the growth of *L. panis* PM1 by providing a NAD^+ regeneration route as well as extra ATP.

[iv] Tae Sun Kang, Darren R. Korber, and Takuji Tanaka. 2013. 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. This chapter is reproduced in this thesis with the permission of Springer.

6.2. Introduction

Citrate is present in many natural products such as fruit, vegetables and milk, and is commonly found as a natural metabolite in living cells. Under aerobic conditions, citrate is always dissimilated via the tricarboxylic acid (TCA) cycle. Various bacterial fermentation pathways are also involved in citrate metabolism under anaerobic conditions (Bott 1997). Citrate metabolism has been extensively studied in some genera of lactic acid bacteria (LAB) utilized as dairy starter cultures: namely, homofermentative *Lactococcus* and heterofermentative *Leuconostoc* species. Two types of citrate utilization routes have been observed in LAB: the first, associated with substrate-level phosphorylation to produce ATP, and the second, responsible for creating a trans-membrane proton motive force (PMF), to generate ATP (Hugenholtz 1993; Konings 2002). In heterofermentative *Leuconostoc* species, the citrate metabolic pathway leads to pyruvate, producing a PMF by oxaloacetate decarboxylation. Pyruvate is further processed to lactate as an end-product, thereby consuming NADH that is provided as redox equivalent of glucose fermentation. This NAD^+ regeneration saves acetyl phosphate from ethanol-producing NADH consumption and instead uses acetyl phosphate to produce acetate and ATP. In homofermentative *Lactococcus* species, the association between citrate metabolism and glycolysis occurs only at the level of end-products (e.g., acetoin and acetate), and lactate is solely the product of glycolysis and citrate is not converted to lactate. Therefore, citrate metabolism is not accompanied by substrate level phosphorylation and thus functions only as a secondary metabolite. In these citrate-utilizing bacteria, citrate permease (CitP) imports citrate and exports lactate using PMF, created by acid end-products, as the driving force that assists citrate utilization in these LAB.

The beneficial effect of citrate metabolism on the growth of LAB at acidic pH is well documented in the literature (Hugenholtz 1993; Konings 2002; Magni et al. 1999; Sanchez et al. 2008). For example, citrate metabolism in *Lactococcus lactis* CRL264 contributes to lactate toxicity resistance at low pH (Sanchez et al. 2008). Lactate produced from glucose increases citrate-lactate exchange through enhancement of citrate CitP capacity, which can reduce ATP expenditure used for pH homeostasis and eventually improves cell growth under certain acidic conditions. Citrate metabolism in *Leuconostoc mesenteroides* is also directly involved in amino acid biosynthesis (Marty-Teysset et al. 1996). In the absence of oxaloacetate decarboxylase

activity, the oxaloacetate pool formed from citrate is directed to aspartate, an essential amino acid in many *Leuconostoc* species. Aspartate is further involved in the synthesis of another amino acid, asparagine, and in the biosynthesis of pyrimidines and purines.

Despite the well-studied citrate metabolism in some lactococci and leuconostocs mentioned above, citrate metabolism in lactobacilli has not yet been fully explored. Only a few lactobacilli have been reported as being citrate-fermenting strains, and considerable variability in citrate end-product profiles have been observed. In the genus *Lactobacillus*, at least two different mechanisms of citrate utilization have been reported. Group I lactobacilli, including *L. rhamnosus* ATCC 11443 and *L. helveticus* ATCC 15807, utilize citrate as the sole carbon source, producing diacetyl, acetoin, and acetate, which have very distinctive aroma properties, as the main metabolic end-products (Medina de Figueroa et al. 2000; Torino et al. 2005). *Lactobacillus pentosus* DSM 20314 and *L. plantarum* WSO, which belong to group II lactobacilli, use a part of the TCA cycle to reduce citrate to succinate and acetate (Chen and McFeeters 1986; Cselovszky et al. 1992). Even within a single species, large variations between strains have been observed. In a strain of *L. plantarum* isolated from orange juice, acetate, but no succinate, was produced from citrate (Kennes et al. 1991); whereas another strain, *L. plantarum* 1919, produced acetate and acetoin as main end-products from citrate fermentation (Palles et al. 1998). While citrate metabolism was investigated in group I and II lactobacilli, citrate utilization by group III lactobacilli remains poorly understood. Citrate utilization has been reported in a few group III strains such as *L. reuteri* and *L. fermentum* (Axelsson and Lindgren 1987; Kaneuchi et al. 1988), but their citrate metabolism has not been investigated in detail to date.

Lactobacillus panis PM1 is an obligatory heterofermentative microorganism belonging to the group III lactobacilli. Early in this thesis (Chapter 3 and 5), this strain's ability to convert citrate to succinate was studied and the role of citrate as an external electron acceptor was proposed. Citrate reduction improved cell growth and was only observed during co-fermentation with a fermentable sugar (e.g., glucose). In this chapter, the roles of citrate utilization and its regulation was investigated using metabolite and *in silico* analyses, in conjunction with qRT-PCR of genes encoding enzymes of citrate metabolism. Also, the role of the transcriptional regulator, PocR, on citrate metabolism was evaluated in over-expressing strains.

6.3 Materials and Methods

6.3.1 Bacterial Strains and Growth Conditions

Lactobacillus panis PM1 was cultured in modified MRS (mMRS) medium at 37 ℃ under microaerobic conditions, as described in section 3.3.1, unless otherwise stated. For acidic media, the initial pH was adjusted to 4.5 through the addition of 10-N HCl. Microaerobic culture of pH 6.5 at 37 ℃ was used as the baseline condition during this study. *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, CA, USA) cells were used for cloning and preparation of the target plasmid, and cultured in LB media at 37 ℃ with vigorous shaking. Selective concentrations of erythromycin (Em) were 10 µg/mL and 300 µg/mL for *L. panis* PM1 and *E. coli*, respectively.

6.3.2 Determination of Glucose and End-products

Optical density and concentration of metabolites (glucose, organic acids, and ethanol) were analyzed by a spectrophotometer and the optimized HPLC method, respectively, as described in section 3.3.2.

6.3.3 RNA Extraction and Reverse Transcription

RNA was extracted by the acid-hot phenol method, as described in section 4.3.6. First-strand cDNA was synthesized using reverse primers of the selected genes (Table 6.1) according to section 4.3.7.

6.3.4 Quantitative Real-time PCR

Real-time PCR amplification was performed in a CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA) using SsoFast EvaGreen Supermix (Bio-Rad). The total volume of the PCR master mixture was 20 µL, to which cDNA template equivalent to 25 ng RNA starting material and 0.5 µM of each primer (Table 6.1) was added. PCR amplification and validation were conducted as described in section 4.3.8. For relative gene expression, the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method, using the 16S rRNA gene for normalization, was performed as described by Livak and Schmittgen (2001). The steps for calculating the expression ratio were as follows:

Table 6.1 The plasmids and primers used in this study

Plasmids	Relevant features	source or reference
pUC18	Cloning vector, Ap^r, 2.7 kb	Invitrogen
pACYC184	Source of chloramphenicol resistance (*cat*) gene	NEB[a]
pSIP411	Source of erythromycin resistance (*ermB*) gene	Sörvig et al. (2005)
pNCKH104	Source of replication origin (*repA*) gene	Heng et al. (1999)
pC	pUC18 derivative, in which *bla* (Ap^r) gene is replaced with the *cat* gene, Cm^r, 2.7 kb	This study
pCER	pC derivative, in which the *ermB-repA* fragment is cloned into the *BamHI-XbaI* sites, Em^r Cm^r, 5.0 kb	This study
pCER-PocR	pCER derivative expressing the *pocR* gene under control of its natural promoter, Em^r Cm^r, 6.5 kb	This study

Primer	Restriction enzymes	T_m (℃)[b]	Nucleotide sequence (5'→3')[c]	Target gene	Function
f16S		58	tggcccaactgatatgac	16S rRNA	16S ribosomal RNA
r16S		58	ctctcatgcacgttcttctt		
fAK		60	acgattgtgttcccagaagg	*ack*	Acetate kinase
rAK		60	agtgaacggcaccagatac		
fCL		60	acttgtggtgggacgacttc	*citF*	Citrate lyase (alpha chain)
rCL		60	cctggagtggtgatggagtt		
fMDH1		60	gttaactggcctgccaaaga	*mdh1*	Malate dehydrogenase 1
rMDH1		60	tcgctcagaagggcattagt		
fPA		60	acgattgtgttcccagaagg	*pta*	Phosphotransacetylase
rPA		60	agtgaacggcaccagatacc		
fME		60	attagtggccctggtgacag	*mae*	Malic enzyme
rME		60	ttcaggtcggtgagcttctt		
fPDH		60	ccgaagccaaagctgtagac	*pdhA*	Pyruvate dehydrogenase (alpha subunit)
rPDH		60	ccgtgttgaatcagttgtgg		
fPocR	*PstI*	60	aactgcagtccaagtgcttcttgatccat	*pocR*	AraC family transcriptional factor
rPocR	*SalI*	60	acgcgtcgaccccactttacccgcaatttgt		

a. New England Biolabs (Beverly, MA, USA). b. T_m, melting temperature. c. Underlined primer sequences represent restriction enzyme sites.

$\Delta C_{T(test)} = C_{T(target\ gene,\ test)} - C_{T(reference\ gene,\ test)}$

$\Delta C_{T(control)} = C_{T(target\ gene,\ control)} - C_{T(reference\ gene,\ control)}$

$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(control)}$

Normalized expression ratio of target genes$_{(test)} = 2^{-\Delta\Delta CT}$.

The Real-Time data were processed using CFX Manager Software (Bio-Rad).

6.3.5 General DNA Techniques, Plasmid Construction, and Bacterial Transformation

DNA work was performed using standard protocols, as described in section 4.3.9. *Lactobacillus panis* PM1 genomic DNA was used as a template, and the primers were designed by Primer3 (http://frodo.wi.mit.edu/primer3/) to include the original promoter and terminator elements of *pocR* gene in the amplified fragment. PCR amplification was carried out using 0.5 μM each of fPocR and rPocR primers (Table 6.1) according to section 4.3.9. The amplified *pocR* gene was digested with *PstI* and *SalI* restriction endonucleases and cloned into the *E. coli-L. panis* PM1 shuttle vector, pCER (Heng et al. 1999; Sörvig et al. 2005) (Table 6.1). The resultant plasmid was transformed into *L. panis* PM1 by the electroporation method, as described in section 4.3.9.

6.3.6 Statistical Analysis

For determinations of end-product concentrations, data were presented as mean values ± standard errors of the means calculated from at least three independent experiments.

6.4. Results and Discussion

6.4.1 *In Silico* Analysis of the Citrate Metabolic Pathway

Candidate genes encoding the enzymes for citrate metabolism were identified in the annotated results of the *L. panis* PM1 draft genome data (unpublished data), and each candidate gene was further analyzed at the UniProt database (http://www.uniprot.org; default parameters were applied) where similarities with other proteins from related bacteria were identified. Critical enzymes in citrate metabolism include: citrate lyase [CL, EC 4.1.3.6, GenBank accession No. KF312441 (alpha subunit), KF312442 (beta subunit), and KF312443 (gamma subunit)], malate dehydrogenase (MDH, EC 1.1.1.37, GenBank accession No. KF312444), fumarate hydratase (FH, EC 4.2.1.2, GenBank accession No. KF312445), succinate dehydrogenase (SDH,

EC 1.3.99.1, GenBank accession No. KF312446), L-lactate dehydrogenase (LDH, EC 1.1.1.27), malic enzyme (ME, oxaloacetate decarboxylase activity, EC 1.1.1.38, GenBank accession No. KF312447), pyruvate dehydrogenase [PDH, EC 1.2.4.1, GenBank accession No. KF312449 (alpha subunit) and KF312448 (beta subunit)], phosphotransacetylase (PTA EC 2.3.1.8, GenBank accession No. KF312450), and acetate kinase (AK, EC 2.7.2.1, GenBank accession No. KF312451). Most genes for these critical enzymes were clustered in a chromosomal locus of the *L. panis* PM1 genome, with the exception of MDH. CL, which catalyzes the initial breakdown of citrate, produces oxaloacetate and acetate, and the presence of CL is unique to citrate-utilizing LAB since it is not found in non-citrate-utilizing LAB (Hugenholtz 1993). The CL enzymes of *L. lactis* and *L. mesenteroides* are a functional complex composed of three proteins: an acyl carrier protein (ACP) (gamma subunit, *citD*), a citryl-S-ACP lyase (beta subunit, *citE*), and a citrate:acetyl-ACP transferase (alpha subunit, *citF*) (Drider et al. 2004). These three genes are organized into a five-gene operon structure (*mae-citCDEF*) coding for ME, citrate lyase ligase, and CL in *L. panis* PM1. The genetic context of the ME gene in *L. panis* PM1 is very similar to that of LAB possessing oxaloacetate decarboxylase activity, including *L. lactis*, *L. mesenteroides*, *Oenococcus oeni*, and *Weissella paramesenteroides* (Drider et al. 2004; Sender et al. 2004) where this gene is coupled with *cit* gene clusters, including *citC* (citrate lyase ligase), *citDEF* (citrate lyase), and *cit(X)G* (unknown function). ME is involved in oxaloacetate decarboxylation coupled to proton consumption, and its role as a secondary PMF-generating pathway has been well-documented in *L. lactis* (Pudlik and Lolkema 2011b). Two genes encoding MDH, which converts oxaloacetate to malate with the consumption of NADH, were found in other loci in the PM1 genome. MDH1 (GenBank accession No. KF312444) and MDH2 showed 91% and 82% identity, respectively, to the MDH of *L. reuteri* at the amino acid level, and *mdh1* was used for further qRT-PCR work. The genes encoding two enzymes involved in the subsequent reduction of malate to succinate were grouped in a four-gene operon structure coding for FH, SDH, LDH, and the 2-oxoglutarate-malate translocator. As an alternate route for acetate production (from pyruvate to acetate), four genes were annotated as alpha (*pdhA*) and beta (*pdhB*) subunits of PDH, PTA, and AK. The cross-membrane assimilation of citrate is a key aspect of citrate utilization. CitP, which is plasmid-encoded, catalyzes the efficient exchange of citrate and lactate, and its amino acid sequence is highly-conserved with over 98% identity in *L. lactis*, *L. lactis*, *W. paramesenteroides*, and *L. mesenteroides* (Drider et al. 2004). This annotation data did not reveal

candidate genes homologous to the *citP* gene. However, the ability of *L. panis* PM1 to metabolize citrate strongly suggested the existence of a CitP-like citrate transporter system.

6.4.2 The Effect of Citrate on Cell Growth and Metabolic Profile

Growth of *L. panis* PM1 is strongly influenced by pH (Khan et al. 2013). HCl acidification of medium resulted in a faster growth rate during the exponential growth phase (i.e., 0.09 h^{-1} at pH 6.5 compared to 0.26 h^{-1} at pH 4.5) (Figure 6.1). During this accelerated growth, the glucose consumption rate also increased from 1.9 to 2.1 $mM \cdot h^{-1}$. Addition of 26 mM citrate decreased the pH of the media to approximately the same acidity (pH 4.5) as the HCl-adjusted medium. The acidification caused by citrate addition affected cell growth and glucose consumption more positively when compared to HCl-adjusted acidic conditions; the growth rate increased from 0.26 to 0.35 h^{-1}, and the rate of glucose consumption accelerated from 2.1 to 3.6 $mM \cdot h^{-1}$.

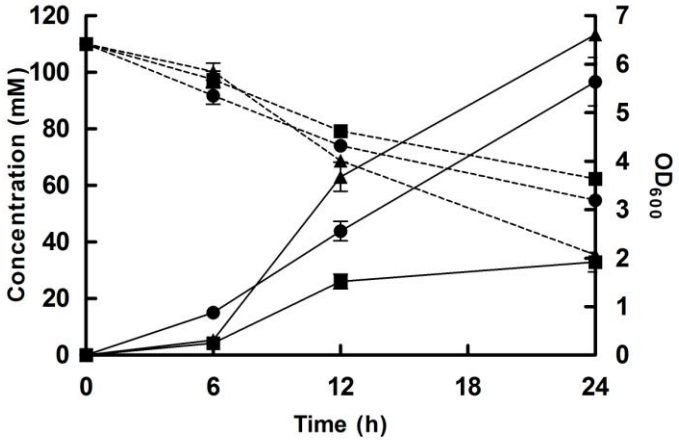

Figure 6.1 Growth and glucose consumption of *L. panis* PM1. *Lactobacillus panis* PM1 was cultured in normal MRS (pH 6.5, squares) or MRS adjusted to pH 4.5 with HCL (circles) or to pH 4.5 with citrate (triangles) for 24 h. Solid and dashed lines represent cell growth and remaining glucose concentration, respectively.

Citrate availability also influenced metabolic profiles of *L. panis* PM1 (Figure 6.2). When *L. panis* PM1 was grown in mMRS containing different concentrations of citrate with a fixed amount of glucose (14 to 62 mM citrate; 55 mM glucose), glucose and citrate were consumed within 48 h. These cultures showed different amounts of end-products (i.e., succinate, lactate, ethanol, and acetate), indicating citrate concentration strongly influenced metabolic activity. The final yields of succinate, lactate, and acetate increased as more citrate was available, while

ethanol production declined. The yield of succinate increased from 11.2 to 40.9 mM over the range of 14 to 62 mM citrate. These yields indicated that the conversion ratio of citrate to succinate decreased from 79% to 66%. Slight increases in the size of the lactate pool were observed at 17 and 23 mM citrate concentrations, compared to 14 mM (from 43.2 to 43.4 and 45.0 mM, respectively); lactate production notably increased at higher citrate concentrations (62.5 and 88.7 mM lactate in the presence of 36 and 62 mM citrate, respectively). In contrast to succinate and lactate, yields of ethanol were reduced by higher initial citrate concentrations. Over the range of 17 to 36 mM citrate, ethanol production became reduced by 15% to 28%, respectively; furthermore, 62 mM citrate reduced ethanol yield by 83% relative to the standard culture condition (48.1 mM vs. 8.2 mM ethanol). Acetate production increased by 4.3, 19.1, 44.6, and 76.1 mM relative to the standard reference culture when citrate concentration was increased from the standard 14 mM to 17, 23, 36 and 62 mM, respectively. It should be noted that the total yield of acetate exceeded the initial citrate concentration, indicating the presence of routes other than CL activity to yield acetate from citrate.

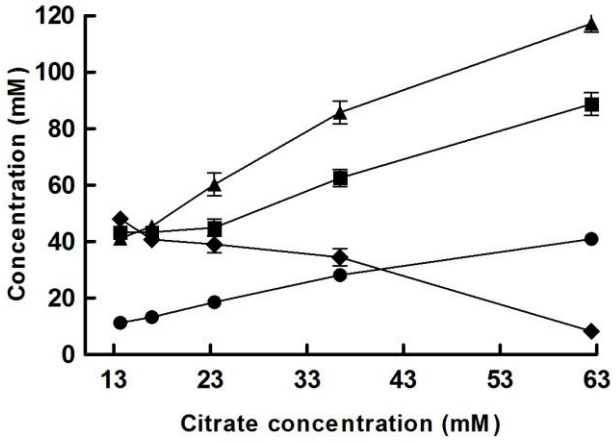

Figure 6.2 The effect of the presence of citrate on the production of end-products by *L. panis* PM1. *Lactobacillus panis* PM1 was cultured in mMRS containing 14, 17, 23, 36, or 62 mM citrate, and production of acetate (triangles), lactate (squares), succinate (circles) and ethanol (diamonds) was analyzed by HPLC from the cultures after 48 h.

The four end-products (lactate, acetate, succinate and ethanol) showed different fates after 48 h culture when glucose was depleted. Figure 6.3 exhibits the changes of metabolite concentration (acetate and lactate) after 48-h culture in the presence of citrate at a variety of concentrations. The reduction of lactate after glucose depletion has previously been observed in *L. panis* PM1 grown in glucose media along with a gradual increase in acetate (Kang et al.

2013c). Figure 6.3 also shows that a 38% decrease in lactate concentration was seen in the standard culture (14 mM citrate) between 48 and 96 h; this lactate disappearance gradually became reduced with increasing citrate concentration (29%, 25%, 8%, and 2% at 17, 23, 36, and 62 mM citrate, respectively). Increases in acetate concentrations were observed between 48 and 96 h and the increases were higher at low citrate concentrations (26%, 31%, 17%, 1%, and 6% at 14, 17, 23, 36, and 62 mM citrate, respectively) (Figure 6.3). Metabolite analyses revealed that succinate and ethanol did not significantly change after 48-h culture (less than 5% at all concentrations), in contrast to lactate and acetate.

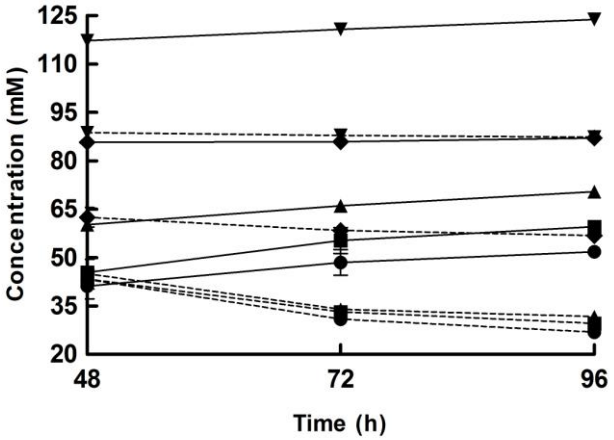

Figure 6.3 The effect of the presence of citrate on acetate and lactate concentration changes by *L. panis* PM1 during stationary phase. *Lactobacillus panis* PM1 was cultured in mMRS containing 14 (circles), 17 (squares), 23 (up-pointing triangles), 36 (diamonds), or 62 (down-pointing triangles) mM citrate for 96 h. The concentrations of acetate (solid lines) and lactate (dashed lines) were monitored for 3 days after glucose depletion.

6.4.3 Transcriptional Analysis of Citrate Metabolism

To further evaluate citrate metabolism by *L. panis* PM1, the expression of the genes encoding AK, CL, MDH1, ME, and PDH (*ack*, *citF*, *mdh1*, *mae*, and *pdhA*, respectively) were quantified in media with and without 23 mM citrate using qRT-PCR (Table 6.2). The expression levels of *ack* and *pdhA* were similar under both conditions. In contrast to these two genes, the genes for CL and MDH1, which are directly involved in citrate metabolism, were significantly up-regulated in the citrate media. Approximately 10- to 30-fold higher expression of *citF* and *mdh1* genes was measured compared to growth in medium without citrate. Citrate also increased the expression of the ME gene, which permits the branching of citrate utilization to pyruvate

production, by approximately 120-fold relative to the standard culture, confirming that citrate can positively-regulate its own metabolism at the transcriptional level.

Table 6.2 The effect of citrate on expression of citrate pathway

Medium[a]	Relative gene expression level				
	***ack* (AK)**	***citF* (CL)**	***mdh1* (MDH)**	***mae* (ME)**	***pdhA* (PDH)**
1	1.00±0.50	1.00±0.25	1.00±0.57	1.00±0.98	1.00±0.27
2	0.67±0.46	12.31±3.13	28.18±17.30	120.92±42.73	1.27±0.28

a. *Lactobacillus panis* PM1 was cultured for 24 h in following media. 1- mMRS, control condition for qRT-PCR. 2- mMRS containing 23 mM citrate.

6.4.4 The Negative Effect of Acetate and Succinate on Citrate Metabolism

The production of succinate underwent significant changes in their metabolic profiles during culture experiments (Table 6.3). In either supplementation condition (167 mM acetate or 21 mM succinate), the initially-available citrate (23 mM) was used up within 24 h; however, the final yield of succinate was different under each condition. Succinate production reached only 12.58 and 9.29 mM in the presence of high initial concentrations of acetate and succinate, respectively; whereas 18.64 mM succinate was observed in the standard culture, i.e., no addition of either succinate or acetate. The molar ratio of succinate to citrate was 81%, 55%, and 40% for the standard culture, acetate-culture and succinate-culture, respectively. Acetate production was similarly affected, but to a lesser degree. The addition of acetate or succinate decreased acetate production by 94% and 88%, respectively, relative to standard culture conditions. Meanwhile, the production of another end-product, ethanol, became increased after the addition of the two metabolites (by 16% and 23% by 167 mM acetate and 21 mM succinate, respectively). While yields of acetate, succinate and ethanol were affected, no significant change was observed for lactate under either supplementation regime. To gain further insight into changes in citrate metabolism, expression levels of the key genes encoding enzymes for the production of acetate and succinate (AK, CL, MDH1, ME, PDH, and PTA) were monitored under these conditions using qRT-PCR. High amounts of acetate and succinate caused down-regulated the expression of *citF* (4-fold) and *ack* genes (5-fold), respectively, relative to the standard conditions (Table 6.3). Also, the presence of the two end-products caused a 4- to 7-fold down-regulation in the *pta* gene.

Table 6.3 The effect of acetate and succinate on expression of the citrate pathway and yield of end-products

Medium[a]	Relative gene expression level						Metabolite production (mM)			
	***ack* (AK)**	***citF* (CL)**	***mdh1* (MDH)**	***mae* (ME)**	***pta* (PTA)**	***pdhA* (PDH)**	**Acetate**	**Ethanol**	**Lactate**	**Succinate**
1	1.00±0.53	1.00±0.53	1.00±0.72	1.00±0.44	1.00±0.53	1.00±0.54	52.10±5.27	28.11±2.95	59.41±7.86	18.64±0.79
2	0.38±0.25	0.25±0.09	0.94±0.39	0.60±0.18	0.24±0.10	0.39±0.18	49.17±3.25	32.49±0.36	58.64±4.49	12.58±1.59
3	0.17±0.07	0.68±0.32	1.26±0.89	0.80±0.25	0.14±0.06	1.15±0.44	45.71±4.11	34.44±3.12	58.94±1.93	9.29±0.08

a. *Lactobacillus panis* PM1 was cultured for 24 h (for qRT-PCR) or 48 h (for HPCL analysis) in the following media.
1- mMRS containing 23 mM citrate, control condition for qRT-PCR
2- mMRS containing 23 mM citrate and 167 mM acetate
3- mMRS containing 23 mM citrate and 21 mM succinate

The *pdh* gene was not affected by either acetate, succinate, or citrate (Tables 6.2 and 6.3). In contrast to the activation by citrate, shown in Table 6.2, acid stress did not cause significant changes at the transcriptional level.

6.4.5. Transcriptional Repression of Citrate Metabolism

Following an extensive screen, it was determined that the transcriptional regulator of the *dha* operon in *L. panis* PM1, composed of glycerol dehydratase and its reactivation factor, could significantly repress the expression of the CL gene as well as final succinate yield. This gene encodes a putative transcriptional regulator (359 amino acid, GenBank accession No. KF312452) of the AraC family that contains typical helix-turn-helix domain as well as a 43% amino acid identity with PocR found in *L. reuteri* in BLAST search. PocR has been reported as a regulatory protein in propanediol utilization and vitamin B_{12} biosynthesis in enteric bacteria (Bobik et al. 1992; Santos et al. 2011). Interestingly, in comparison with a PM1 strain transformed with pCER (an empty plasmid), over-expression of this gene (PocR) strongly inhibited succinate production (16.41 vs. 2.60 mM) (Table 6.4), leaving most of the available citrate untouched (20.37 mM). In addition, the over-expressing strain displayed a significant decrease in acetate yield from 104.76 to 11.63 mM, which was accompanied by an approximately 200% increase in ethanol concentration; whereas a significant change was not observed in lactate production. To probe the transcriptional role of the PocR of *L. panis* PM1 on citrate metabolism, the expression of the *citF* gene, encoding the first enzyme for citrate dissimilation, was evaluated by qRT-PCR using RNA obtained from the PM1-pCER and over-expressing strains. The expression of this gene was repressed (10-fold) in the over-expressing strain relative to the PM1-pCER strain (Table 6.4), suggesting a transcriptional repressor role of the PocR gene.

Table 6.4 The effect of over-expression of PocR gene on expression of the CL gene and yield of end-products

Strains[a]	Relative gene expression level	Metabolite production (mM)				
	***citF* (CL)**	**Acetate**	**Ethanol**	**Lactate**	**Succinate**	**Citrate[b]**
pCER	1.00±0.08	104.76±0.62	23.79±0.24	52.15±0.73	16.41±0.53	N.D.
pCER-PocR	0.10±0.01	11.63±0.19	69.87±0.85	56.05±0.07	2.60±0.08	20.37±0.07

a. Each strain was cultured in mMRS medium containing 23 mM citrate for 24 h (for qRT-PCR) or 48 h (for HPCL analysis). b. Remaining citrate after 48 h. N.D., not detected.

Citrate utilization has been studied in only a few strains of group III lactobacilli. The first evidence of succinate production from citrate was observed in two strains of *L. reuteri* (Kaneuchi et al. 1988). However, succinate production by *L. reuteri* appears to vary among subspecies since it was observed that *L. reuteri* DSM20016 strain did not produce succinate (Chapter 3). The metabolism of citrate to succinate consumes NADH; therefore, the existence of this system may influence sugar metabolism by influencing the availability of reducing equivalents. In Chapter 3, it was demonstrated that *L. panis* PM1 has the unique ability to consume citrate through the non-stoichiometrical production of various end-products. In the present study, pathways of citrate metabolism in a group III lactobacillus (*L. panis* PM1) and its regulation by the presence of citrate, its metabolic end-products (acetate and succinate), and the over-expression of PocR was investigated.

Possible citrate metabolic pathways were examined from the metabolite profiles and *in silico* analysis of the genome sequence of *L. panis* PM1 (Figure 6.4). Important genes necessary for the production of succinate, acetate, and lactate from citrate were annotated, except for citrate permease (CitP). Medina de Figueroa et al. (2000) reported that 13 lactobacilli strains capable of utilizing citrate possessed no homologues to the *citP* of *L. lactis* in their plasmids or chromosomal DNA, indicating the essential presence of citrate transport systems with little or no homology to *citP*. Like those lactobacilli, citrate consumption by *L. panis* PM1 suggests the existence of a citrate-importing system.

Citrate metabolic patterns are mostly determined by the activities of MDH and ME in LAB. The succinate pathway has been reported in a few group II lactobacilli strains during co-fermentation of citrate and fermentable sugars (e.g., glucose or lactose), and stoichiometric amounts of succinate and acetate produced from citrate via the reductive TCA cycle (CL, MDH, FH, and SDH) were seen in these strains (Chen and McFeeters 1986; Cselovszky et al. 1992; Dudley and Steele 2005). In many citrate-fermenting LAB, including *L. lactis*, *L. mesenteroides* and group I and II lactobacilli, citrate is converted to pyruvate by oxaloacetate decarboxylase activity, which leads to an altered end-product pattern (e.g., acetoin, diacetyl, and acetate) (Kennes et al. 1991; Marty-Teysset et al. 1996; Palles et al. 1998; Pudlik and Lolkema 2011a; Torino et al. 2005). The annotated *L. panis* PM1 genome revealed the presence of genes encoding both ME and MDH; however, 66% to 79% of the initial citrate was converted to

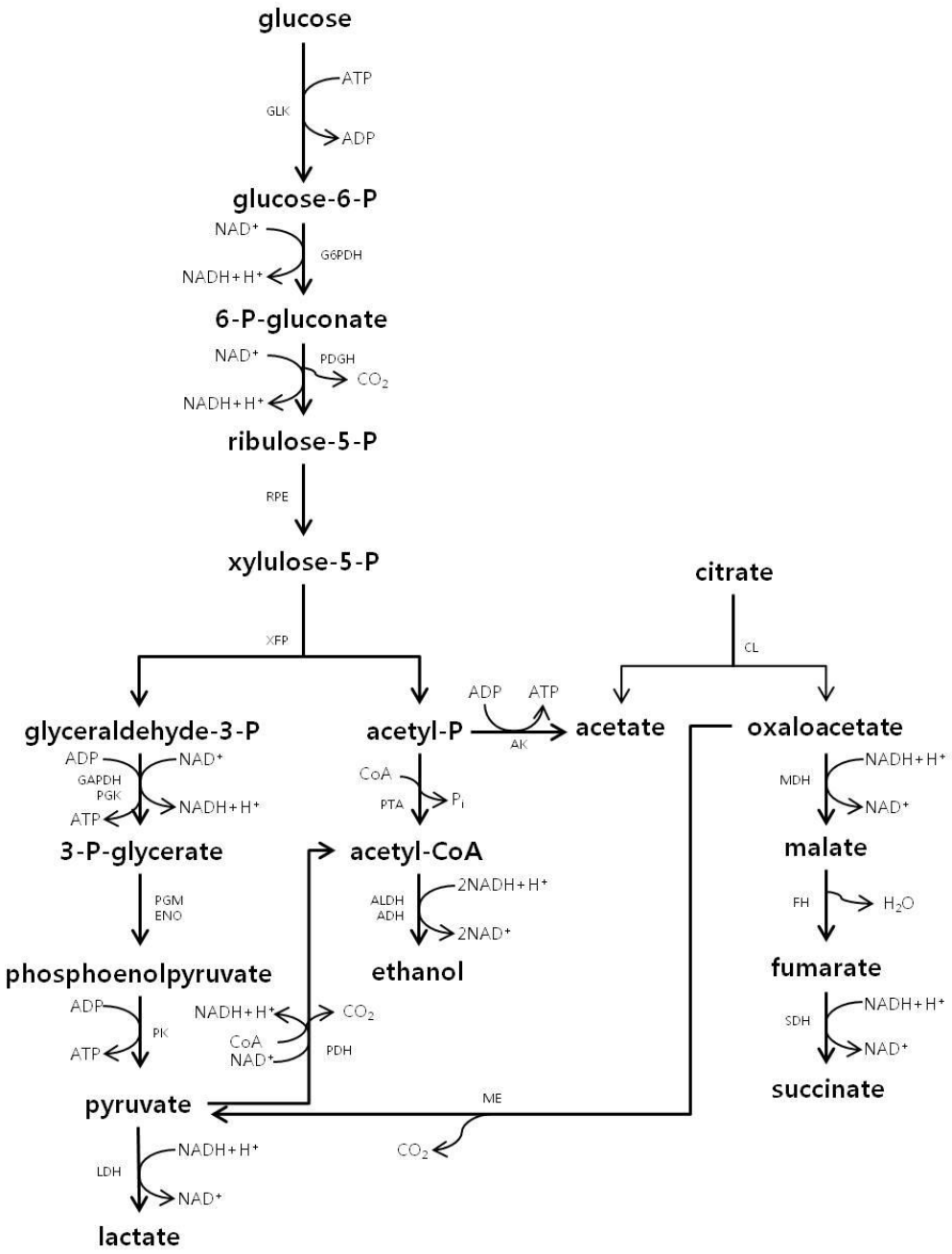

Figure 6.4 Schematic diagram of the citrate metabolic pathway in *L. panis* PM1. Abbreviation, ADH, alcohol dehydrogenase; AK, acetate kinase; ALDH, acetaldehyde dehydrogenase; CL, citrate lyase; CoA, coenzyme A; ENO, enolase; FH, fumarate hydratase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLK, glucokinase; G6PDH, glucose 6-phosphate dehydrogenase; LDH, lactate dehydrogenase; ME, malic enzyme; MDH, malate dehydrogenase; PDH, pyruvate dehydrogenase; PGDH, 6-phosphogluconate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglyceromutase; PK, pyruvate kinase; PTA, phosphotransacetylase; RPE, ribulose 5-phosphate 3-epimerase; SDH, succinate dehydrogenase; and XFP, xylulose 5-phosphate phosphoketolase.

succinate, and acetoin and diacetyl were not detected in *L. panis* PM1 citrate fermentation broth, indicating unique pathways for citrate metabolism by this organism. The lactate pool was also enlarged by citrate availability in the culture media until glucose depletion (Figure 6.2). The observation of these three end-products (succinate, acetate, and lactate) and their molar ratios suggested that a part of oxaloacetate was diverted to pyruvate (by ME activity), and then further directed to the production of lactate or acetate.

Pathways for the conversion of pyruvate to acetate were considered on the basis of decreases in lactate and increases in acetate after glucose depletion (Figure 6.3). Three different routes for the conversion of pyruvate to acetyl phosphate have been reported in LAB, which is then converted to acetate (Pudlik and Lolkema 2011a). Pyruvate oxidase (POX) can directly convert pyruvate to acetyl phosphate; however, the absence of this enzyme in *L. panis* PM1 was previously discussed in Chapter 5. The other two routes are via acetyl coenzyme A that is formed from pyruvate by pyruvate-formate-lyase (PFL) or PDH; subsequently, this acetyl coenzyme A is converted to acetyl phosphate by phosphotransacetylase (PTA). The annotation data of the *L. panis* PM1 genome sequence showed only genes for PDH and PTA. Moreover, formate that is produced by the activity of PFL was not detected as an end-product in my metabolite analyses. Therefore, if the pyruvate pool produced from oxaloacetate is directed to acetate production, a combination of PDH and PTA enzymes would contribute to the acetate production. It should be noted, though, that the PDH-PTA pathway could be less beneficial to the cell in terms of overall redox balance since acetate production via this route can generate one NADH; whereas, lactate formation by LDH consumes one NADH. This would lead to additional NADH production that would likely exceed the capacities for NAD^+ regeneration (e.g., via ethanol pathway) during rapid glucose metabolism (Richter et al. 2003; Richter et al. 2001; Zaunmuller et al. 2006). Therefore, unless LDH activity becomes limited for the pyruvate pool produced from glucose metabolism, the pyruvate from citrate metabolism would increase the lactate pool rather than produce acetate via the PDH-PTA pathway. This explains why lactate tended to accumulate until glucose was used up, and later, why this lactate pool was converted into acetate after depletion of the available energy (Figure 6.3).

Under acidic conditions (pH 4.5), the growth of *L. panis* PM1 was accelerated in combination with more rapid glucose consumption (Figure 6.1). The pH gradient created by this acidity (pH 4.5) could reduce ATP expenditure for initial glucose uptake through glucose

importation via a sugar:H^+ symporter, which eventually would stimulate glucose consumption and growth (Khan et al. 2013). Although citrate cannot serve as the sole carbon source for strain PM1 (Figure 3.2), 26 mM citrate enabled faster growth and glucose consumption at pH 4.5 than in the absence of citrate (i.e., HCl-adjusted acidic conditions), indicating the beneficial effect of citrate during energy metabolism. Similar stimulatory effects have been found in some heterofermentative lactobacilli, leading to the production of acetate and ATP instead of ethanol (i.e., redirection of acetyl phosphate) (Drinan et al. 1976; Kennes et al. 1991; Medina de Figueroa et al. 2000; Pudlik and Lolkema 2011a; Torino et al. 2005; Zaunmuller et al. 2006). The citrate-to-succinate pathway of *L. panis* PM1 can be used as an alternate route for regeneration of NAD^+ through the activities of two dehydrogenases (Figure 6.4); thus, the presence of citrate also contributed to changes in metabolite profiles. The production of succinate and acetate increased along with increased citrate availability, and were accompanied by decreases in ethanol (Figure 6.2). These observations clearly demonstrate that citrate acted as an external electron acceptor through the citrate-to-succinate route, thereby assisting in the production of extra ATP during glucose metabolism. Also, when considering the reduced degradation of the lactate pool in the presence of higher citrate concentrations (36 and 62 mM) after glucose depletion (Figure 6.3), citrate metabolism could produce sufficient additional ATP for extended survival during the stationary phase.

The regulation of citrate metabolism in LAB has a number of physiological consequences and depends on the organism in question, as well as their surrounding environment. CitP and CL are constitutively expressed in *L. mesenteroides*, but their expression levels are up-regulated by citrate. On the other hand, oxaloacetate decarboxlase is not expressed in the absence of citrate, redirecting oxaloacetate into aspartate by transamination (Marty-Teysset et al. 1996). The citrate pathway of *L. lactis* is constitutively expressed at neutral pH and induced by acidic pH, but not by citrate; thus, expression of the *citQRP* operon is 14-fold higher at pH 4.5 than at pH 6.5 in *L. lactis* (Magni et al. 1999). Furthermore, the *citMCDEFGRP* operon of *L. paramesenteroides* is induced by citrate, independent of the medium pH, at the transcriptional level (Drider et al. 2004; Martin et al. 2000).

The genes encoding CL, MDH1, and ME enzymes of the citrate metabolic pathway of *L. panis* PM1 are significantly up-regulated by the presence of citrate (Table 6.2). However, the acid stress caused by acetate (167 mM, 1%) or succinate (21 mM, 0.25%) was not sufficient to activate

citrate metabolism in *L. panis* PM1 at the transcriptional level; whereas final yields of acetate and succinate were reduced by acid stress (Table 6.3). Smaller decreases (6% to 12%) in yields of acetate than for succinate (33% to 50%) under acidic conditions could be related to the down-regulation of the *ack* and *pta* genes (Table 6.3). However, the 4-fold down-regulation of the *citF* gene induced by acetate stress would not be a logical factor responsible for reducing final concentrations of succinate and acetate when considering its up-regulation by citrate (12-fold; Table 6.2) along with the complete depletion of initial citrate. The transcriptional regulator, PocR, of the *dha* operon of *L. panis* PM1 could act as a transcriptional repressor of the citrate pathway. A global regulatory role of PocR has been reported in *L. reuteri*, and the over-expression of this gene differentially-regulates 120 genes related to coenzymes, secondary metabolites and energy production (Santos et al. 2011). The over-expression of PocR in *L. panis* PM1 strongly repressed the expression of the *citF* gene along with the final yields of succinate and acetate (Table 6.4). This observation clearly indicates the transcriptional repressor role of PocR on the *mae-citCDEF* operon of *L. panis* PM1.

6.5 Conclusions

In this study, citrate metabolism in *L. panis* PM1 was investigated and it showed a unique feature that resulted in the co-production of succinate and pyruvate (and ultimately lactate). This metabolic pathway can provide the PM1 strain with an alternate NAD^+ regeneration route and additional ATP for stationary phase survival. Its regulation at the transcriptional level, as well as activation by the presence of citrate and repression by PocR, can efficiently control the routes of NAD^+ regeneration (either the citrate-to-succinate route or the acetyl phosphate-to-ethanol route). Also, the two main end-products of citrate metabolism, succinate and acetate, function in the system's regulation. The acid stress caused by the two end-products feedback-inhibits their final yield without significant changes at transcriptional level, suggesting the presence of another protein-level regulatory system. Citrate metabolism and its fine-regulatory control system provide *L. panis* PM1 strain with a more competent ability to adapt to a wide spectrum of environmental and nutritive conditions.

6.6 Connection to the Next Study

Citrate metabolism was a key NAD^+ regeneration route, with direct links to central metabolism, affecting overall energy efficiency and end-product patterns of *L. panis* PM1. As

evidenced in Chapter 3, the conversion of glycerol to 1,3-PDO is a key feature of *L. panis* PM1 in terms of fundamentally understanding the metabolism of this strain. This glycerol-processing route is especially interesting from the standpoint of industrial applications, including whether 1,3-PDO production from low-price glycerol is feasible. Thus, in the following Chapter (Chapter 7), the physiological roles and regulatory mechanisms of the glycerol-to-1,3-PDO pathway of *L. panis* PM1 were studied in detail. This deeper understanding of glycerol metabolism by *L. panis* PM1 would contribute to the application of this strain in cost-effective processes utilizing low-price glycerol to produce 1,3-PDO.

7. GLYCEROL AND ENVIRONMENTAL FACTORS: EFFECTS ON 1,3-PROPANEDIOL PRODUCTION AND NAD^+ REGENERATION IN *LACTOBACILLUS PANIS* PM1[v]

7.1 Abstract

This study was aimed to understand influences of fermentation factors on NADH recycling and mechanisms of 1,3-propanediol (1,3-PDO) production in *Lactobacillus panis* PM1. Metabolite analyses, qRT-PCR of the glycerol reductive pathway [glycerol dehydratase (DhaB) and 1,3-PDO dehydrogenase (DhaT)], and DhaT activity assays were conducted at different pH values, temperatures, and initial glycerol concentrations. The supplementation of 150 mM glycerol caused a shift in NADH flux from ethanol to 1,3-PDO production; whereas 300 mM glycerol negatively-affected the regeneration of NAD^+ via 1,3-PDO production. This retardation decreased transcript levels and specific activities of DhaT. The decreased DhaT activity eventually caused a shutdown of 1,3-PDO production. Temperature and pH did not significantly affect the specific activity of DhaT; whereas DhaB and DhaT gene expression became activated under acidic conditions. Moreover, fresh glucose addition after its depletion could not restart glycerol reduction, but rather led to increased ethanol production. The tested environmental factors affected 1,3-PDO production in different ways, including changing the expression level of enzymes and shifting the NAD^+ regeneration pathways. These findings offer key targets for optimizing 1,3-PDO production by *L. panis* PM1 for improvement of 1,3-PDO manufacturing efficiencies.

[v] Tae Sun Kang, Darren R. Korber, and Takuji Tanaka. 2013. 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. This chapter is reproduced in this thesis with the permission of Wiley-Blackwell.

7.2 Introduction

A key feature of lactobacilli metabolism is efficient carbohydrate fermentation coupled with substrate-level phosphorylation through the production of lactate and/or ethanol. To adapt to various environmental conditions, lactobacilli shift their metabolism, which leads to significantly different end-product profiles (Axelsson and Lindgren 1987). In homolactic fermentation, NADH formed during early glycolysis is mainly reoxidized by lactate dehydrogenase, thereby producing lactate as the only end-product; whereas heterolactic fermentation achieves primary carbohydrate fermentation through substrate-level phosphorylation in the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway wherein NADH is mainly recycled back to NAD^+ through the production of lactate and ethanol (Kandler 1983; Stiles and Holzapfel 1997) (Figure 7.1).

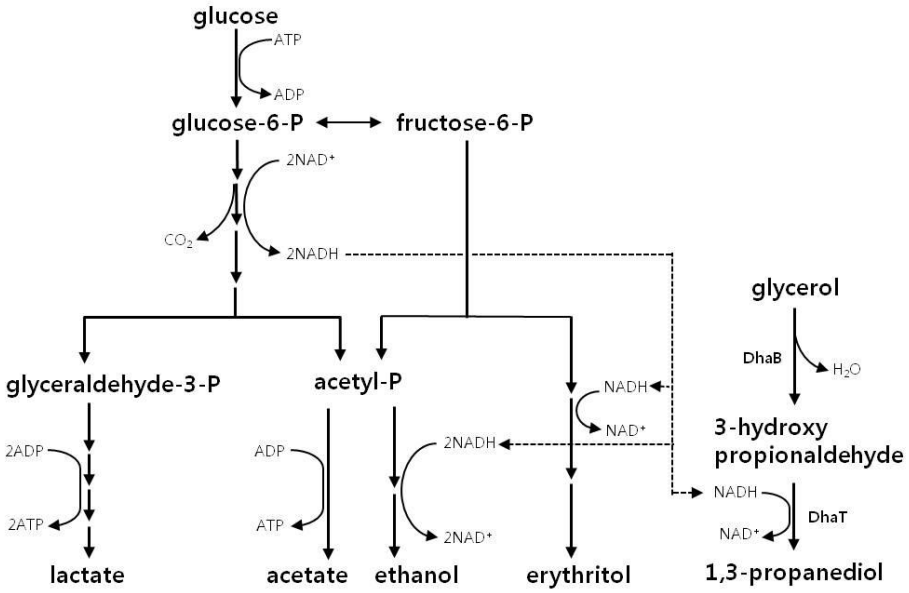

Figure 7.1 Schematic diagram of the glycerol and glucose metabolic pathways and a possible erythritol formation route of *L. panis* PM1. Glucose metabolism via the 6-phosphogluconate pathway produces two NADH molecules, and the fate of these NADH molecules is finely-regulated through the production of various end-products, including acetate lactate, ethanol, 1,3-PDO and erythritol. DhaB and DhaT represent glycerol dehydratase and 1,3-propanediol dehydrogenase, respectively.

The difference in the manner by which NADH is recycled is closely related to energy metabolism. The homofermentative pathway generates two moles of ATP per mole of glucose; whereas the heterofermentative pathway yields one ATP per glucose (Poolman 1993; Zaunmuller et al. 2006). Besides this main route of metabolic energy production,

heterofermentative lactic acid bacteria (LAB) can use external electron acceptors, such as citrate, fructose, and oxygen, to reoxidize NADH in conjunction with the ethanol production pathway, and the phosphoketolase/external electron acceptor pathways can, for example, yield one or two extra ATP per glucose (Zaunmuller et al. 2006).

Lactobacillus panis PM1 belongs to obligatory heterofermentative lactobacilli and is of interest due to its ability to produce 1,3-propanediol (1,3-PDO) during anaerobic glycerol fermentation (Khan et al. 2013). Although glycerol cannot be used as the sole carbon source by *L. panis* PM1, glycerol fermentation plays an important role as an NAD^+ regeneration route. *Lactobacillus panis* PM1 ferments glycerol via the reductive pathway where glycerol is first converted to 3-hydroxypropionaldehyde (3-HPA) catalyzed by glycerol dehydratase (DhaB), after which 3-HPA is reduced to 1,3-PDO by 1,3-propanediol dehydrogenase (DhaT) (Figure 7.1). In this process, DhaT plays a key role in the regeneration of NAD^+. However, it has been reported that this enzyme is sensitive to its substrate, 3-HPA (Celinska 2010; Hao. et al. 2008). Therefore, the accumulation of this compound triggers decreased DhaT activity, which in turn accelerates further buildup of 3-HPA in *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Enterobacter agglomerans* (Barbirato et al. 1997; Celinska 2010).

Various factors affecting the fine regulation of the DhaBT pathway have been reported for *Klebsiella*, *Citrobacter*, and *Lactobacillus* (Biebl et al. 1999; Borch et al. 1991; Celinska 2010; Hao. et al. 2008; Torino et al. 2001). For example, aeration can influence glycerol metabolism differently in these bacteria. While aerobic conditions are more favored than anaerobic conditions for *K. pneumoniae* (Hao. et al. 2008), *L. panis* PM1 primarily produces 1,3-PDO under anaerobic or microaerobic conditions (Khan et al. 2013). Another influential factor is pH. In acidic media, *K. pneumoniae* produces 1,3-PDO, along with 2,3-butanediol, as characteristic end-products. Grahame et al. (2013) also demonstrated that alkaline pH-stimulated *L. panis* PM1 produces acetate and 1,3-PDO under specific conditions. Carbon source is another crucial factor. Glycerol metabolism is stimulated by the existence of glucose and lactate in resting cells of *L. reuteri* (Lüthi-Peng et al. 2002).

In spite of the importance of the glycerol reductive pathway for NADH recycling, the factors affecting this pathway have not yet been fully explored. Therefore, the metabolic profiles of 1,3-PDO and ethanol were examined at different pH values, temperatures, and initial glycerol concentrations to improve the understanding of NADH recycling and mechanisms of

1,3-PDO production in *L. panis* PM1. The observed metabolic shifts were also further examined through qRT-PCR analysis of the glycerol reductive pathway genes and detemination of DhaT activity, demonstrating how *L. panis* PM1 changes its metabolic profiles in response to environmental factors.

7.3 Materials and Methods

7.3.1 Bacterial Strains and Growth Conditions

Lactobacillus panis PM1 was cultured in modified MRS (mMRS) medium at 37 ℃ under microaerobic conditions, as described in section 3.3.1, unless otherwise stated. To determine the effects of initial glycerol concentration on 1,3-PDO production and DhaT activity, *L. panis* PM1 was cultured in mMRS containing 0, 150, or 300 mM glycerol, and to elucidate the effects of pH and temperature on NAD^+ regeneration and DhaT activity, *L. panis* PM1 was cultured at four combinations of temperature (30 or 37 ℃) and pH (4.5 or 6.5) in mMRS containing 150 mM glycerol. For acidic media, the initial pH, 6.5, was adjusted to a pH of 4.5 through the addition of 10-N HCl. These pH values were chosen as they reflected the optimal growth condition (pH 4.5) of *L. panis* PM1 (Khan et al. 2013), as well as the pH of the general lactobacilli culture medium, MRS (pH 6.5).

7.3.2 Preparation of Crude Cell Extracts

Lactobacillus panis PM1 cells, grown as described above, were disrupted by sonication, and protein concentration in the crude extract was determined by the Bradford method, as described in section 4.3.2.

7.3.3 Enzyme Assay

DhaT activity in crude cellular extract was determined at 340 nm (ε^{NADH} = 6,220 M^{-1} cm^{-1}) (Johnson and Lin 1987). The assay mixture contained 2 mM NAD^+, 100 mM 1,3-PDO, and 30 mM ammonium sulfate in 100 mM potassium carbonate buffer at pH 9.0. The assay was carried out at 37 ℃ for 10 min. In these determinations, one unit of activity corresponds to the generation of 1 µmol of NADH per min. Specific activity was expressed as units per milligram of protein.

7.3.4 Determination of Glucose and End-products

Optical density and concentration of metabolites (glucose, glycerol, organic acids, 1,3-PDO, and ethanol) were analyzed by a spectrophotometer and the optimized HPLC method, respectively, as described in section 3.3.2.

7.3.5 Quantification of H_2O_2 and 3-HPA

Hydrogen peroxide in culture media was determined as described in section 5.3.2, and 3-HPA quantification was carried out using a colorimetric method (Doleyres et al. 2005).

7.3.6 RNA Extraction and Reverse Transcription

RNA was extracted by the acid-hot phenol method, as described in section 4.3.6. First-strand cDNA was synthesized using reverse primers of the selected genes (Table 7.1) according to section 4.3.7.

Table 7.1 The primers used in this study

Target gene	Function	Primer	T_m (℃)[a]	Nucleotide sequence (5' →3')
16S rRNA	16S ribosomal RNA	f16S	58	tggcccaactgatatgac
		r16S	58	ctctcatgcacgttcttctt
dhaB	Glycerol dehydratase large subunit	fGDH	60	tggttggttcacaaactgga
		rGDH	60	ccggcatcgaaatacaactt
dhaT	1,3-PDO dehydrogenase	fPDDH	60	gtgccggcattattttgact
		rPDDH	60	tgtggtcaacggaaacgtaa

a. T_m, melting temperature.

7.3.7 Quantitative Real-time PCR

Real-time PCR amplification was performed in a CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA) using SsoFast EvaGreen Supermix (Bio-Rad). The total volume of the PCR master mixture was 20 µL, to which cDNA template equivalent to 25 ng RNA starting material and 0.5 µM of each primer (Table 7.1) was added. PCR amplification and validation were conducted as described in section 4.3.8. For relative gene expression, the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method, using the 16S rRNA gene for normalization, was performed as described by Livak and Schmittgen (2001). The steps for calculating the expression ratio were as follows:

$\Delta C_{T(test)} = C_{T(dhaB \text{ and } dhaT, \text{ test})} - C_{T(16S \text{ rRNA, test})}$

$\Delta C_{T(control)} = C_{T(dhaB \text{ and } dhaT, \text{ control})} - C_{T(16S \text{ rRNA, control})}$

$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(control)}$

Normalized expression ratio of $dhaB_{(test)}$ and $dhaT_{(test)} = 2^{-\Delta\Delta CT}$.

The RT-PCR data were processed using CFX Manager Software (Bio-Rad).

7.3.8 Statistical Analysis

For determinations of end-product concentration and DhaT enzyme activities, data were presented as mean values ± standard errors of the means calculated from at least three independent experiments. Differences of final end-products were analyzed by the *t* test (Mann-Whitney test) for two groups, or the one-way ANOVA test (Kruskal-Wallis test) for three groups, using GraphPad Prism, version 5.0, software (GraphPad Software, Inc., San Diego, CA, USA). A *P* value of < 0.05 was considered significant.

7.4. Results and Discussion

7.4.1 The Effects of Initial Glycerol Concentration on 1,3-PDO Production

External glycerol was used as an electron acceptor *by L. panis* PM1, which resulted in the production of 1,3-PDO (Table 7.2). This reduction reaction also caused a shift in the NAD^+ regeneration system. At 48 h, cells grown in 150 mM glycerol medium produced 87.52 mM 1,3-PDO along with 41.71 mM ethanol that represented the primary NAD^+ regeneration system; whereas in the absence of glycerol, 48-h *L. panis* PM1 culture yielded 53.57 mM ethanol. Although the growth of *L. panis* PM1 was not affected by up to 8% (870 mM) of glycerol (Khan et al. 2013), the production of 1,3-PDO was negatively-affected by high glycerol (300 mM) concentration (Table 7.2). When glycerol was increased from 150 to 300 mM, the final yield of 1,3-PDO and acetate in the 300 mM glycerol medium was reduced from 87.52 to 64.72 mM and from 68.51 to 57.45 mM, respectively, while ethanol production was increased from 41.71 to 48.08 mM. Glucose fermentation via the 6-PG/PK pathway can produce equimolar amounts of acetyl phosphate, the intermediate at the fork of metabolic pathways to acetate and ethanol. Thus, this result demonstrated that ethanol production was in competition with the production of 1,3-

PDO and acetate and glycerol fermentation served as an alternate route for NAD^+ regeneration in *L. panis* PM1.

Table 7.2 The effect of initial glycerol concentration on the expression of *dhaB* and *dhaT* and on the yield of end-products

Glycerol (mM)[a]	Relative gene expression level		Metabolite production (mM)				
	dhaB	***dhaT***	**1,3-PDO**	**Ethanol**	**Lactate**	**Acetate**	**Succinate**
0	1.00±0.39	1.00±0.30	-	53.57±0.94	56.21±3.01	33.56±2.30	10.97±0.02
150	1.11±0.41	1.25±0.35	87.52±0.52	41.71±0.37	58.39±0.23	68.51±0.59	11.38±0.02
300	0.79±0.13	0.17±0.04	64.72±0.46	48.08±0.67	59.91±0.24	57.45±1.87	11.58±0.36

a. *Lactobacillus panis* PM1 was cultured in mMRS containing 0, 150, or 300 mM glycerol for 24 h (for qRT-PCR) and 48 h (for HPLC); 0 mM glycerol was used as control condition for relative gene expression by qRT-PCR.

7.4.2 The Effects of Glucose Supplementation on 1,3-PDO Production

The reduction of glycerol to 1,3-PDO and ethanol is absolutely dependent on the availability of reducing equivalents from glucose metabolism (Chapter 3). Therefore, the effect of glucose supplementation on NADH flux after glucose depletion was expected to increase both 1,3-PDO and ethanol production. *Lactobacillus panis* PM1 was cultured in mMRS medium containing 55 mM glucose and 300 mM glycerol at 30 ℃ under microaerobic conditions, and this first batch culture reached the stationary phase within 48 h. Additional glucose (32 and 24 mM) was then fed directly into the medium after 48- and 96-h culture, respectively, where available glucose had already been depleted. Interestingly, metabolite production profiles indicated that glucose supplementation after initial glucose depletion (at 48 h) did not increase 1,3-PDO production despite the fact that added glucose was used up within 24 h after feeding. After 120-h culture, ethanol and acetate production increased by 42% and 56% of the 48-h culture values, to concentrations of 76.06 and 96.62 mM, respectively. The sum of ethanol and acetate was almost equal (on a molar basis) to that of added glucose (total 56 mM; Figure 7.2).

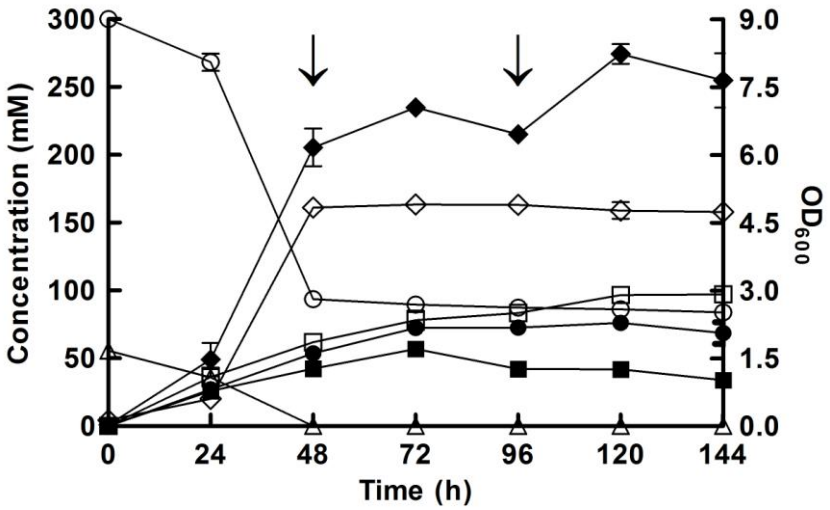

Figure 7.2 The effect of glucose supplementation on further 1,3-PDO production by *L. panis* PM1. *Lactobacillus panis* PM1 was cultured in mMRS (pH 6.5) containing 55 mM glucose and 300 mM glycerol at 30 ℃. Arrows represent additional glucose supplementation (32 mM at 48 h and 24 mM at 96 h). The concentrations of glycerol (white circles), glucose (white up-pointing triangles), ethanol (black circles), acetate (white squares), lactate (black squares), and 1,3-PDO (white diamonds) are provided on the left-hand *y*-axis, and the cell density (black diamonds) is provided on the right-hand *y*-axis.

7.4.3 The Effects of Initial pH and Temperature on 1,3-PDO and Other End-Product Production

Culture pH (4.5 or 6.5) significantly affected both 1,3-PDO and ethanol production during growth at 37 ℃ ($P < 0.05$), where the lower pH condition decreased the production of 1,3-PDO and ethanol by 12% (87.52 to 77.45 mM) and 44% (41.71 to 23.52 mM), respectively (Table 7.3). At 37 ℃, *L. panis* PM1 growth was almost double that observed at 30 ℃ under either pH conditions, while the lower temperature (30 ℃) yielded more 1,3-PDO at each pH (12% more at pH 6.5 and 23% more at pH 4.5) compared to culture at 37 ℃. The lowest yields of 1,3-PDO and ethanol were observed during growth under the optimal condition (37 ℃ and pH 4.5). In other studies (Kang et al. 2013a; Kang et al. 2013c; Khan et al. 2013), other NAD^+ regeneration routes (i.e., pyruvate to lactate, citrate to succinate, and molecular oxygen to hydrogen peroxide) were reported to maintain redox balance in *L. panis* PM1. The range of lactate yield was comparable among all conditions (from 52.11 to 58.39 mM), indicating that the NADH pool should be similar in size among the tested conditions [glucose (55 mM) was used to produce similar amounts of lactate with consumption of NADH, Table 7.3]. Significant

differences in hydrogen peroxide accumulation were not observed in any of the samples (from 6.2 to 4.1 μM, $P > 0.05$, Table 7.3). However, pH and temperature affected succinate and erythritol pathways. The production of succinate decreased under pH 4.5 conditions; whereas the final yields of erythritol significantly increased under acidic conditions ($P < 0.05$) (Table 7.3).

7.4.4 Transcription Levels and Enzyme Activities of 1,3-PDO Pathway

To further evaluate the effects of pH, temperature, and glycerol concentration on the regeneration of NAD^+ through the glycerol-1,3-PDO reductive pathway, candidate genes for DhaB and DhaT were identified through analysis of the annotated *L. panis* draft genome sequence data (unpublished data). The expression levels of these two genes were examined by qRT-PCR under the conditions used for metabolite analyses presented in the previous sections, i.e., different pH (4.5 and 6.5), temperature (30 ℃ and 37 ℃), and glycerol concentrations (0, 150, and 300 mM). The different glycerol concentrations resulted in similar expression levels of *dhaB* under all conditions. The expression of *dhaT* occurred at similar levels under both control (0 mM glycerol) and 150 mM glycerol conditions; however, its expression in the cultures amended with 300 mM glycerol was one-sixth that of the control condition (Table 7.2). In addition, this transcriptional data was well-correlated with DhaT enzyme assay results at 24 h (Figure 7.3). Specific activities of this enzyme were comparable among all conditions until 12-h culture; however, DhaT activity under 300 mM glycerol did not increase at 24 h, in contrast to the other two assay conditions (0 and 150 mM glycerol). While DhaT activities varied, the yields of 1,3-PDO and ethanol at qRT-PCR sampling time (24-h culture) were comparable among all conditions (1,3-PDO; 0, 19.39, and 19.69 mM, ethanol; 22.45, 21.78, and 19.35 mM in 0, 150, and 300 mM glycerol samples, respectively). In contrast to glycerol concentration, pH significantly influenced the expression of the two genes, as shown in Table 7.3. Under the optimal growth condition (37 ℃ and pH 4.5), 15-fold higher expression of *dhaB* and *dhaT* were observed relative to the control condition (37 ℃ and pH 6.5). However, the specific activity of DhaT was not induced by the acidic conditions and was thus comparable among all conditions (Table 7.3), indicating high glycerol concentration to be the main controlling factor of DhaT activity.

Table 7.3 The effect of initial pH and culture temperature on the expression of *dhaB* and *dhaT* and on the yield of end-products

Conditions[a]	Relative gene expression level[b]		Specific activity[b,c]	Metabolite production[b] (mM or μM)[d]						
	dhaB	***dhaT***	**DhaT**	**1,3-PDO**	**Ethanol**	**Lactate**	**Succinate**	**Acetate**	**Erythritol**	**H_2O_2**
pH6.5 / 37 ℃[e]	1.00±0.10	1.00±0.54	16.88±0.40	87.52±0.52	41.71±0.37	58.39±0.23	11.38±0.02	68.51±0.59	1.04±0.01	5.8±0.8
pH6.5 / 30 ℃	3.07±0.10	1.68±0.10	15.00±0.53	99.54±0.53	40.10±1.58	52.11±0.06	11.49±0.08	59.97±0.31	0.99±0.02	4.1±0.6
pH4.5 / 37 ℃	15.33±1.74	16.10±0.10	17.51±0.11	77.45±0.68	23.52±0.46	55.41±0.27	10.31±0.04	63.51±0.37	1.23±0.03	6.2±0.6
pH4.5 / 30 ℃	8.49±0.96	8.28±0.54	15.43±0.59	100.78±0.48	34.80±0.60	55.93±0.31	10.15±0.05	66.32±0.11	1.56±0.06	4.6±1.0

a. *Lactobacillus panis* PM1 was cultured in mMRS containing 150 mM glycerol at each pH and temperature condition. b. Samples for qRT-PCR, enzyme activity and HPLC were withdrawn from the 37 ℃ cultures after 24 h and from the 30 ℃ cultures after 48 h. c. $\mu mol \cdot min^{-1} \cdot mg^{-1}$. d. Unit of H_2O_2 is μM and the others are mM. e. Control condition for relative gene expression by qRT-PCR.

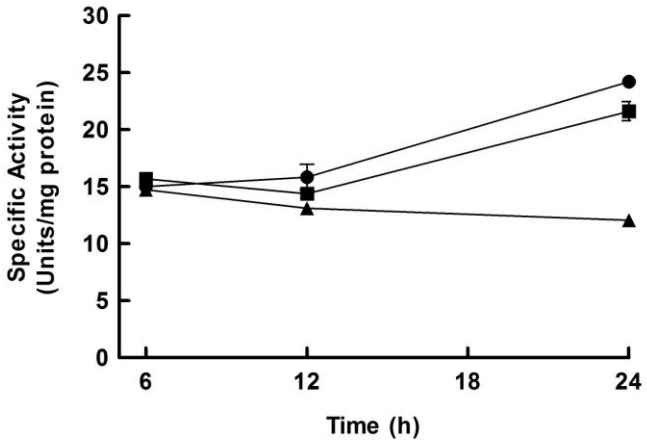

Figure 7.3 The effect of glycerol concentration on DhaT activity of *L. panis* PM1. *Lactobacillus panis* PM1 was cultured in mMRS (pH 6.5) containing 55 mM glucose and 0 (circles), 150 (squares), or 300 (triangles) mM glycerol, respectively. DhaT activity was measured after 6-, 12-, and 24-h of culture.

Only a few lactobacillus strains, including *L. panis PM1*, *L. buchneri*, *L. reuteri, L. hilgardii* and *L. dilovorans,* have been reported to be 1,3-PDO producers, and the reductive pathway in those heterofermentative strains plays a key role in the production of 1,3-PDO from glycerol (Biebl et al. 1999; Khan et al. 2013; Saxena et al. 2009). Chapter 3 proposed that the conversion of glycerol to 1,3-PDO was an important auxiliary pathway to maintain the redox balance in *L. panis* PM1. However, more detailed information of the factors influencing NADH recycling during end-product formation is necessary in order that *L. panis* PM1 be used for glycerol conversion as part of engineered biofuel by-product management applications.

As *L. panis* PM1 belongs to the group III LAB, NADH recycling under anaerobic conditions depends on the production of lactate and ethanol through the 6-PG/PK pathway (Lüthi-Peng et al. 2002; Pedersen et al. 2004; Veiga da Cunha and Foster 1992a). As shown in Table 7.2, the presence of an external source of glycerol shifted NAD^+ regeneration from ethanol production to the reduction of glycerol to 1,3-PDO, decreasing ethanol production by 22% in the presence of 150 mM glycerol. In other words, the decrease in ethanol production provided surplus NADH that was then utilized for the production of 1,3-PDO. In Chapter 3, extremely-high glycerol concentrations actually decreased the conversion of glycerol to 1,3-PDO, and the ratio of glucose to glycerol was a key factor for optimal 1,3-PDO production (Grahame et al. 2013). The present results agree with this decrease, as providing higher glycerol concentration (300 mM) altered the route of NADH recycling, consequently reducing the yield of 1,3-PDO by

26% and increasing ethanol production by 15% compared to the production levels seen at 150 mM glycerol. This result demonstrated that both ethanol and 1,3-PDO production are in competition for the same NADH pool, and that at a high glycerol concentration (300 mM), part of the NADH pool was redirected to the production of ethanol. This observation also confirmed that the conversion of glycerol to 1,3-PDO acted as an auxiliary pathway for the NAD^+ regeneration system.

In the presence of glycerol under microaerobic conditions, NADH from initial glucose metabolism was directed to 1,3-PDO and ethanol production. However, the availability of NADH was not a determining factor in shifting between ethanol and 1,3-PDO. As exhibited in Figure 7.2, glucose depletion caused a shutdown of the reductive pathway for 1,3-PDO production, and NADH flux was directed to ethanol production after providing additional glucose; i.e., 1,3-PDO production did not resume. The yield of 1,3-PDO increased until initial glucose depletion at 48 h (20 mM at 24 h and 160 mM at 48 h), and total glycerol consumption at each time point was higher than these amounts (32 mM at 24 h and 205 mM at 48 h). Similar results were observed in Table 3.2 where this gap (between glycerol consumed and 1,3-PDO produced) increased at high glycerol concentration (52 and 24 mM in 326 and 163 mM glycerol media, respectively) along with a reduced 1,3-PDO conversion rate (65% and 81% in 326 and 163 mM glycerol media, respectively). These observations are indicative of the accumulation of the intermediate 3-HPA (the only intermediate in the glycerol reductive pathway).

qRT-PCR results revealed that the expression of *dhaB* and *dhaT* were mainly activated under acidic conditions; whereas *dhaT* expression was repressed at the high glycerol concentration (300 mM) (Tables 7.2 and 7.3). This data matched the result of a low final 1,3-PDO yield in the presence of high concentrations of glycerol. At 24 h, glucose consumption and ethanol and 1,3-PDO production values were similar at all glycerol concentrations (0, 150 and 300 mM; except 0 mM glycerol, where 1,3-PDO was not observed). As shown in Figure 7.3, the activities of the DhaT enzyme were similar at each glycerol concentration until 12 h of culture. However, at 24 h, the transcription level and specific activity of this enzyme in mMRS medium supplemented with 300 mM glycerol was reduced by 83% and 50%, respectively, from than observed in mMRS alone (Table 7.2 and Figure 7.3). This observation clearly indicated that decreased DhaT activity, which was negatively-affected by the progress of growth, was a main reason for reduced 1,3-PDO production in the presence of 300 mM glycerol compared to 150

mM glycerol. Also, considering that significant amounts of 3-HPA were not detected by the quantification method from media of all glycerol concentrations (data not shown), the accumulation of 3-HPA inside of the cells offers a logical explanation for the decreased transcription level and activity of DhaT enzyme, which eventually negatively-impacted 1,3-PDO production and necessitated the redirection of NADH recycling.

However, total redox balance cannot be completely explained solely by the above 1,3-PDO and ethanol producing routes. The total amounts of 1,3-PDO and ethanol production were similar among the treatments pH 6.5-37 ℃, pH 6.5-30 ℃, and pH 4.5-30 ℃, ranging from 129.23 to 135.58 mM; whereas under the optimal condition (pH 4.5-37 ℃), less 1,3-PDO and ethanol production was observed (100.97 mM), indicating a shift of NADH flow through ethanol and 1,3-PDO production and leaving some NADH untouched under the optimal condition. However, the yield of lactate and hydrogen peroxide were comparable among all conditions, and succinate production decreased during culture under pH 4.5 conditions (Table 7.3), suggesting those pathways did not reoxidize the remaining NADH resulting from decreased ethanol or 1,3-PDO production. These results suggest the existence of an alternative, unexplored NADH recycling route.

Temperature and pH were also significant factors affecting the yield of 1,3-PDO. Although the lowest 1,3-PDO yield was observed when the optimal growth condition was employed, the expression of the *dhaB* and *dhaT* were found to be highest. Higher 1,3-PDO production was observed for 30 ℃ cultures compared with 37 ℃ cultures, showing no significant change in transcription levels of those two genes. Furthermore, the tested environmental factors did not significantly affect the specific activity of the DhaT enzyme (Table 7.3). It therefore appears that the decreased production of 1,3-PDO under the optimal condition was not associated with reduced activity of DhaT enzyme, in contrast with the high glycerol (300 mM) situation. The different growth rates seen at 37 ℃ and 30 ℃ does offer a clue. Generally, the activities of NADH-generating enzymes from the 6-PG/PK pathway (i.e., glucose-6-phosphate and 6-phosphogluconate dehydrogenases) largely exceed the activities of the NADH-reoxidizing enzymes from the ethanol pathway (i.e., acetaldehyde and alcohol dehydrogenases) in typical glucose fermentation by heterofermentative LAB (Richter et al. 2003; Richter et al. 2001; Zaunmuller et al. 2006). Therefore, at the high growth rate observed under the optimal condition, an excess NADH pool formed by rapid glucose metabolism and limited capacities of

the ethanol and 1,3-PDO pathways to process this excess should force a partial shift of NADH reoxidation toward other pathways, even though the transcription level of the glycerol reductive pathway was up-regulated.

The erythritol pathway has been observed in various heterofermentative LAB as a main alternate route for the limited NADH-reoxidation of ethanol pathway during rapid growth (Richter et al. 2001; Stolz et al. 1995; Veiga da Cunha and Foster 1992a). Although *L. panis* PM1 could not ferment erythritol as the sole carbon source (Khan et al. 2013), it was detected as a minor end-product (Table 7.3). The final yields of erythritol increased under acidic conditions, unlike the production of ethanol or succinate, suggesting a possible role of erythritol production as a minor NADH-reoxidizing route in *L. panis* PM1. Different routes of NAD^+ regeneration have been reported for many end-product-yielding pathways (e.g., mannitol, erythritol, and glycerol), and multiple combinations of those pathways, in conjunction with the typical ethanol pathway, are used by heterofermentative LAB according to their environmental circumstances (Richter et al. 2003). Therefore, these multiple combinations, including the erythritol pathway, could be used to complement the decreased NAD^+ regeneration capacity seen during ethanol and 1,3-PDO production in *L. panis* PM1 grown under the optimal growth condition.

7.5 Conclusions

The glycerol reductive pathway of *L. panis* PM1 is a main route for NADH recycling in the presence of glycerol. Various environmental factors, including temperature, pH, and electron acceptors, can affect 1,3-PDO production via this pathway. However, those factors affect the yield of 1,3-PDO in different ways. High glycerol concentration negatively-affected DhaT activity, which resulted in less 1,3-PDO production, as well as shifting of NADH's fate; whereas temperature and pH indirectly affected 1,3-PDO production by changing the NADH reoxidation route (e.g., erythritol pathway).

7.6 Connection to the Next Study

This chapter showed that the glycerol reductive pathway was directly regulated by 3-HPA accumulation through decreases in the specific activity of DhaT that was repressed at the transcript level. However, the regulation of the first enzyme, glycerol dehydratase, in the conversion of glycerol to 1,3-PDO, was not evident in this chapter. Thus, in the following Chapter (Chapter 8), the regulation of glycerol dehydratase was investigated at both the

transcriptional and enzymatic levels. This detailed regulatory model of the glycerol reductive route contributes to a more complete understanding of the 1,3-PDO bioconversion mechanism and enabled further engineering work in *L. panis* PM1.

8. TRANSCRIPTIONAL REPRESSOR ROLE OF PocR ON THE GLYCEROL REDUCTIVE PATHWAY BY *LACTOBACILLUS PANIS* PM1[vi]

8.1 Abstract

Lactobacillus panis PM1 can utilize a 1,3-propanediol (1,3-PDO) biosynthetic pathway, consisting of diol dehydratase (PduCDE) and 1,3-PDO dehydrogenase (DhaT), as a NADH recycling system, to survive under various environmental conditions. In this study, a key transcriptional repressor (PocR), which was annotated as a transcriptional factor of AraC family, was identified as part of the 1,3-PDO biosynthetic pathway of *L. panis* PM1. The over-expression of the PocR gene resulted in the significant repression (81%) of *pduC* (PduCDE large subunit) transcription, and subsequently, the decreased activity of PduCDE by 22%. As a result of the regulation of PduCDE, production of both 3-hydroxypropionaldehyde and 1,3-PDO in the PocR over-expressing strain was significantly decreased by 40% relative to the control strain. These results clearly demonstrate the transcriptional repressor role of PocR in the 1,3-PDO biosynthetic pathway.

[vi] Tae Sun Kang, Darren R. Korber, and Takuji Tanaka. 2014. Transcriptional repressor role of PocR on the 1,3-propanediol biosynthetic pathway by *Lactobacillus panis* PM1. Biotechnology Letters doi: 10.1007/s10529-014-1477-6. This chapter is reproduced in this thesis with the permission of Springer.

8.2 Introduction

Lactobacillus panis PM1 belongs to the obligatory heterofermentative group of lactobacilli and is of interest due to its ability to produce 1,3-propanediol (1,3-PDO) during anaerobic glycerol fermentation. In the 1,3-PDO biosynthetic pathway of this strain, glycerol is initially converted to 3-hydroxypropionaldehyde (3-HPA) by glycerol dehydratase (DhaBCE), after which 3-HPA is reduced to 1,3-PDO by 1,3-propanediol dehydrogenase (DhaT) (Kang et al. 2013b). The second step of this process oxidizes NADH (by the DhaT reaction), regenerating NAD^+ consumed during glucose metabolism. Through this NADH oxidation, the pathway shifts the fate of acetyl phosphate from ethanol production to acetate production, thereby generating an extra ATP per glucose (Kang et al. 2013b).

The compound 1,3-PDO has been widely utilized in industrial applications, including laminates, adhesives, resins, films, solvents, detergents, cosmetics, engine coolants, fibers, and carpets due to its unique features that include biodegradability and higher light stability (Kaur et al. 2012; Saxena et al. 2009). Recently, 1,3-PDO production by microbial fermentation has received increased attention as a safer alternative (i.e., mild pressure, temperature, and fewer toxic intermediates) compared with conventional chemical production methods. High levels of 1,3-PDO have been achieved in fermentations by the genera *Klebsiella* (*K. pneumoniae* and *K. oxytoca*) and *Clostridium* (*C. butyricum and C. pasteurianum*). A final 1,3-PDO concentration of 963 mM (yield of 0.48 mol/mol) was achieved by *K. pneumoniae* ATCC 25995 and *C. butyricum* DSM *5431* produced 625 mM of 1,3-PDO (yield of 0.62 mol/mol) in their fed-batch fermentation of glycerol (Cameron et al. 1998; Reimann and Biebl 1996). In the 1990s, expensive glycerol costs relative to sugars (e.g., glucose) forced 1,3-PDO production strategies by fermentation to focus on the utilization of glucose (Biebl et al. 1999; Cameron et al. 1998). In an effort to engineer a single microorganism capable of direct conversion of glucose to 1,3-PDO, a high amount of 1,3-PDO production (1803 mM) was achieved by using an engineered *E. coli* strain utilizing *K. pneumoniae* DhaT and *E.coli* NADPH-dependent aldehyde reductase (YqhD) (Nakamura and Whited 2003). However, tremendous demand for biofuels (e.g., biodiesel and bioethanol) has caused a sharp drop in crude glycerol prices over recent years (Clomburg and Gonzalez 2013). Thus, current engineering strategies for 1,3-PDO production have shifted toward the utilization of the now lower-priced glycerol. Introduction of the 1,3-PDO pathway of

C. butyricum into *C. acetobutylicum* enabled the recombinant strain to produce 1104 mM of 1,3-PDO (yield of 0.65 mol/mol) along with low levels of other end-products [i.e., butyrate (193 mM), acetate (67 mM), and lactate (22 mM)] in fed-batch fermentation of glycerol (Gonzalez-Pajuelo et al. 2005). Inactivation of aldehyde dehydrogenase gene in *K. pneumoniae* YMU2 resulted in a higher concentration (928 mM) of 1,3-PDO and yield (0.70 mol/mol) of 1,3-PDO than those of the parent strain (699 mM and 0.36 mol/mol), along with nearly no ethanol formation (37 mM vs. 201 mM), which is a major competitor for 1,3-PDO production (Zhang et al. 2006).

However, the fact that these major 1,3-PDO producers are opportunistic pathogens presents a considerable drawback in terms of potential industrial applications, particularly in the food, cosmetic and biomedical sectors (Gaspar et al. 2013; Kaur et al. 2012; Saxena et al. 2009). In this respect, the generally recognized as safe (GRAS) feature of natural 1,3-PDO producers from the lactobacilli (e.g., *L. reuteri*) offer an attractive option for use as potential cell factories for 1,3-PDO production.

The accumulation of 3-HPA, which is the only intermediate in 1,3-PDO biosynthetic pathway, negatively-affected both enzyme activity and transcription of *dhaT*, resulting in a decrease in the 1,3-PDO production (Kang et al. 2013b). In this study, a transcriptional regulator (PocR) of the glycerol dehydratase gene was identified by comparison of transcript levels and enzyme activities of this gene, as well as by changes in end-product patterns between wild-type and PocR over-expressing strains.

8.3 Materials and Methods

8.3.1 Bacterial Strains and Growth Conditions

Lactobacillus panis PM1 was cultured in modified MRS (mMRS) medium at 30 ℃ under microaerobic conditions, as described in section 3.3.1, unless otherwise stated. For the 1,3-PDO fermentation, the initial pH, 6.5, was adjusted to a pH of 4.5 through the addition of 10-N HCl.

8.3.2 Preparation of Crude Cell Extracts

Lactobacillus panis PM1 cells grown in mMRS containing glucose and glycerol were disrupted by sonication, and protein concentration in the crude extract was determined by the Bradford method, as described in section 4.3.2.

8.3.3 Enzyme Assays

Glycerol dehydratase activity was determined as described by Kwak et al. (2012). Briefly, the reaction mixture consisted of 100 mM potassium phosphate pH 7.0, 10 mM of glycerol, 20 µM coenzyme B_{12}, and 0.05 mg of protein. The reaction mixture was incubated at 37 ℃ for 20 min, and the enzyme reaction was terminated by addition of the same volume of 100 mM citrate solution. The quantification of 3-HPA in the reaction solution was carried out using a colorimetric method (Doleyres et al. 2005). In this determination, one unit of activity corresponds to the production of 1 mmol of 3-HPA per min. The activity of 1,3-propanediol dehydrogenase in crude cellular extract was determined at 340 nm (ε^{NADH} = 6,220 M^{-1} cm^{-1}), according to section 7.3.3. One unit of DhaT activity corresponds to the generation of 1 µmol of NADH per min. Specific activities of glycerol dehydratase and 1,3-PDO dehydrogenase were expressed as units per milligram of protein.

8.3.4 Determination of Glucose and End-products

Optical density and concentration of metabolites (glucose, glycerol, 3-HPA 1,3-PDO, and ethanol) were analyzed by a spectrophotometer and the optimized HPLC method, respectively, as described in section 3.3.2.

8.3.5 RNA Extraction and Reverse Transcription

RNA was extracted by the acid-hot phenol method, as described in section 4.3.6. First-strand cDNA was synthesized using reverse primers of the selected genes (Table 8.1) according to section 4.3.7.

8.3.6 Quantitative Real-time PCR

Real-time PCR amplification was performed in a CFX96 real-time detection system (Bio-Rad, Hercules, CA, USA) using SsoFast EvaGreen Supermix (Bio-Rad). The total volume of the PCR master mixture was 20 µL, to which cDNA template equivalent to 25 ng RNA starting

material and 0.5 μM of each primer (Table 8.1) were added. PCR amplification and validation were conducted as described in section 4.3.8. For relative gene expression, the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method, using the 16S rRNA gene for normalization, was performed as described by Livak and Schmittgen (2001). The steps for calculating the expression ratio were as follows:

$\Delta C_{T(test)} = C_{T(pduC \text{ and } dhaT, \text{ test})} - C_{T(16S \text{ rRNA, test})}$

$\Delta C_{T(control)} = C_{T(pduC \text{ and } dhaT, \text{ control})} - C_{T(16S \text{ rRNA, control})}$

$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(control)}$

Normalized expression ratio of $pduC_{(test)}$ and $dhaT_{(test)} = 2^{-\Delta\Delta CT}$.

The Real-Time PCR data were processed using CFX Manager Software (Bio-Rad).

Table 8.1 The plasmids and primers used in this study

Plasmids	Relevant features	Reference
pCER	*Lactobacillus panis* PM1 expression vector, Em^r Cm^r, 5.0 kb	Kang et al. (2013a)
pCER-PocR	pCER derivative expressing the PocR gene under control of its natural promoter, Em^r Cm^r, 6.5 kb	Kang et al. (2013a)

Target gene	Function	Primer	T_m (°C)[a]	Nucleotide sequence (5'→3')
16S rRNA	16S ribosomal RNA	f16S	58	tggcccaactgatatgac
		r16S	58	ctctcatgcacgttcttctt
pduC	Diol dehydratase large subunit	fGDH	60	tggttggttcacaaactgga
		rGDH	60	ccggcatcgaaatacaactt
dhaT	1,3-PDO dehydrogenase	fPDDH	60	gtgccggcattattttgact
		rPDDH	60	tgtggtcaacggaaacgtaa

Cm^r and Em^r represent chloramphenicol and erythromycin resistance, respectively. a. T_m, melting temperature.

8.3.7 Statistical Analysis

For determinations of end-product concentration, enzyme activities and transcriptional levels of *pduC* and *dhaT*, data were presented as mean values ± standard errors of the means calculated from at least three independent experiments. Differences in two groups were analyzed by the unpaired *t* test using GraphPad Prism, version 5.0, software (GraphPad Software, Inc., San Diego, CA, USA). A *P* value of < 0.05 was considered significant.

8.4 Results and Discussion

8.4.1 Glycerol Dehydratase Operon Structure of *Lactobacillus panis* PM1

The draft genome of *L. panis* PM1 (unpublished data) showed that the genes encoding glycerol dehydratase were organized as a part of a nine-gene operon structure (*pduABCDEGHKJ*), suggesting that this type of dehydratase is likely a diol dehydratase (EC 4.2.1.28, PduCED) (Daniel et al. 1998). This *pdu* operon encoded for propanediol utilization proteins [GenBank accession Nos. KF515976 (PduA), KF515977 (PduB), KF515983 (PduK), and KF515984 (PduJ)], propanediol dehydratase [GenBank accession Nos. KF515978 (PduC, large subunit), KF515979 (PduD, medium subunit), and KF515980 (PduE, small subunit)], and propanediol dehydratase reactivation factor [GenBank accession Nos. KF515981 (PduG, large subunit] and KF515982 (PduH, small subunit)] (Figure 8.1); whereas the DhaT gene was located in a different chromosomal locus of the *L. panis* PM1 genome as a single gene operon. A putative transcriptional regulator (PocR, 359 amino acid, GenBank accession No. KF312452) was found immediately upstream of the *pduABCDEGHKJ* operon and oriented in the opposite coding direction, and the putative promoters of the *pocR* and *pduABCDEGHKJ* operon were both located in a 218 bp intergenic gap between the translational start codons of the PocR and PduA genes (Figure 8.1). In the BLAST search of the UniProt database (http://www.uniprot.org; default parameters were applied), the putative protein sequence of PocR showed a high sequence identity to the AraC family of transcriptional factors that contain a typical helix-turn-helix domain and mainly stimulate transcription from cognate promoters (Gallegos et al. 1997). In addition, the stimulatory effects of PocR on vitamin B_{12}, 1,3-PDO, and reuterin synthesis have been reported for *L. reuteri* JCM 1112 (Santos et al. 2011). In Chapter 6, PocR acted as a transcriptional repressor on citrate metabolism in *L. panis* PM1, and the over-expression of the PM1 strain's PocR gene significantly decreased the expression level of the citrate lyase gene, resulting in significant decreases in succinate and acetate that are the major end-products of citrate metabolism (Table 6.4). Furthermore, the PocR of *L. panis* PM1 showed 43% and 37% amino acid identity with PocR found in *L. reuteri* JCM 1112 and *L. brevis* ATCC 367, respectively, which were reported as natural 1,3-PDO producers, during this BLAST search.

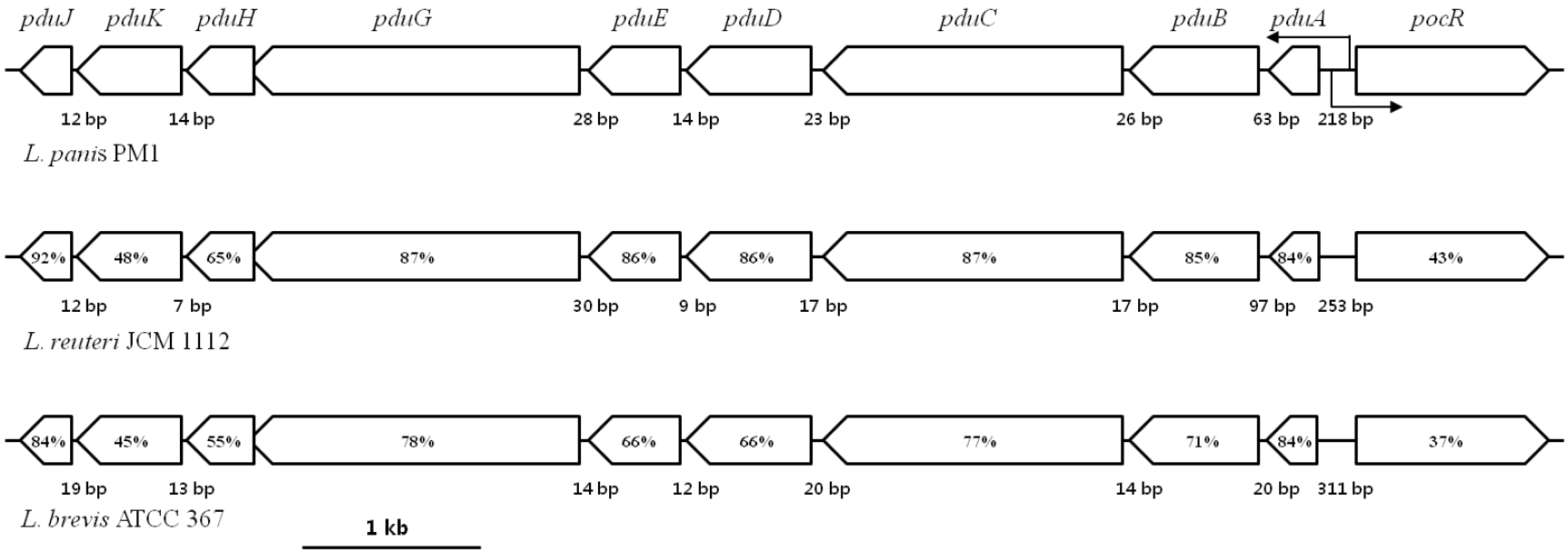

Figure 8.1 Organization of the *pduABCDEGHKJ* operon and *pocR* gene in *L. panis* PM1 and closely-related species. PduABKJ; propanediol utilization proteins, PduCDE: propanediol dehydratase - large, medium, and small subunits, PduGH; propanediol dehydratase reactivation factor - large and small subunits, and PocR; AraC-family transcriptional factor. The percentage amino acid identity with strain PM1 and the sizes of the intergenic gaps are indicated in, and below, each gene, respectively. Arrows represent putative promoters for the *pduABCDEGHKJ* operon and *pocR*, as predicted by Virtual footprint (http://prodoric.tu-bs.de/vfp/vfp_promoter.php).

Whereas, genes encoding the *pduABCDEGHKJ* operon in the PM1 strain showed higher amino acid identity (55% to 92%) with those of *L. reuteri* JCM 1112 and *L. brevis* ATCC 367, except for PduK (48% and 45%, respectively) (Figure 8.1). These observations raised the question of whether PocR can function as a transcriptional repressor for the 1,3-PDO biosynthetic pathway in *L. panis* PM1.

8.4.2 Transcriptional Repressor Role of PocR on *pduABCDEGHKJ* Operon

To demonstrate the transcriptional repressor role of PocR of *L. panis* PM1 on the 1,3-PDO biosynthetic pathway, the expression levels of *pduC* and *dhaT* were evaluated by qRT-PCR using RNA obtained from the PocR over-expressing strain, PM1-pCER-PocR (the control strain used was transformed with the empty plasmid, PM1-pCER) (Table 8.1). The over-expression of the PocR gene strongly-repressed the transcription of *pduC* (81% decrease; $P < 0.01$); whereas *dhaT* was not significantly regulated relative to the control strain in the presence of glycerol ($P > 0.05$), suggesting a transcriptional repressor role of the PocR gene on the *pduABCDEGHKJ* operon (Table 8.2). Furthermore, the over-expression of PocR significantly decreased the specific activity of PduCDE from 1.80 to 1.41 ($P < 0.01$), and was accompanied by an approximate 12% increase in DhaT activity ($P > 0.05$) (Table 2). This enzyme assay result well-matched the expression level of the PduC and DhaT genes.

These changes in transcriptional levels and specific activities of PduCDE and DhaT significantly affected end-product formation. After 96-h fermentation, available glucose was depleted in both strains, while concentrations of 1,3-PDO and 3-HPA were significantly decreased from 162.62 and 15.42 mM to 98.09 and 3.56 mM, respectively, by the over-expression of PocR. The decreases in 1,3-PDO and 3-HPA were accompanied by an approximate 31% increase in ethanol concentration ($P < 0.01$) (Table 8.2). These changes in end-product production were in accordance with data seen in Table 7.2 that showed the competitive metabolism between ethanol and 1,3-PDO for available NADH and a shift of acetyl-phosphate (the precursor of ethanol) to acetate. However, the production of 1,3-PDO exceeded the increase in ethanol as seen in Table 8.2, suggesting parts of NADH can be recycled by other pathways (e.g., citrate and erythritol) as discussed in Chapters 4 and 6. As displayed in Table 7.2 and Figure 7.3, high glycerol conditions resulted in the accumulation of 3-HPA, which decreased the

Table 8.2 The transcriptional repressor role of PocR on the 1,3-propanediol biosynthetic pathway

Strains[a]	Relative gene expression level		Specific activity		Metabolite production (mM)			
	pduC[b]	*dhaT*	PduCDE[c]	DhaT[d]	1,3-PDO	3-HPA	Ethanol	Acetate
pCER[e]	1.00±0.06	1.00±0.39	1.80±0.08	13.75±0.71	162.62±0.22	15.42±0.28	43.65±0.38	54.26±0.36
pCER-PocR	0.19±0.04	2.26±0.76	1.41±0.05	15.35±0.57	98.09±2.86	3.56±0.34	57.09±1.89	38.33±0.77

a. For qRT-PCR and enzyme assays, each strain was cultured in mMRS (pH 4.5) medium containing 60 mM glucose and 150 mM glycerol at 30 ℃ for 24 h, and for HPCL analysis, each strain was cultured in mMRS (pH 4.5) medium containing 60 mM glucose and 300 mM glycerol at 30 ℃ for 96 h. b. Diol dehydratase (PduCDE) large subunit. c. $mmol \cdot min^{-1} \cdot mg^{-1}$. d. $\mu mol \cdot min^{-1} \cdot mg^{-1}$. e. Control condition for relative gene expression by qRT-PCR.

transcription level and specific activity of the DhaT enzyme. Eventually, the decreased DhaT activity negatively-impacted 1,3-PDO production. The culture conditions used in the present study were similar to Chapter 7 where 55 mM glucose was depleted within the first 48 h, and the production of end-products (e.g., 1,3-PDO and ethanol) and the consumption of glycerol halted along with glucose depletion. In this study, high amounts of glycerol (100 to 180 mM) at 96 h were observed, indicating that the amount of glycerol cannot be the limiting factor for 1,3-PDO and 3-HPA production. All in all, This present study clearly shows that PocR over-expression decreased the transcription and specific activity of PduCDE, resulting in a decrease in 3-HPA accumulation; this decrease in 3-HPA levels would be expected to release the DhaT enzyme from the restriction caused by 3-HPA accumulation, leading to the higher activity observed in the PM1-pCER-PocR strain (Table 8.2). However, the decrease in PduCDE activity could not supply sufficient 3-HPA for NAD^+ regeneration by DhaT, which consequently redirected NADH to ethanol production (thus resulting in the production of low levels of 1,3-PDO and high levels of ethanol). Therefore, these observations strongly support a repressor role of PocR on the 1,3-PDO biosynthetic pathway of *L. panis* PM1, presumably functioning as part of a regulatory system for 1,3-PDO production.

The activity of glycerol dehydratase is a limiting step for the biotechnological 1,3-PDO production, and relates with the catalytic role of coenzyme B_{12} and its inactivation by glycerol (Daniel et al. 1998). Accordingly, many previous studies have focused on increasing the activities of glycerol dehydratase and/or 1,3-PDO dehydrogenase in natural 1,3-PDO producers by over-expressing these genes (Hao et al. 2008b; Zhao et al. 2009; Zheng et al. 2006). However, the excessive over-expression of these two endogenous genes did not improve the yield and/or concentration of 1,3-PDO in the recombinant *K. pneumoniae* strains mostly due to the

accumulation of 3-HPA and shortage of NADH. In this regard, the PocR gene of *L. panis* PM1 could be a good target to improve 1,3-PDO production through its deletion. Unlike the direct over-expression of glycerol dehydratase, the deletion of its repressor, PocR, can result in a mild increase in the activity of this enzyme, leading to less accumulation of 3-HPA. In addition, the over-expression of *E. coli* YqhD improved the conversion of 3-HPA to 1,3-PDO, resulting in an increase in 1,3-PDO in *L. panis* PM1 (Kang et al. 2014). Thus, these findings suggest that introduction of the YqhD gene into a PocR-deficient PM1 strain will further improve the biotechnological 1,3-PDO production by recombinant PM1 strains.

8.5 Conclusions

Combining the present results with those of the previous study (Chapter 7), the 1,3-PDO biosynthetic pathway of *L. panis* PM1 is apparently regulated at the transcriptional level by two factors, PocR and 3-HPA accumulation, which repress PduCDE and DhaT, respectively. Thus, this finding provides the *L. panis* PM1 strain with a fine-regulation system for metabolism via the 1,3-PDO route. In addition, the fact that PocR acts as a repressor of citrate metabolism in *L. panis* PM1 (Chapter 6) suggests that it can efficiently control the routes for NAD^+ regeneration to adapt to a wide spectrum of environmental and nutritive conditions.

8.6 Connection to the Next Study

This chapter confirmed that the presence of PocR and its effects on 3-HPA handling were a part of regulatory mechanisms for 1,3-PDO biosynthesis by *L. panis* PM1. Along with the knowledge obtained from the previous chapters, the metabolic activity of this strain confirmed the potential for this strain to be utilized in glycerol processing. Bioconversion of 1,3-PDO by *L. panis* PM1 using thin stillage-based media offers a new application of low-price glycerol. In the following Chapter (Chapter 9), 1,3-PDO production by *L. panis* PM1 was evaluated in thin stillage under various culture conditions. To prevent the accumulation of 3-HPA, the wild-type PM1 strain was genetically engineered. An engineered PM1 strain has potential as a cost-efficient platform for industrial conversion of low-price glycerol to 1,3-PDO.

9. BIOCONVERSION OF GLYCEROL TO 1,3-PROPANEDIOL IN THIN STILLAGE-BASED MEDIA BY ENGINEERED *LACTOBACILLUS PANIS* PM1[vii]

9.1 Abstract

Thin stillage (TS) is a major by-product that remains after bioethanol production, and its disposal reflects the high cost of bioethanol production. Thus, the development of cost-effective ways to process TS is a pending issue in bioethanol plants. The aim of this study was to evaluate the utilization of TS for the production of the valuable chemical, 1,3-propanediol (1,3-PDO), by *Lactobacillus panis* PM1. Different fermentation parameters, including temperature, pH and strains (wild-type and a recombinant strain expressing the YqhD gene) were tested in batch and fed-batch cultivations. The highest 1,3-PDO concentration (12.85 g/L) and yield (0.84 g/g) were achieved by batch fermentation at pH-4.5/30 ℃ by the YqhD-recombinant strain. Furthermore, pH-controlled batch fermentation reduced the total fermentation period, resulting in the maximal 1,3-PDO concentration of 16.23 g/L and yield of 0.72 g/g in TS without expensive nutrient or nitrogen (e.g., yeast extract, beef extract, and peptone) supplementation. The addition of two trace elements, Mg^{2+} and Mn^{2+}, in TS increased 1,3-PDO yield (0.74 g/g) without 3-hydroxypropionaldehyde production, the only intermediate in the 1,3-PDO biosynthetic pathway of *L. panis* PM1. These results suggest that *L. panis* PM1 can offer a cost-effective process that utilizes the TS to produce a value-added chemical, 1,3-PDO.

[vii] Tae Sun Kang, Darren R. Korber, and Takuji Tanaka. 2014. Bioconversion of glycerol to 1,3-propanediol in thin stillage-based media by engineered *Lactobacillus panis* PM1. Journal of Industrial Microbiology and Biotechnology 41(4): 629-635 . This chapter is reproduced in this thesis with the permission of Springer.

In this chapter, the amounts and conversion of metabolites are mostly expressed in g/L or g/g as the above original article uses these units. The conversion factors of g/L to mM are: 5.56 for glucose, 10.87 for glycerol, 13.16 for 1,3-PDO, and 21.74 for ethanol. The conversion factor of g/g to mol/mol is: 1.21 for glycerol to 1,3-PDO.

9.2 Introduction

Bioethanol is widely used as an alternative transportation fuel and, according to the U.S. Energy Information Administration, its production from corn grain in the U.S. has exceeded 13 billion gallons since 2010 (Annual Energy Review 2011). Dry-grind processes are currently used for the production of bioethanol, leaving the non-volatile components (i.e., whole stillage) after ethanol distillation from fermented beer (Kim et al. 2008). Whole stillage is usually separated by centrifugation into a solid fraction (wet distillers' grains, WDG) and a liquid fraction (thin stillage, TS). The separated TS is further condensed using an evaporator and then mixed with the WDG to produce wet distillers' grains with solubles (WDGS or DDGS, dried form of WDGS) that is sold as an animal feed. Dry-grind process includes two energy intensive procedures, distillation of ethanol and evaporation of TS, which reflect high costs of bioethanol production. Therefore, bioethanol production should be coupled with cost-effective processes that utilize the TS to produce more valuable materials than that of animal feed. Since TS contains glucan oligomers (12.4 g/L) and glycerol (14.4 g/L) as major components along with monomer sugars (glucose, xylose, and arabinose), and various minerals and amino acids as minor components (Kim et al. 2008), several attempts to use TS as a carbon or nutrient supplement have been made in microbial fermentation for the production of eicosapentaenoic acid by *Pythium irregulare* (Liang et al. 2012), butanol by *Clostridium pasteurianum* (Ahn et al. 2011), and lactate by *Lactobacillus rhamnosus* (Djukic-Vukovic et al. 2013).

The compound 1,3-PDO is a linear aliphatic glycol with two terminal hydroxyl groups, and its unique features, biodegradability and higher light stability, allow its use in a wide range of industrial applications, including laminates, adhesives, resins, films, solvents, detergents, cosmetics, engine coolants, fibers, and carpets (Kaur et al. 2012; Saxena et al. 2009). Conventionally, 1,3-PDO is produced by catalytic chemical reactions, hydration of acrolein or hydroformylation of ethylene oxide to 3-hydroxypropionaldehyde (3-HPA), followed by hydrogenation to 1,3-PDO, a process that requires high pressure, temperature, and expensive catalysts along with the release of toxic intermediates. Therefore, production of 1,3-PDO through the process of microbial fermentation has been received more attention as a safer alternative. Bacteria able to convert glycerol to 1,3-PDO have been identified to include the genera *Klebsiella* (*K. pneumoniae* and *K. oxytoca*), *Enterobacter* (*E. agglomerans*), *Citrobacter* (*C.*

freundii), *Clostridium* (*C. butyricum and C. pasteurianum*), and *Lactobacillus* (*L. brevis*, *L. reuteri*, and *L. buchneri*) (Kaur et al. 2012; Saxena et al. 2009).

Lactobacillus panis PM1 is a natural 1,3-PDO-producing organism originally isolated from TS (Khan et al. 2013). In this bacterium, glycerol fermentation occurs via a reductive pathway where glycerol is first converted to 3-HPA catalyzed by glycerol dehydratase (DhaB), after which 3-HPA is reduced to 1,3-PDO by 1,3-propanediol dehydrogenase (DhaT). While this strain can reduce glycerol, it lacks glycerol oxidative pathways to utilize glycerol as a carbon source. In general, lactobacilli are harmless and non-pathogenic microorganism with a broad field of application in the food industry, but require complex nutrient sources for their growth, including amino acids, vitamins, specific minerals, fatty acids, purines, and pyrimidines (Konings 2002). In addition, yeast and beef extracts are usual nutrients for lactobacilli growth; however, their high price is a significant limitation that hampers industrial scale application of lactobacillus strains (Li et al. 2010).

The goal of this study was to test the feasibility of utilizing TS as a culture medium, and specifically utilizing the substrate, glycerol, for bioconversion into 1,3-PDO by *L. panis* PM1. For improved 1,3-PDO production using TS, optimal conditions (pH and temperature) and fermentation methods (batch and fed-batch fermentation) were examined. Furthermore, the expression of an exogenous NADPH-dependent aldehyde reductase (YqhD) of *Escherichia coli* in pH-controlled batch fermentation under optimized culture conditions suggested that *L. panis* PM1 and TS were an ideal host and medium, respectively, for 1,3-PDO bioconversion.

9.3 Materials and Methods

9.3.1 Thin Stillage Source and Liquid Stillage Preparation

Thin stillage (TS) remaining after bioethanol distillation was obtained from North West Bio-Energy Ltd. (Unity, SK, Canada). After centrifugation (6,084 × *g*, 20 min, 15 ℃) by a RC-5C centrifuge (Du Pont Instruments, Hoffman Estates, IL, USA), solid matter was removed from TS, and the resultant liquid part (liquid stillage; LS) was sterilized at 121 ℃ for 20 min.

9.3.2 Bacterial Strains and Growth Conditions

Lactobacillus panis PM1 was cultured in modified MRS (mMRS) medium or LS at 30 ℃ under microaerobic conditions, as described in section 3.3.1, unless otherwise stated. For

acidic media, the initial pH, 6.5, was adjusted to a pH of 4.5 through the addition of 10-N HCl. *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, CA, USA) cells were used for cloning and preparation of the target plasmid, and cultured in LB medium at 37 ℃ with vigorous shaking. Concentrations of erythromycin (Em) for selective plating were 10 μg/mL and 300 μg/mL for *L. panis* PM1 and *E. coli*, respectively.

9.3.3 Plasmid Construction and Bacterial Transformation

DNA work was performed by standard protocols described in section 4.3.9. The genomic DNA of *E. coli* JM109 was used as a template for the coding region of the YqhD gene and the primers were designed by Primer3 (http://frodo.wi.mit.edu/primer3/). PCR amplification was carried out using 0.5 μM each of fYqhD and rYqhD primers (Table 9.1) according to section 4.3.9. The amplified YqhD fragment gene was digested with *NotI* and *SalI* restriction endonucleases and then cloned into the *L. panis* PM1 expression vector, pCER-PocR (Table 9.1). The resultant plasmid was transformed into *L. panis* PM1 by the electroporation method, as described in section 4.3.9.

Table 9.1 The plasmids and primers used in this study

Plasmids		Relevant features		source or reference
pCER		*E. coli-L. panis* PM1 shuttle vector, Em^r Cm^r, 5.0 kb		Kang et el. (2013a)
pCER-PocR		pCER derivative expressing the PocR gene under control of its natural promoter, Em^r Cm^r, 6.5 kb		Kang et el. (2013a)
pCER-YqhD		pCER-PocR derivative, in which the PocR gene is replaced with the YqhD gene, Em^r Cm^r, 6.7 kb		This study
Primer	**Restriction enzymes**	**T_m (℃)[b]**	**Nucleotide sequence (5' →3')[a]**	**Target gene**	**Function**
fYqhD	*NotI*	50.6	ataagaatgcggccgcatgaacaactttaatctgcac	*yqhD*	NADPH-dependent aldehyde reductase
rYqhD	*SalI*	63.5	acgcgtcgacttagcgggcggcttcgtatat		

a. Underlined primer sequences represent restriction enzyme sites. b. T_m, melting temperature.

9.3.4 Batch, Fed-batch, and pH-controlled Batch Fermentation

Batch, or fed-batch, 1,3-PDO fermentations were performed in 50-mL conical tubes containing 40 mL of fermentation media. For batch fermentation, the concentrations of glucose

and glycerol in the LS (initial 0.9 g/L glucose and 8.5 g/L glycerol concentrations) were set at 20 g/L glucose and 28 g/L glycerol, and 28 g/L glycerol were added into mMRS medium, including 10 g/L glucose. For fed-batch fermentation, 10 g/L glucose was initially added to the LS and additional glucose and glycerol were fed into the culture media after 48 h (5.0 g/L glucose and 9.8 g/L glycerol), 72 h (3.3 g/L glucose and 6.5 g/L glycerol), and 96 h (1.7 g/L glucose and 3.3 g/L glycerol). The overall molar ratio of glucose to glycerol was 0.37 in both batch and fed-batch cultures using LS. The glucose to glycerol molar ratio in batch fermentation using mMRS was 0.18. The fermentation tests were conducted at 30 ℃ for 6 days. Batch fermentations (pH-controlled) were performed in a 5-L fermentor (Bioflow III; New Brunswick Scientific (NBS) Co. Inc., Edison, NJ, USA) with 3 L of LS containing 30 g/L glucose and 16.5 g/L glycerol for a 0.61 mol glucose/mol glycerol ratio, and a 50-mL seed culture was used as inoculum. Fermentation temperature was maintained at 30 ℃, pH was set at 4.5 using 5-N NaOH, and agitation to mix the pH-control agent was regulated at 150 rpm. No air or other gas was supplied during the fermentation.

9.3.5 Determination of Glucose, Glycerol and End-products

Optical density and concentration of metabolites (glucose, glycerol, organic acids, 3-HPA, 1,3-PDO, and ethanol) were analyzed by a spectrophotometer and the optimized HPLC method, respectively, as described in section 3.3.2.

9.3.6 Statistical Analysis

For determinations of end-product concentrations, data were presented as mean values calculated from at least two independent experiments. Differences of 1,3-PDO concentrations produced from different fermentation conditions and strains were analyzed by the unpaired *t* test using GraphPad Prism, version 5.0, software (GraphPad Software, Inc., San Diego, CA, USA). A *P* value of < 0.05 was considered significant.

9.4 Results and Discussion

9.4.1 Optimizing 1,3-PDO Production Conditions

In Chapter 7, two factors, temperature and pH, contributed to a shift in NADH recycling routes, significantly affecting 1,3-PDO production in *L. panis* PM1. Thus, in this study with a

new recombinant variant of *L. panis* PM1, batch fermentations for 1,3-PDO production was examined using four combinations of temperature (30 or 37 ℃) and pH (4.5 or 6.5) for 96 h to confirm the optimal conditions for the recombinant strain (Figure 9.1). Temperature was a significant factor affecting 1,3-PDO production ($P < 0.01$). Under low temperature (30 ℃) conditions, 1,3-PDO concentration was increased by 75% at pH 4.5 and by 82% at pH 6.5. Fermentation pH did not significantly affect 1,3-PDO production at each different temperature; however, at low pH (4.5), ethanol production, which generally competes with 1,3-PDO production for the NADH pool, was significantly reduced (2.22 g/L at pH 6.5 to 1.80 g/L at pH 4.5). Therefore, it was determined that pH-4.5/30 ℃ was the optimal condition for 1,3-PDO fermentation by *L. panis* PM1. This combination was employed for further studies of the recombinant variant.

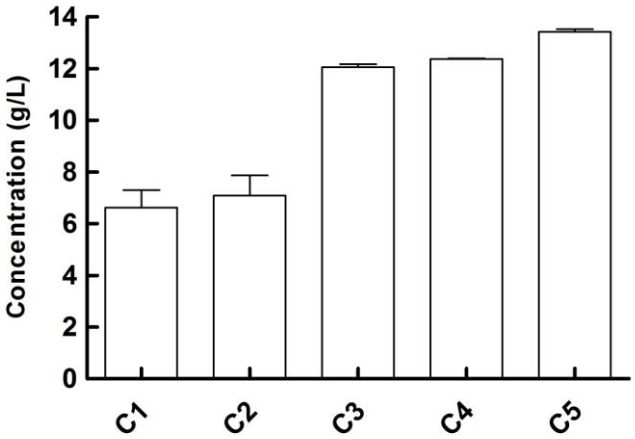

Figure 9.1 Concentration of 1,3-PDO resulting from different fermentation conditions and strains. *Lactobacillus panis* PM1 was cultured in mMRS medium containing 28 g/L glycerol for 96 h under following conditions: pH 6.5-37 ℃ (C1), pH 4.5-37 ℃ (C2), pH 6.5-30 ℃ (C3), or pH 4.5-30 ℃ (C4). 1,3-PDO fermentation by the recombinant strain (C5), PM1-pCER-YqhD, was conducted under the C4 condition.

The *E. coli*-derived enzyme, YqhD, is a NADPH-dependent aldehyde reductase. According to Nakamura and Whited (2003), the recombination of 1,3-PDO producing pathway (*K. pneumoniae* DhaB and DhaT) showed lower 1,3-PDO concentration compared to the utilization of YqhD (*K. pneumoniae* DhaB and *E. coli* YqhD) in an engineered *E. coli* strain. This positive result of YqhD drives more attention to this gene in various metabolic engineering projects, including 1,3-PDO, 1,2-PDO, and isobutanol (Jarboe 2011). *Lactobacillus panis* PM1 has been reported as a natural 1,3-PDO producer via the glycerol reductive pathway. However, the accumulation of 3-HPA caused by a lack of sufficient NADH represses the activity of DhaT, the

second step of the reductive pathway, leading to a decrease in 1,3-PDO concentration (Kang et al. 2013b). To compensate for the reduced activity of DhaT during glycerol fermentation, the *E. coli* YqhD gene was expressed in *L. panis* PM1 under the control of the PocR gene promoter (Kang et al. 2013a). The ability of the resultant recombinant, PM1-pCER-YqhD, was tested under the optimal 1,3-PDO fermentation condition. In comparison with a PM1 strain containing the empty plasmid (PM1-pCER), the PM1-pCER-YqhD strain produced 8.5% more 1,3-PDO (12.37 vs. 13.43 g/L, $P < 0.01$) (Figure 9.1). In both strains, available glucose was depleted within 72 h, which resulted in the cessation of further 1,3-PDO production in the PM1-pCER strain. However, the PM1-pCER-YqhD strain produced 6.1% more 1,3-PDO after this glucose depletion, suggesting the NADPH-dependent role of YqhD in the conversion of 3-HPA to 1,3-PDO. Hummel (1999) reported the production of (R)-phenylethanol by a new NADPH-dependent ADH coupled with an NADP-dependent formate dehydrogenase in *Lactobacillus kefir*. In Chapter 6, an auxiliary system for NADH recycling using citrate was revealed. One of the enzymes in this system (malate dehydrogenase, EC 1.1.1.37) can utilize NADPH as cofactor. In addition, the draft genome of *L. panis* PM1 (unpublished data) revealed a candidate gene for NAD(P) transhydrogenase (EC 1.6.1.2). Thus, these enzymes and unidentified enzymes may contribute to reduction of $NADP^+$ to NADPH, assuming to assist the YqhD reaction. As such, each pathway may contribute a small portion; together, many anabolic and auxiliary pathways can recycle NADPH for overall redox balance.

9.4.2 Growth and 1,3-PDO Production in LS

The acidic characteristic of TS (pH range of 4.4 to 5.0), various unfermented components derived from grains (e.g., minerals and amino acids), yeast cells, and large glycerol contents (up to 2%) in TS could provide *L. panis* PM1 with the necessary conditions for 1,3-PDO fermentation (Kim et al. 2008). However, low concentrations of fermentable sugars (e.g., below 0.9 g/L glucose) eventually caused the cessation of cell growth at mid-log phase along with low 1,3-PDO yield (figure 9.2). During batch fermentation using mMRS (Figure 9.1), only one half of initially-available glycerol (28 g/L) was used for the production of 1,3-PDO before glucose depletion under the optimal condition where the molar ratio of glucose to glycerol was set at 0.18. Therefore, in order to maximize the fermentation yield, additional glucose and glycerol were supplemented into LS at a ratio of 0.37 mol glucose/mol glycerol. For 1,3-PDO

fermentation, two strains, PM1-pCER and PM1-pCER-YqhD, were cultured using batch and fed-batch methods under the optimal condition (pH 4.5 at 30 ℃).

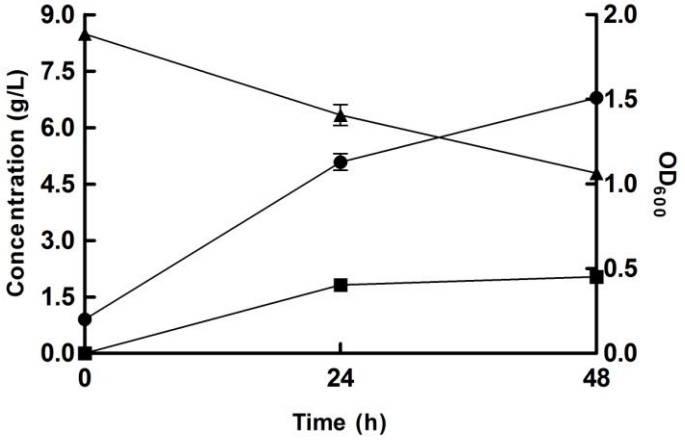

Figure 9.2 Growth, glycerol consumption, and 1,3-PDO production in batch fermentation of the liquid stillage by *L. panis* PM1. The concentrations of glycerol (triangles) and 1,3-PDO (squares) are provided on the left-hand *y*-axis, and the cell density (circles) is provided on the right-hand *y*-axis.

Figure 9.3 shows 1,3-PDO production, glucose and glycerol consumption, and cell density during the respective fermentation runs. Batch fermentation by both strains produced 11% to 12% more 1,3-PDO compared with fed-batch fermentation, and the over-expression of the YqhD gene increased 1,3-PDO concentration from 10.35 to 11.61 g/L in fed-batch fermentation and from 11.56 to 12.85 g/L in batch fermentation. The highest 1,3-PDO concentration (12.85 g/L) was achieved in batch culture of PM1-pCER-YqhD strain, where a 1,3-PDO yield of 0.84 g/g was observed in conjunction with the consumption of 56% glycerol. One-half of the total glucose (10 g/L) was mostly consumed during the first 48 h of culture; whereas the consumption of the other half of glucose required almost twice the amount of time (additional 96 h or 144 h), thereby resulting in reduced 1,3-PDO conversion and growth rates. The molar ratio of consumed glucose to consumed glycerol for 1,3-PDO production increased from 0.37 in mMRS medium to 0.61 in LS. This result suggested that after 48-h culture in LS, limited nutrient sources (e.g., beef extract, yeast extract, and peptone) relative to MRS medium under low pH conditions (below 4.0) negatively-affected the overall 1,3-PDO fermentation (e.g., prolonged fermentation time). These negative factors should be minimized for applications of TS and *L. panis* PM1 in glycerol-bioconversion processes, and pH-control could be a significant cost-effective solution at the industrial scale.

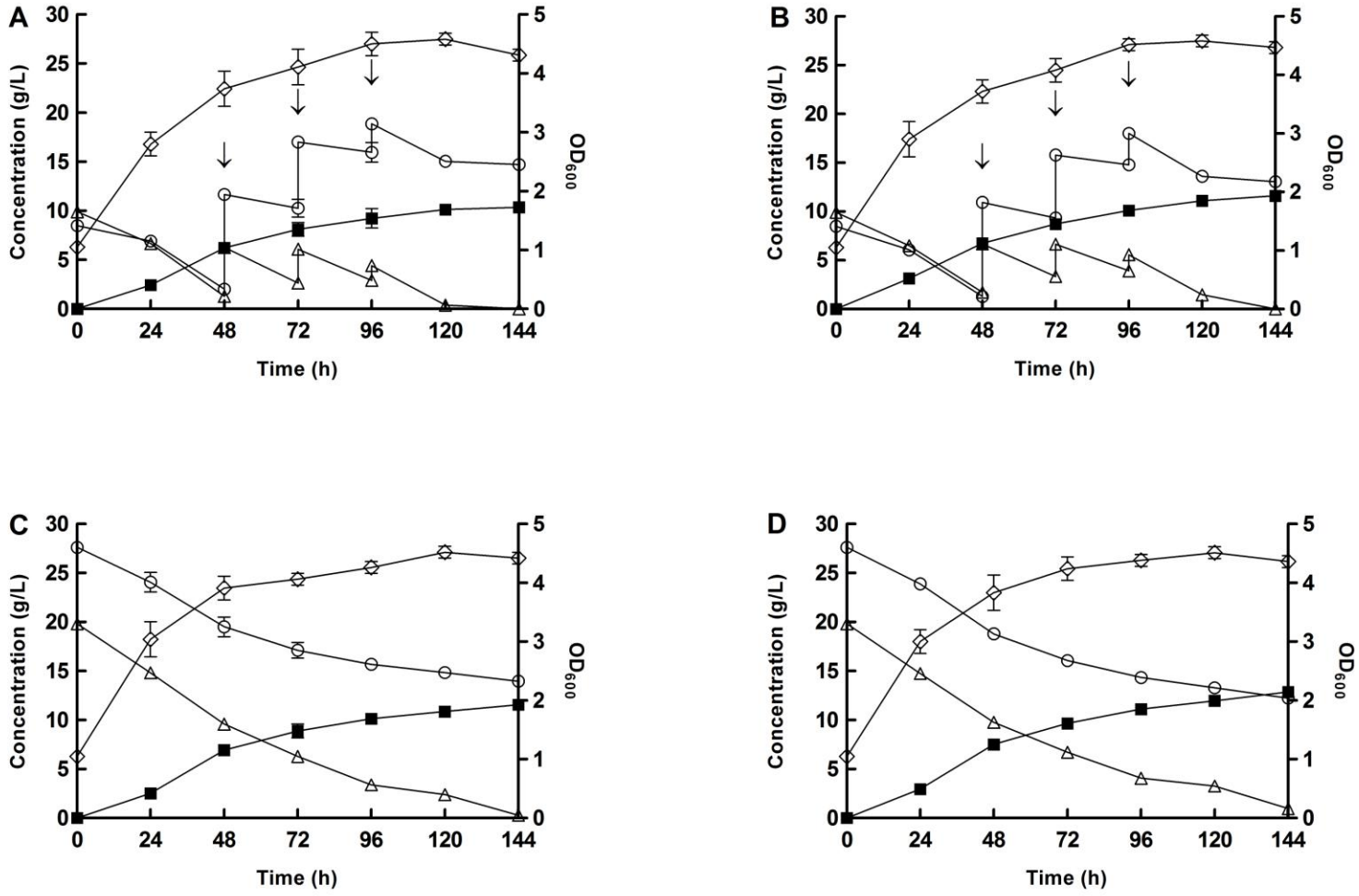

Figure 9.3 Production of 1,3-PDO by PM1-pCER (A, C) and PM1-pCER-YqhD (B, D) using different fermentation methods. A, B: fed-batch fermentation and C, D: batch fermentation. The concentrations of glycerol (circles), glucose (up-pointing triangles), and 1,3-PDO (black squares) are provided on the left-hand *y*-axis, and the cell density (diamonds) is provided on the right-hand *y*-axis. Arrows show time of additional glucose and glycerol supplementation.

9.4.3. pH-controlled Batch Fermentation

Based on the results shown in Figure 9.3, the molar ratio of glucose to glycerol was adjusted to 0.61 in LS with the addition of glucose, and the PM1-pCER-YqhD strain was further used for pH-controlled batch 1,3-PDO fermentation. Under the pH-controlled condition (at pH 4.5), 20 g/L glucose was consumed within 100 h, reflecting an increased glucose consumption rate compared with the pH-uncontrolled condition reported in section 9.4.2 (0.2 $g \cdot L^{-1} \cdot h^{-1}$ vs. 0.14 $g \cdot L^{-1} \cdot h^{-1}$), and maximal 1,3-PDO production (16.23 g/L) was achieved along with low ethanol concentration (below 1 g/L) (Figure 9.4A). However, after 120-h culture, 1,3-PDO production was significantly reduced even though sufficient glucose and glycerol remained in the culture medium, and was accompanied with a decrease in glycerol consumption, resulting in a decrease in average 1,3-PDO yield (0.72 g/g).

In *L. panis* PM1, glycerol is initially converted to 3-HPA by coenzyme B_{12}-dependent glycerol dehydratase, and the glycerol-inactivated enzyme is rapidly reactivated by its reactivating factor in the presence of ATP and Mg^{2+} or Mn^{2+} (Daniel et al. 1998). Even though *L. panis* PM1 can synthesize vitamin B_{12}, limited trace elements (e.g., Mg^{2+} and Mn^{2+}) in LS during fermentation could be causative factors for reduced 1,3-PDO yield and prolonged fermentation time. To enhance 1,3-PDO fermentation, 100 mg/L $MgSO_4 \cdot 7H_2O$ and 50 mg/L $MnSO_4$ were added into LS and 1,3-PDO fermentation was conducted under the same pH-controlled condition. The presence of the two trace elements increased the glucose consumption rate, and the available glucose (30 g/L) was depleted within 140 h. Although the 1,3-PDO concentration was slightly reduced (15.93 g/L), 1,3-PDO yield increased to 0.74 g/g without evidence of 3-HPA accumulation, attaining the balance of 3-HPA formation and consumption (Figure 9.4B). After 120-h culture, 1,3-PDO production was also significantly reduced; however, this reduction was mostly related with glucose depletion. In Chapter 7, glucose depletion was shown to result in the shutdown of the 1,3-PDO pathway and additional glucose feeding after initially-available glucose depletion did not cause resumption of 1,3-PDO production. Thus, this result suggested a positive role of Mg/Mn trace elements addition on the glycerol reductive pathway, and it means if more glucose is added in the LS, additional 1,3-PDO can be produced during the same time period.

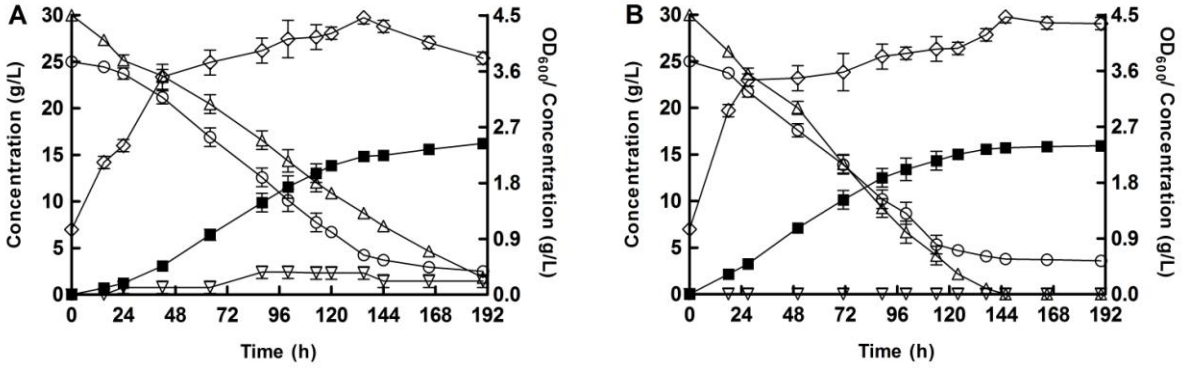

Figure 9.4 Production of 1,3-PDO in pH-controlled batch fermentation of the liquid stillage by PM1-pCER-YqhD. LS, containing 30 g/L glucose and 25 g/L glycerol, supplemented without (A) or with (B) 100 mg/L $MgSO_4 \cdot 7H_2O$ and 50 mg/L $MnSO_4$, was used as fermentation media. The concentrations of glycerol (circles), glucose (up-pointing triangles), and 1,3-PDO (black squares) are provided on the left-hand *y*-axis, and the cell density (diamonds) and 3-HPA concentration (down-pointing triangles) are provided on the right-hand *y*-axis.

Klebsiella pneumoniae and *Clostridium butyricum* are the best natural 1,3-PDO producers, and their concentration (35 to 59 g/L) and productivity (0.6 to 2.4 $g \cdot L^{-1} \cdot h^{-1}$) of 1,3-PDO in batch fermentation are superior to those of *L. panis* reported in this chapter (Saxena et al. 2009). However, 1,3-PDO fermentation by these two bacteria requires yeast extracts and various trace elements in their culture media which substantially increases the cost of fermentation. Recently, Pflügl et al. (2012) reported that high concentrations of 1,3-PDO production (85 g/L in 190 h) were produced by *Lactobacillus diolivorans*, which belongs to the same lactobacilli group (III) as *L. panis* PM1, in fed-batch culture using MRS as a medium. However, this attempt also required expensive culture media, supplementation of high concentrations of glucose (approximately 60 g/L), and the addition of vitamin B12 (5 mg/L). Furthermore, this strain produced 14.4 g/L or 23.8 g/L 1,3-PDO after 147 h in batch fermentation on MRS with 3% glucose and either 2% or 3% glycerol. These amounts are comparable to the result (16.2 g/L) obtained from the pH-controlled batch fermentation on LS supplemented with 3% glucose and 2.5% glycerol (Figure 9.4A). Thus, this suggests that LS is a good culture medium for 1,3-PDO fermentation by *L. panis* PM1 compared with expensive MRS medium, and that 1,3-PDO fermentation by *L. panis* PM1 could be further improved by increasing inoculum volume, as 50-mL seed culture was used for 3-L main culture work in this study, increasing total fermentable sugar, as well as by optimizing trace elements in TS. Therefore, these findings demonstrate the possibility of a cost-effective way to produce 1,3-PDO using TS.

9.5 Conclusions

This study showed that an engineered *Lactobacillus panis* PM1 is an ideal host for cost-effective disposal of TS that provides sufficient nutrient sources for 1,3-PDO conversion for this strain. Under the optimal condition, the recombinant PM1 strain produced 16.23 g/L 1,3-PDO and 0.72 g/g yield in LS without expensive nutrient source supplementation. The engineered strain provides a cost-effective method to convert glycerol to 1,3-PDO in the thin stillage-based culture media. These findings offer a new platform for the production of value-added chemicals from TS from ethanol plants.

9.6 Connection to the Next Study

In this Chapter, the successful production of 1,3-PDO was achieved at a very high efficiency in thin stillage-based media by an engineered PM1 strain. However, this strategy involved an inherent drawback due to the basic characteristics of the PM1 strain; it cannot utilize glycerol without NADH provision by the 6-PG/PK pathway (i.e., glucose metabolism). The supplementation of high amounts of fermentable sugar to thin stillage cannot satisfy the cost efficiencies necessary for industrial applications. To overcome this drawback, the following Chapter (Chapter 10) utilized genetic techniques to engineer the PM1 strain on the basis of the improved metabolic knowledge obtained from Chapters 3 to 8. If an artificial glycerol oxidative pathway can directly connect glycerol to the central metabolism (6-PG/PK pathway) of *L. panis* PM1, the resultant engineered strain would not require glucose supplementation (to the thin stillage) during conversion of glycerol to 1,3-PDO. This metabolic engineering strategy would enable more cost-effective disposal of thin stillage from bioethanol plants.

10. METABOLIC ENGINEERING OF A GLYCEROL OXIDATIVE PATHWAY IN *LACTOBACILLUS PANIS* PM1 TO UTILIZE BIOETHANOL THIN STILLAGE: POTENTIAL TO PRODUCE PLATFORM CHEMICALS FROM GLYCEROL[viii]

10.1 Abstract

Lactobacillus panis PM1 has ability to produce 1,3-propanediol (1,3-PDO) from thin stillage (TS, major waste material after bioethanol production) and is therefore of significance. However, the fact that *L. panis* PM1 cannot use glycerol as a sole carbon source presents a considerable problem in terms of utilization of this strain in a wide range of industrial applications. Accordingly, *L. panis* PM1 was genetically engineered to directly utilize TS as a fermentable substrate for the production of valuable platform chemicals without the need for exogenous nutrient supplementation (e.g., sugars and nitrogen sources). An artificial glycerol oxidative pathway, comprised of glycerol facilitator, glycerol kinase, glycerol 3-phosphate dehydrogenase, triosephosphate isomerase, and NADPH-dependent aldehyde reductase genes of *Escherichia coli*, was introduced into *L. panis* PM1 in order to directly utilize glycerol for the production of energy and valued-added chemicals. A pH 6.5 culture converted glycerol to mainly lactic acid (85.43 mM); whereas a significant amount of 1,3-propanediol (59.96 mM) was achieved at pH 7.5. Regardless of the pH, ethanol (82.16 to 83.22 mM) was produced from TS fermentations, confirming that the artificial pathway metabolized glycerol for energy production and converted it into lactate or 1,3-PDO and ethanol in a pH-dependent manner. This study demonstrates the cost-effective conversion of TS to value-added chemicals by the engineered PM1 strain cultured under industrial conditions. Thus, these findings can contribute to reduced costs of reduction in over bioethanol production cost.

[viii] This chapter is submitted to Biotechnology for Biofuels. Tae Sun Kang, Darren R. Korber, and Takuji Tanaka. 2014. Metabolic engineering of a glycerol oxidative pathway in *Lactobacillus panis* PM1 to utilize bioethanol thin stillage: Potential to produce platform chemicals from glycerol.

10.2 Introduction

Thin stillage (TS) is a major fermentation residue from dry-grind processes of bioethanol production. Its downstream treatment, including making a condensed form for animal feed, is an energy- and cost-intensive process (Kim et al. 2008), while its utilization as feed is hardly a high-value application of this industrial by-product and hence does not significantly reduce the cost of ethanol production. TS contains a variety of complex nutrients, including various carbohydrates, minerals, and amino acids. Glycerol (up to 2%) is generated in TS during bioethanol production regardless of feedstock (i.e., sugar cane or corn) (Clomburg and Gonzalez 2013). Glycerol is at a higher reduced state compared to carbohydrates (i.e., fermentable sugars). The reduced nature of glycerol allows synthesis of fuels and other reduced chemicals at higher yields with minimal byproducts compared to common sugars (e.g., glucose and xylose); however, the high degree of reduction in glycerol makes it difficult for microorganisms to utilize under anaerobic conditions. A few bacteria, including *Klebsiella*, *Enterobacter*, and *Clostridium*, have been exploited to bioconvert glycerol to industrially-relevant chemicals (e.g., 1,3-propanediol (1,3-PDO), 3-hydroxypropionic acid, butanol and ethanol) (Ashok et al. 2013; Gonzalez-Pajuelo et al. 2006; Ito et al. 2005; Mu et al. 2006; Scheel and Lutke-Eversloh 2013). Since they are classified as opportunistic pathogens, industrial applications of these strains may pose issues, particularly regarding worker biosafety.

Lactobacilli have been long considered as generally recognized as safe (GRAS) microorganisms. Because of their small genomes and relatively simple metabolic pathways, lactobacilli have been intensively utilized as cell factories for the production of platform chemicals, including lactate, 2,3-butanediol, 1,3-PDO, ethanol, food flavors, sweeteners, and complex polysaccharides, through metabolic engineering approaches (Gaspar et al. 2013). *Lactobacillus panis* PM1 is a natural 1,3-PDO-producing organism originally isolated from TS. This strain achieves primary carbohydrate fermentation through substrate-level phosphorylation via the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway wherein NADH is mainly recycled back to NAD^+ through the production of lactate and ethanol (Figure 10.1). In the presence of glycerol, the glycerol reductive pathway (glycerol to 1,3-PDO) is a major NADH recycling system used in conjunction with the ethanol production pathway.

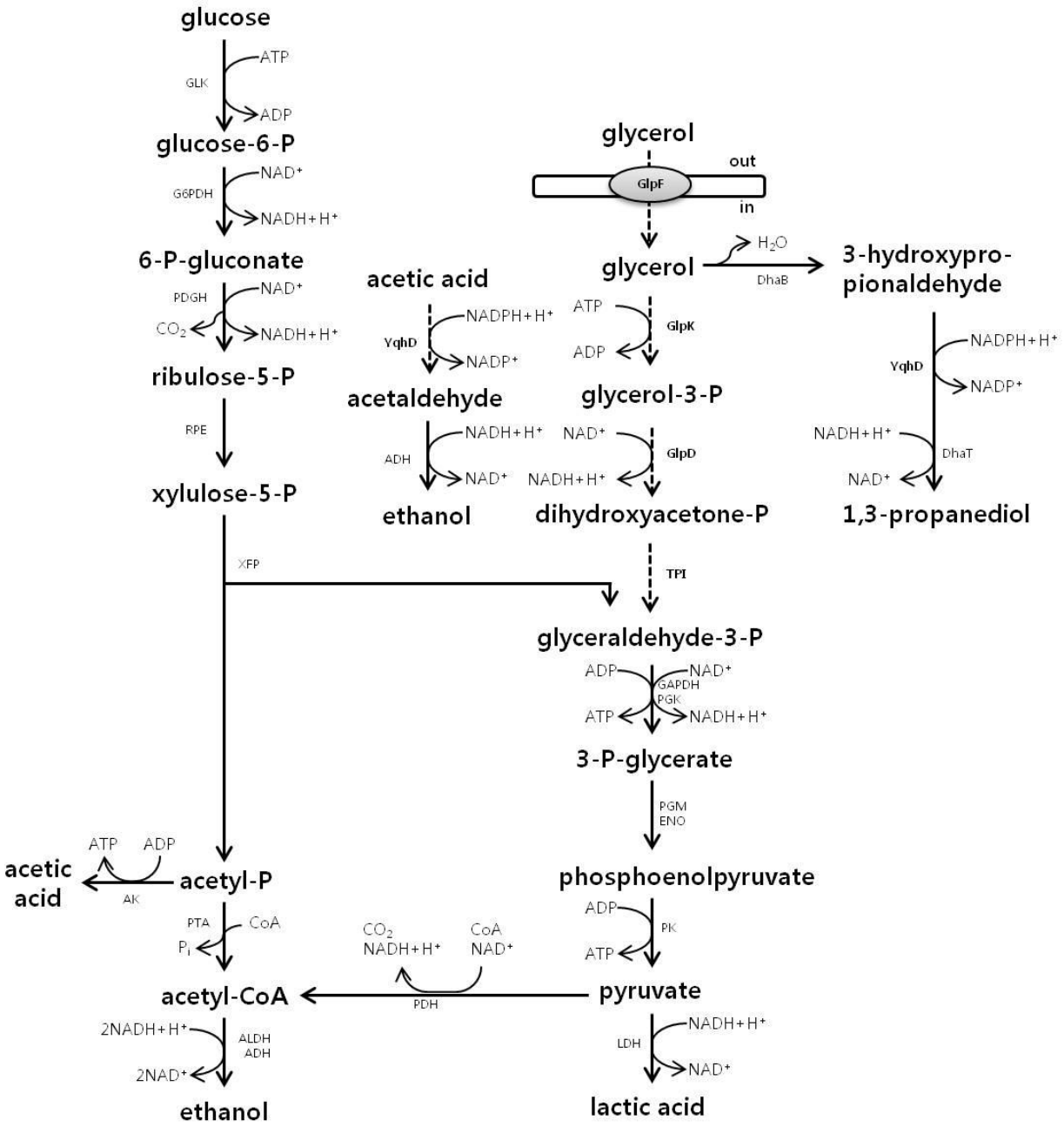

Figure 10.1 Schematic diagram of the glycerol metabolic pathway in the engineered PM1 strain. Dashed lines, metabolically-engineered pathways. Abbreviation, ADH, alcohol dehydrogenase; AK, acetate kinase; ALDH, acetaldehyde dehydrogenase; CoA, coenzyme A; DhaB, glycerol dehydratase; DhaT, 1,3-propanediol dehydrogenase; ENO, enolase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GlpD, glycerol 3-phosphate dehydrogenase; GlpF, glycerol facilitator; GlpK, glycerol kinase; LDH, lactate dehydrogenase; PDH, pyruvate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglyceromutase; PK, pyruvate kinase; PTA, phosphotransacetylase; TPI, triosephosphate isomerase; and YqhD, NADPH-dependent aldehyde reductase.

The consumption of common cofactor, NADH, is shifted from ethanol to 1,3-PDO production, yielding one extra ATP per glucose (Kang et al. 2013b). However, glycerol metabolism in *L. panis* PM1 is not connected to the central metabolic (6-PG/PK) pathway, and thus strain PM1 cannot utilize glycerol as a sole source for energy. As seen in Figure 9.4, TS is a good culture medium for 1,3-PDO fermentation by *L. panis* PM1, providing a final 1,3-PDO concentration of 16.23 g/L and the yield of 0.72 g/g from TS supplemented to 30 g/L glucose and 25 g/L glycerol; however, low indigenous fermentable sugar content (e.g., glucose below 0.9 g/L) in TS is the main bottleneck for the cost-effective glycerol bioconversion by the wild-type PM1 strain (Chapter 9). Therefore, the absence of a glycerol oxidative pathway (glycerol to glyceraldehyde 3-phosphate) indeed hampers a wide range of industrial applications using this strain.

In an effort to develop a cost-effective approach to processing glycerol based on utilization of TS, *Lactobacillus panis* PM1 was metabolically-engineered for the production of more valuable platform chemicals from glycerol in TS. In this chapter, the successful expression of an artificial glycerol oxidative pathway, composed of glycerol facilitator (GlpF), glycerol kinase (GlpK), glycerol 3-phosphate dehydrogenase (GlpD), triosephosphate isomerase (TPI), and NADPH-dependent aldehyde reductase (YqhD) genes of *Escherichia coli*, in *L. panis* PM1 is described. The engineered strain can produce value-added chemicals, including lactate, ethanol, and 1,3-PDO, from TS without the need for additional fermentable sugar and nutrient supplementation (e.g., glucose and yeast extract, beef extract, peptone, and vitamin B_{12}).

10.3 Materials and Methods

10.3.1 Thin Stillage Source and Liquid Stillage Preparation

Thin stillage (TS) remaining after bioethanol production was obtained from Pound-Maker Agventures Ltd. (Lanigan, SK, Canada). Liquid stillage (LS) from the TS was prepared as described in section 9.3.1.

10.3.2 Bacterial Strains and Growth Conditions

Lactobacillus panis PM1 and its engineered strains were cultured in modified MRS (mMRS) medium or LS at 37 ℃ under microaerobic conditions, as described in section 3.3.1, unless otherwise stated. For acidic media, the pH was adjusted from 6.5 to 4.5 through the addition of 10-N HCl. *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, CA, USA) cells were used

for cloning and preparation of the target plasmid, and cultured in LB media at 37 ℃ with vigorous shaking. Concentrations of erythromycin (Em) for selective plating were 10 μg/mL and 300 μg/mL for *L. panis* PM1 and *E. coli*, respectively.

10.3.3 General DNA Techniques, Plasmid Construction, and Bacterial Transformation

The genomic DNA of *E. coli* JM109 was used as a template for the genes encoding GlpFK, GlpD, TPI, and YqhD, and the corresponding primers were designed by Primer3 (http://frodo.wi.mit.edu/primer3/). PCR amplification was carried out using 0.5 μM each of primers (Table 10.1) according to section 4.3.9. PCR fragments for GlpFK (introduced restriction sites; *SalI/XbaI*), GlpD (introduced restriction sites; *XbaI/SacI*), TPI (introduced restriction sites; *NcoI/SacI*), and YqhD (introduced restriction sites; *SacI*) were consecutively cloned into pUC18 to yield pUC18-GlpFK, pUC18-GlpFKD, pUC18-GlpFKD-TPI, and pUC18-GlpFKD-TPI-YqhD, respectively. The GlpFKD-TPI (4.7 kb) and GlpFKD-TPI-YqhD (5.9 kb) fragments from each construct were amplified using fGlpFK and rM13 primers, digested with *NotI* and *SalI* restriction endonucleases, and cloned into the *L. panis* PM1 expression vector, pCER-EGFP (Table 4.1) to yield the pCER-GlpFKD-TPI and pCER-GlpFKD-TPI-YqhD plasmids (Table 10.1 and Figure 10.2), respectively. These plasmids were then transformed into *L. panis* PM1 by the electroporation method, as described in section 4.3.9.

10.3.4 Batch and pH-controlled Batch Fermentation

Batch lactate fermentation was performed in 50-mL conical tubes containing 40 mL of mMRS medium. The concentration of glycerol in mMRS with, or without, sodium acetate was set at 160 mM, and the fermentation tests were conducted at 37 ℃. Batch fermentation (pH-controlled) was performed in a 5-L fermentor (Bioflow III; New Brunswick Scientific (NBS) Co. Inc., Edison, NJ, USA) with 3 L of LS (initial glycerol concentration at 160 mM), into which 50-mL mid-log phase culture was inoculated. Fermentation temperature was adjusted to 37 ℃, and pH was controlled at 6.5 (for lactate fermentation) or 7.5 (for 1,3-PDO fermentation) with 5-N NaOH, and agitation was set at 80 rpm to mix the pH-control agent. No air or other gas was supplied during the fermentation.

Table 10.1 The plasmids and primers used in this study

Plasmids	Relevant features	source or reference
pUC18	Cloning vector, Ap^r, 2.7 kb	Invitrogen
pUC18-GlpKF	pUC18 derivative carrying the GlpF and GlpK genes from *E. coli* JM109	This study
pUC18-GlpKFD	pUC18 derivative carrying the GlpF, GlpK, and GlpD genes from *E. coli* JM109	This study
pUC18-GlpKFD-TPI	pUC18 derivative carrying the GlpF, GlpK, GlpD, and TPI genes from *E. coli* JM109	This study
pUC18-GlpKFD-TPI-YqhD	pUC18 derivative carrying the GlpF, GlpK, GlpD, TPI, and YqhD genes from *E. coli* JM109	This study
pCER-EGFP	*Lactobacillus panis* PM1 expression vector, in which the EGFP[a] gene was expressed under control of the P*orfX* promoter, Em^r Cm^r, 6.0 kb	Kang et al. 2013d
pCER-GlpKFD-TPI	pCER-EGFP derivative, in which the EGFP gene was replaced with the GlpKFD-TPI fragment, Em^r Cm^r, 10.8 kb	This study
pCER-GlpKFD-TPI-YqhD	pCER-EGFP derivative, in which the EGFP gene was replaced with the GlpKFD-TPI-YqhD fragment, Em^r Cm^r, 12.0 kb	This study

Primer	Restriction enzymes	T_m (℃)[b]	Nucleotide sequence (5'→3')[c]	Target gene	Function
fGlpFK	*SalI/NotI*	54	acgcgtcgacgcggccgcatgagtcaaacatcaaccttg	*glpF and glpK*	Glycerol facilitator and Glycerol kinase
rGlpFK	*XbaI*	59	tgctctagattattcgtcgtgttcttccca		
fGlpD	*XbaI*	60	gctctaga**gaaggaga**atggaaaccaaagatctgattgtg	*glpD*	Glycerol 3-phosphate dehydrogenase
rGlpD	*SacI/NcoI*	63	tccgagctcccatggttacgacgccagcgataacct		
fTPI	*NcoI*	56	catgccatg**ggagaaaga**atgcgacatcctttagtgatg	*tpi*	Triosephosphate isomerase
rTPI	*SacI*	59	tccgagctcttaagcctgtttagccgcttc		
fYqhD	*SacI*	51	tccgagctc**gaaggaga**atgaacaactttaatctgcac	*yqhD*	NADPH-dependent aldehyde reductase
rYqhD	*SacI*	64	tccgagctcttagcgggcggcttcgtatat		
rM13	*SalI*	45	acgcgtcgaccaggaaacagctatgac		pUC/M13 sequencing reverse primer

a. EGFP, enhanced green fluorescent protein. b. T_m, melting temperature. c. Underlined and bold sequences represent restriction enzyme sites and artificial ribosome binding sites, respectively.

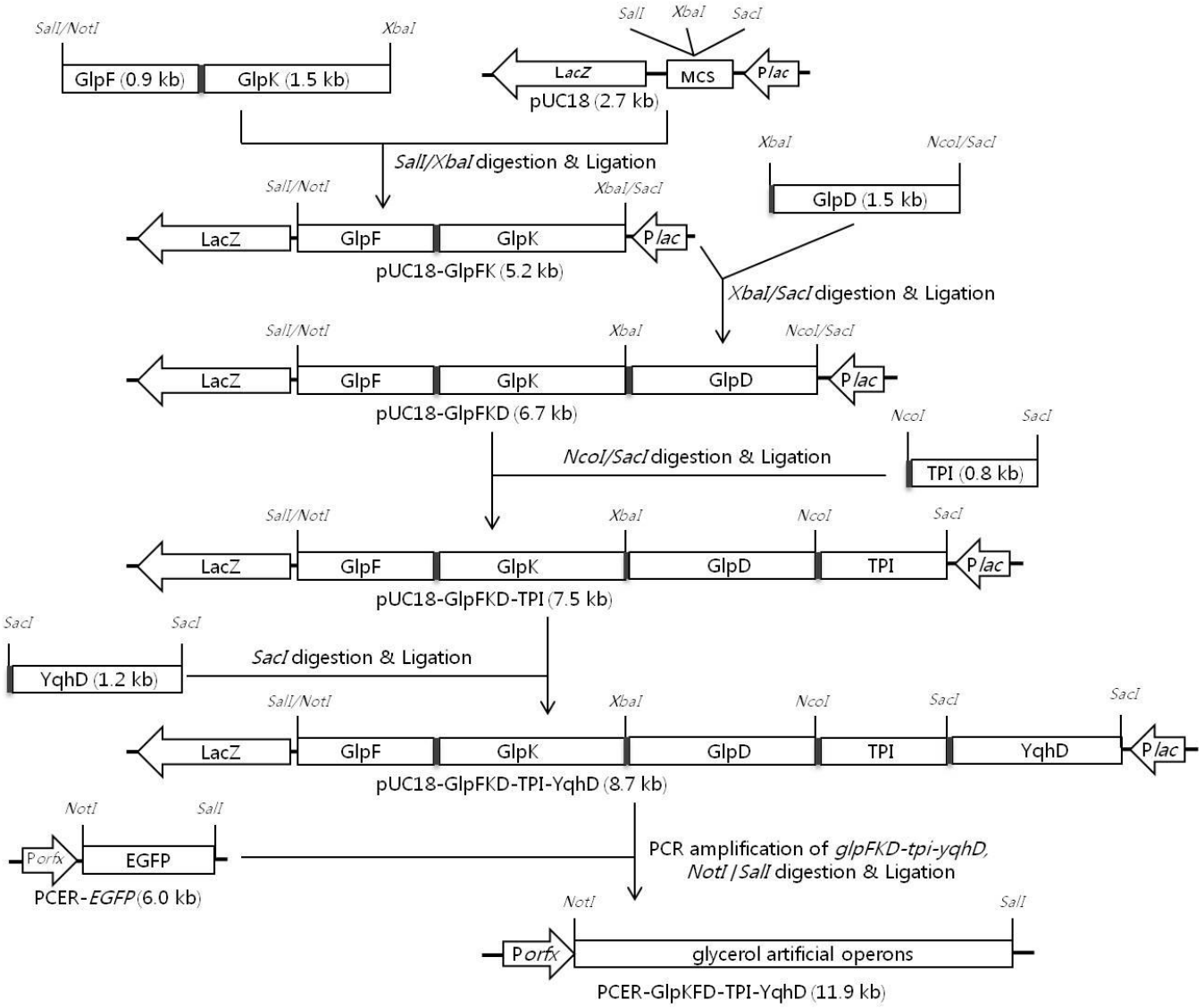

Figure 10.2 Construction of the glycerol artificial operons. Dark gray regions shown between each gene represent ribosomal binding sites.

10.3.5 Determination of Glycerol and End-products

Optical density and concentration of metabolites (glycerol, organic acids, 3-HPA, 1,3-PDO, and ethanol) were analyzed by a spectrophotometer and the optimized HPLC method, respectively, as described in section 3.3.2.

10.3.6 Preparation of Crude Cell Extracts

Lactobacillus panis PM1 and its engineered cells grown in mMRS containing different carbon sources (glycerol or glucose) or LS at pH 4.5-7.5 were disrupted by sonication, as described in section 4.3.2. Protein concentration in the crude culture extract was determined by the Bradford method, as described in section 4.3.2.

10.3.7 Enzyme Assays

Acetaldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) assays were spectrophotometrically evaluated at 37 ℃ by measuring the changes in absorbance at 340 nm (ε^{NADH} = 6,220 M^{-1} cm^{-1}). One unit of activity corresponds to the generation or consumption of 1 μmol of NAD(P)H per min. For ALDH (acetaldehyde to acetate) activity, the reaction mixture consisted of 50 mM potassium carbonate (pH 9.0), 5 mM DTT, 0.2 mM $NADP^+$, and 0.1 mg protein. The mixture was pre-incubated for 10 min at room temperature and the reaction was started by the addition of 10 mM acetaldehyde (a stock solution of 100 mmol/L acetaldehyde diluted with methanol). ADH (acetaldehyde to ethanol) was measured in 100 mM potassium carbonate (pH 9.0), 5 mM DDT, 0.2 mM NADH, and 0.1 mg protein. After 10 min pre-incubation at room temperature, the reaction was started by addition of 10 mM acetaldehyde (a stock solution of 100 mmol/L acetaldehyde diluted with methanol). Glycerol dehydratase (DhaB) and 1,3-propanediol dehydrogenase (DhaT or YqhD) activities were determined by the 3-HPA-quantification method. Briefly, for DhaB (glycerol to 3-HPA) activity, the reaction mixture consisted of 100 mM potassium phosphate (pH 7.0), 10 mM glycerol, 20 μM coenzyme B_{12}, and 0.1 mg protein (Kwak et al. 2012). The reaction mixture was incubated at 37 ℃ for 20 min, and the enzyme reaction was terminated by the addition of the same volume of 100 mM citrate solution. Quantification of 3-HPA in the reaction solution was carried out using a colorimetric method (Doleyres et al. 2005). For DhaT or YqhD (1,3-PDO to 3-HPA) activity, the assay mixture contained 2 mM NAD^+ (for DhaT) or $NADP^+$ (for YqhD), 100 mM 1,3-PDO, 30 mM

ammonium sulfate, and 0.1 mg protein in 100 mM potassium carbonate buffer at pH 9.0 (Kang et al. 2013b). The assay was carried out at 37℃ for 20 min, and reaction termination and 3-HPA quantification were conducted as described above. In these determinations, one unit of activity corresponds to the production of 1 mmol of 3-HPA per min. Specific activities of the enzymes were expressed as units per milligram of protein.

10.3.8 Statistical Analysis

For determinations of end-product concentrations and enzyme activities, data were presented as mean values calculated from at least two independent experiments. The specific activities of DhaB, DhaT, and YqhD were analyzed by two-way analysis of variance with Bonferroni posttests using GraphPad Prism, version 5.0, software (GraphPad Software, Inc., San Diego, CA, USA). A P value of < 0.05 was considered significant.

10.4 Results and Discussion

10.4.1 Metabolic Engineering Strategy for a Glycerol Oxidative Pathway

The glycerol metabolic pathway of *L. panis* PM1 is limited to a reductive pathway, and thus glycerol fermentation is restricted to NADH re-oxidation (for 1,3-PDO production) rather than its use as an energy source (Kang et al. 2013b). This inability implies that TS contains little readily-utilizable carbon for *L. panis* PM1 growth (Figure 9.2). For more cost-effective biotechnological applications involving TS, a glycerol oxidative pathway was constructed in *L. panis* PM1 to enable glycerol to serve as the sole carbon source for energy and growth (Figures 10.1 and 10.2). *Escherichia coli* possesses two glycerol oxidative pathways, GlpK/GlpD and glycerol dehydrogenase (DhaD)/dihydroxyacetone kinase (DhaK), and mostly uses the DhaDK route under anaerobic conditions (Durnin et al. 2009); however, the DhaDK route did not work properly in *L. panis* PM1 under microaerobic conditions since DhaK is a phosphotransferase system -dependent kinase (Bächler et al., 2005), and group III lactobacilli generally tend to lack activity of this system (Wisselink et al., 2002). Alternatively, the GlpKD route, which contains an ATP-dependent kinase, was chosen as the backbone of the artificial glycerol oxidative pathway. This oxidative pathway consisted of *glpF*, *glpK* and *glpD* that are required to import glycerol and to oxidize the imported glycerol to dihydroxyacetone phosphate (DHAP). DHAP would then be converted into glyceraldehyde 3-phosphate (GAP) via TPI activity as part of the

typical Embden-Meyerhof (EM) pathway. However, as previously described in Chapter 4, the absence of TPI activity in *L. panis* PM1 resulted in an accumulation of DHAP to the extent that its toxicity influenced cell growth and eventually lead to early growth cessation. Consequently, the TPI gene from *E. coli* was cloned into an artificial glycerol oxidative pathway to prevent DHAP build-up and toxicity. This artificial operon, GlpFKD-TPI, was sub-cloned into the *L. panis* PM1 expression vector, pCER-EGFP (Table 4.1). In this plasmid, the expression of the GlpFKD-TPI fragment was controlled under the P*orfX* promoter that was derived from the sakacin P regulon of *L. sake* LTH673 (Risøen et al. 2000) and was developed as an inducible promoter (by sakacin P) for *L. sakei* and *L. plantarum* (Sörvig et al. 2005). However, the basal activity of this promoter was significantly high in *L. sakei* and *L. plantarum,* suggesting potential high expression in *L. panis* without the need for specific induction (Sörvig et al. 2005). Previously, Chapter 4 demonstrated the successful expression of the exogenous TPI gene in *L. panis* PM1 using the same expression system in the absence of its inducer.

The resultant plasmid was transformed into the wild-type PM1 strain, and the resultant recombinant strain (PM1-pCER-GlpFKD-TPI) was cultured in mMRS supplemented with glycerol (24 mM) to test whether glycerol could be used as the sole carbon source (Figure 10.3).

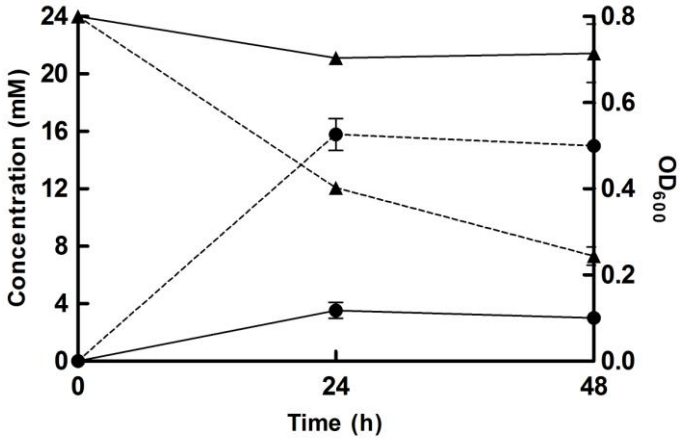

Figure 10.3 Growth and glycerol consumption of wild type and recombinant PM1 strains. PM1-pCER (solid lines) and PM1-pCER-GlpFKD-TPI (dashed lines) strains were cultured in mMRS medium containing 24 mM glycerol. Symbols: circles - cell density (OD_{600}) and triangles - glycerol concentration.

The PM1-pCER-GlpFKD-TPI strain consumed 10-times more glycerol relative to the control strain containing the empty plasmid (PM1-pCER) over the first 24 h, indicating that the artificial operon functioned to metabolize glycerol. However, the growth of this engineered strain ceased

after 24 h and only 17 mM of glycerol was consumed, resulting in a cell density (OD_{600}) of only 0.5 (Figure 10.3). End-product analyses showed that most of the consumed glycerol was directed towards lactate, and not 1,3-PDO production, which is a typical NAD^+ regeneration route in *L. panis* PM1 (Kang et al. 2013b), indicating that NADH formed from the new artificial pathway (i.e., by the GlpD reaction) was not recycled sufficiently through the indigenous glycerol reductive pathway. During anaerobic fermentation, the overall redox balance within the cell is a critical determinant governing overall end-product profiles (Clomburg and Gonzalez 2013). How NADH is recycled was likely responsible for the poor growth and low glycerol consumption rates observed in Figure 10.3, suggesting the necessity for an additional, or supplemental, NADH-recycling route.

10.4.2. Construction of a New NADH Recycling System

The reduced growth and glycerol utilization of PM1-pCER-GlpFKD-TPI suggested that the NADH recycling system did not function as hypothesized, and therefore an alternative NADH recycling system appeared necessary to enable TS utilization. Accordingly, a new NADH recycling system was constructed by subcloning the *E. coli* YqhD gene into the glycerol oxidative pathway, generating pCER-GlpFKD-TPI-YqhD (Figure 10.2). The *E. coli* YqhD is a NADPH-dependent aldehyde reductase and its activity contributes to a variety of reactions involving butyraldehyde, glyceraldehyde, malondialdehyde, isobutyraldehyde, methylglyoxal, propanealdehyde, acrolein, furfural, glyoxal, 3-hydroxypropionaldehyde, glycolaldehyde, acetaldehyde, and acetol (Jarboe 2011). It is expected that YqhD can process acetate in the media into ethanol in conjunction with NADH-dependent ADH, oxidizing NAD(P)H. It would also be beneficial to produce more 1,3-PDO as, previously, the expression of the exogenous YqhD gene in *L. panis* PM1 could compensate for the reduced DhaT activity caused by 3-HPA accumulation, resulting in higher 1,3-PDO production (Figures 9.1 and 9.3). This previous result also indicated that the NADPH pool would not be a limiting step to enable YqhD as an auxiliary system. The final recombinant strain, PM1-pCER-GlpFKD-TPI-YqhD, was cultured in mMRS supplemented with glycerol (160 mM) as sole carbon source to test how the YqhD gene could contribute to overall NADH recycling and glycerol consumption. The PM1-pCER-GlpFKD-TPI-YqhD strain achieved 4-times greater growth on glycerol than the PM1-GlpFKD-TPI strain did, reaching a final cell density (OD_{600}) of 2.0.

To detect the activities of both ADH and ALDH, which comprised the new NADH recycling system, the PM1-pCER-GlpFKD-TPI-YqhD strain was cultured in mMRS medium supplemented with 160 mM glycerol as the sole carbon source for 24 h. As a reference, the PM1-pCER strain (which cannot use glycerol as a carbon source) was cultured in mMRS with 60 mM glucose. The specific activity of the ADH enzyme observed in the PM1-pCER and the PM1-pCER-GlpFKD-TPI-YqhD strains was 13.59±0.24 and 5.95±0.64 units/mg protein, respectively. This difference in the specific activity would be originated from different carbon sources. The 6-PG/PK pathway is a primary metabolism for glucose fermentation in *L. panis* PM1 where ethanol is produced as a major end-product for NADH recycling. No detectable activity of the ALDH enzyme was detected in the PM1-pCER strain; whereas 3.19±0.03 units/mg protein was detected for the PM1-pCER-GlpFKD-TPI-YqhD strain. This observation indicated that the ALDH activity originated from the exogenous YqhD gene. Obviously, the YqhD system is dependent on the recycling of NAPDH. While the exact NADPH recycling system has not been identified, the reduction of $NADP^+$ for YqhD activity may be achieved by NADP-dependent dehydrogenases, including the citrate auxiliary pathway examined in Chapter 6, and NAD(P) transhydrogenase, as discussed in Chapter 9. With the new NADH recycling system, NADH produced from the oxidative glycerol pathway could now be reoxidized via the acetate-to-ethanol route by the activities of YqhD and the indigenous ADH, consuming NAD(P)H (Figure 10.1). As a result, the presence of acetate, a component of the MRS medium (from 5 g/L sodium acetate), positively-affected the cell growth of the PM1-GlpFKD-TPI-YqhD strain in the mMRS medium containing glycerol as the sole carbon source. In the presence of acetate, the PM1-pCER-GlpFKD-TPI-YqhD strain increased glycerol consumption by 75% and lactate production by 77% compared to the culture in the absence of acetate (Table 10.2).

Table 10.2 The effect of acetate on NADH recycling system

Medium[a]	OD_{600}	Lactate	Glycerol[b]	Acetate	Ethanol
with acetate (50 mM)	1.53±0.07	46.15±0.38	57.24±0.77	29.8±0.30[c]	29.02±0.28
without acetate	0.59±0.01	26.05±0.89	32.79±0.53	6.07±0.08	11.70±0.08

a. PM1-pCER-GlpFKD-TPI-YqhD strain was cultured in mMRS media containing 160 mM glycerol with or without acetate for 24 h. End-product unit is mM. b. Consumed amount. c. Initially-available acetate amount (50 mM) was reduced to 29.8 mM.

In the absence of acetate, the new recycling system could not function due to substrate limitation, and consequently the glycerol reductive pathway was the main NAD^+ regeneration route. This shift in NADH recycling could force the recombinant strain to consume glycerol with the production of 1,3-PDO, leading to carbon loss of glycerol for ATP generation via pyruvate production (Figure 10.1). The acetate-to-ethanol route, on the other hand, could allow most glycerol to be used for lactate production in the presence of acetate, generating one net ATP per glycerol. Thus, more ATP yield in the presence of acetate could be a factor for the improved growth. However, the growth and glycerol consumption of the PM1-GlpFKD-TPI-YqhD strain halted during the mid- to late-log phase even though sufficient acetate for the new recycling system remained in the culture medium [approximate cell density (OD_{600}) of 2.0].

Table 10.3 shows the negative effect of acidic culture conditions on the new NADH recycling system that was introduced as a part of the recombinant plasmid. In pH 6.5 medium, the PM1-GlpFKD-TPI-YqhD strain consumed more glycerol (2.9-fold) and acetate (6.9-fold) and produced more lactate (3.8-fold) and ethanol (7.3-fold) relative to the pH 4.5 medium. Lactate was a major glycerol metabolism end-product in the engineered strain, and its accumulation during fermentation caused the pH of the media to drop from the initial 6.5 to below 5.0 within 24 h. Thus, growth-related acidification negatively affected the new NADH recycling system, which eventually resulted in the cessation of growth and glycerol consumption during the mid- to late-log phase.

Table 10.3 The effect of pH on NADH recycling system

Initial pH[a]	Lactate	Glycerol[b]	Acetate[c]	Ethanol
6.5	56.19±0.31	59.47±0.23	22.62±0.46	37.77±1.65
4.5	14.98±0.08	20.59±0.46	46.04±0.56	5.14±0.08

a. PM1-pCER-GlpFKD-TPI-YqhD strain was cultured in mMRS medium containing 160 mM glycerol for 24 h at pH 4.5 or 6.5. End-product unit is mM. b. Consumed amount. c. Initially-available acetate amount (50 mM) was reduced to 22.62 and 46.04 mM, respectively.

10.4.3 pH-controlled Batch Culture of the Recombinant PM1 in LS

To prevent low pH growth-limitation, a pH-controlled batch culture method was adopted. Considering acetate consumption via the new NADH recycling system of the PM1-GlpFKD-TPI-YqhD strain (acetate-to-ethanol route), 4 g/L sodium acetate (approximately 40 mM acetate) was added to LS. End-product formation and glycerol consumption by the recombinant strain

were monitored over 4 days. The initially-available glycerol (160 mM) in LS was mostly consumed within 72-h during culture under the pH 6.5 condition, producing 85.43 mM of lactate with a yield of 0.61 mol/mol (Figure 10.4A).

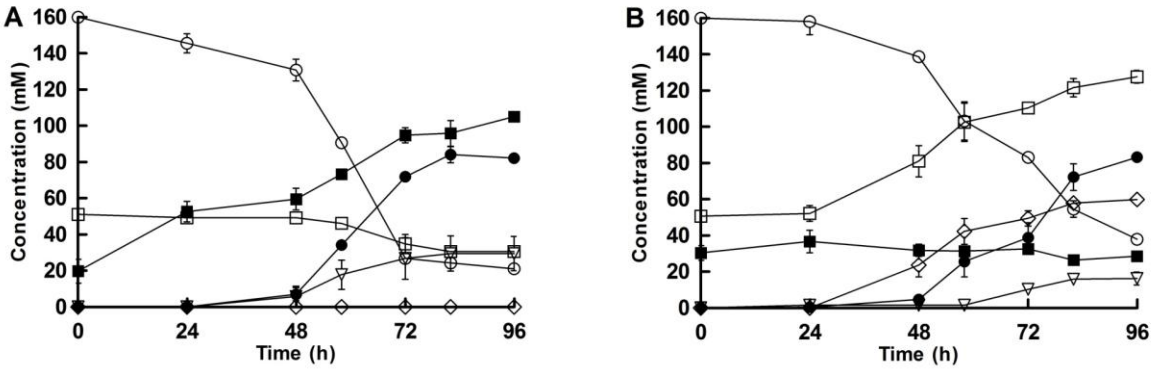

Figure 10.4 pH-controlled batch fermentation of the engineered PM1 strain in the liquid stillage. The engineered strain, PM1-pCER-GlpFKD-TPI-YqhD, was cultured at pH 6.5 (A) and 7.5 (B) for lactate and 1,3-PDO fermentation, respectively. The concentrations of glycerol (white circles), ethanol (black circles), acetate (white squares), lactate (black squares), 3-HPA (white down-pointing triangles) and 1,3-PDO (white diamonds) are provided on the left-hand *y*-axis. Note: the lactate concentrations are not standardized among the batch of TS.

Over the same period, acetate decreased from 51.19 to 30.54 mM, which led to an increase in ethanol (82.16 mM); i.e., ethanol production exceeded the decrease in acetate. This data indicated that the acetate-to-ethanol route was insufficient for NAD^+ regeneration during the rapid glycerol consumption period (from 48 h to 72 h). Ethanol production is a typical NAD^+ regeneration route and the pyruvate-to-ethanol route (via acetyl coenzyme A) has previously been reported in *L. panis* PM1 (Kang et al. 2013a). Thus, a part of the pyruvate formed from glycerol could be directed to ethanol production due to the limited capacity of lactate dehydrogenase for NAD^+ regeneration (Figure 10.1; Kang et al. 2013d). Lactate production was severely reduced after 72-h of culture, along with 3-HPA accumulation (26.84 mM). The compound 3-HPA is a sole intermediate of the glycerol reductive pathway and its toxicity to cell growth has been well-studied in various bacteria (Barbirato et al. 1997; Celinska 2010; Kang et al. 2013b). It is suggested that 3-HPA accumulation was a major factor hampering high yields during lactate fermentation.

In section 10.4.2, the negative effect of acidic pH on glycerol consumption was mentioned; whereas mild alkaline conditions were a significant factor in determining the fate of

glycerol utilization by the engineered strain. In pH 7.5-controlled fermentation, lactate was not produced, and instead, higher amounts of 1,3-PDO were produced relative to the pH 6.5 condition, with a concentration of 59.96 mM and a yield of 0.49 mol/mol (these molar ratios were calculated as the concentration of 1,3-PDO produced over the concentration of glycerol consumed; Figure 10.4B). In addition, acetate (added for NADH recycling) increased from 50.68 to 127.63 mM during fermentation, and was accompanied by an increase in ethanol (83.22 mM).

The specific activities of DhaB, DhaT, and YqhD (for 3-HPA-to-1,3-PDO conversion) were determined from the engineered strain cultured at pH 6.5 and 7.5 for 48 h to further elucidate how 1,3-PDO production was affected by changes in culture pH. At pH 7.5, the specific activities of DhaT and YqhD were significantly higher than those at pH 6.5 ($P < 0.001$); whereas DhaB activity was comparable under both pH conditions (Figure 10.5). These results could explain the higher accumulation of 3-HPA, which would further repress DhaT, eventually resulting in little 1,3-PDO production (Kang et al. 2013b) under the pH 6.5 condition. In contrast, under the pH 7.5 condition, YqhD was centrally-involved in the conversion of 3-HPA to 1,3-PDO, in conjunction with DhaT, and the pyruvate pool formed by the new glycerol oxidative pathway was directed to acetate and ethanol for extra ATP formation and NADH recycling, respectively, suggesting that pH can shift NAD^+ regeneration routes (Figure 10.1).

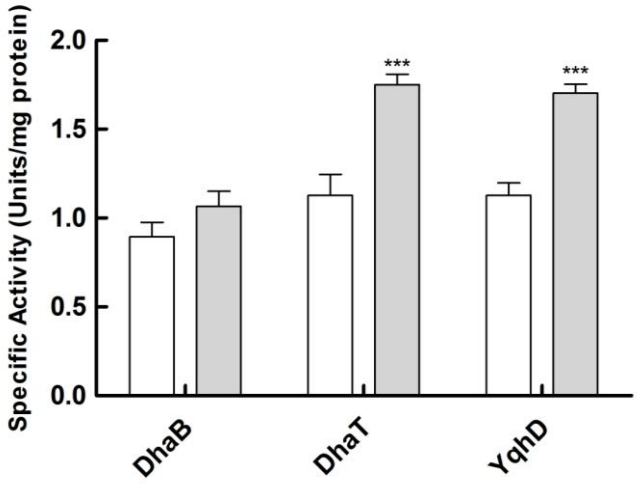

Figure 10.5 The effect of pH on the glycerol reductive pathway. The specific activities of DhaB, DhaT, and YqhD were measured from the 48-h samples obtained during pH-controlled fermentation. White and gray bars represent pH 6.5 and 7.5 conditions, respectively. *** $P < 0.001$

Recently, the search for new technologies capable of producing commodity chemicals from crude glycerol has been renewed due to the rising production of bioethanol and biodiesel (Clomburg and Gonzalez 2013; da Silva et al. 2009). For the production of several chemicals, including lactate and 1,3-PDO, microbial fermentation has been considered to be a safer alternative, i.e., mild pressure, temperature, and fewer toxic intermediates, compared with conventional chemical methods (Gao et al. 2011; Gaspar et al. 2013). Recently, *Lactobacillus diolivorans*, which belongs to the same lactobacilli group (III) as *L. panis* PM1, successfully produced a high amount of 1,3-PDO (75 g/L) in a fed-batch fermentation (Pflügl et al., 2014). In their study, the authors used crude glycerol from biodiesel production and lignocellulosic hydrolysate for 1,3-PDO production to reduce the material costs, instead of using purified glycerol and glucose. However, such a high 1,3-PDO concentration was obtained using expensive MRS-based media, vitamin B_{12} supplementation, and pretreatment by physicochemical and enzymatic methods for lignocellulosic hydrolysate. TS is the largest source of low-price glycerol and complex nutrients, and several studies have focused on the utilization of TS for the production of value-added chemicals, such as 1,3-PDO by *L. panis* PM1 (Kang et al. 2014) and butanol by *Clostridium pasteurianum* DSM 525 (Ahn et al. 2011). Djukic-Vukovic et al. (2013) reported that high amounts of lactate production (42 g/L) were produced by *Lactobacillus rhamnosus* immobilized on zeolite using TS supplemented with 50 g/L glucose as a medium. However, these attempts required the supplementation of high amount of fermentable sugars or expensive nutrient sources (e.g., yeast extract).

To the best of my knowledge, there have been no reports showing the production of platform chemicals solely from TS. Thus, the attempts in this Chapter show more economic conditions in TS utilization than those of other previous studies, suggesting the feasibility of value-added chemical production by *L. panis* PM1 under simulated industrial conditions. Although TS was a possible culture medium for the engineered PM1 strains, relatively low levels of glycerol (up to 2%) in TS could be a limitation for high amounts of value-added chemical production. The supplementation of additional low-price glycerol to TS-based medium could be a simple solution for this limitation. However, the accumulation of toxic intermediates (e.g., 3-HPA or DHAP) and the instability of the rolling circle replicon used for the artificial operon expression are other issues during prolonged fermentation. Thus, to achieve this final goal (I) cloned enzymes may need to be controlled by separate promoters for fine tuning of their

activities, which would reduce the accumulation of the toxic intermediates, (II) a theta-type replicon suitable for *L. panis* PM1 strain should be identified for the stability of the artificial operon, and (III) inoculum volume and feeding system for additional glycerol (e.g., continuous or fed-batch method) need to be optimized to reduce fermentation time and to maximize productivity.

In this study, to reduce the costs of fermentable substrates and nutrient sources, genetic engineering strategies were applied to *L. panis* PM1 strain. The artificial glycerol oxidative pathway could direct glycerol to equimolar GAP that was converted to lactate by the phosphoketolase pathway, and the exogenous YqhD contributed to direct 1,3-PDO production and NADH recycling system in the recombinant strain. In addition, ethanol was a major end-product regardless of whether lactate or 1,3-PDO fermentation by the recombinant PM1 strain occurred, providing a cost-efficient option to recover more ethanol from TS in bioethanol plants. Overall, these findings clearly demonstrate the significant feasibility of direct platform chemical production from TS without the need for expensive fermentable sugar and nutrient source supplementation.

10.5 Conclusions

In this study, metabolic engineering techniques were applied to the wild-type PM1 strain to enable the utilization of glycerol from TS as a fermentable substrate for the production of valuable platform chemicals. Introduction of an artificial glycerol oxidative pathway and NADH recycling system enabled the engineered strain to use glycerol as the sole energy source and to produce ethanol, lactate, and/or 1,3-PDO solely from TS. These developments demonstrate the significant feasibility for directly utilizing TS for the production of value-added products from *L. panis* PM1.

11. GENERAL DISCUSSION

The host strain used in this thesis, *L. panis* PM1, is a novel microorganism isolated from bioethanol thin stillage (TS) (Khan et al. 2013), and is of particular interest due to its ability to convert glycerol in TS to 1,3-propanediol (1,3-PDO), an increasingly valuable commodity chemical. This ability has been documented in other lactobacilli, including *L. reuteri*, *L. brevis*, *L. buchneri*, *L. hilgardii*, and *L. diolivorans* (Lüthi-Peng et al. 2002; Pasteris and Strasser de Saad 2009; Pflügl et al. 2012; Veiga da Cunha and Foster 1992a). They all belong to group III lactobacilli. As discussed in Chapters 3, 7 and 8, this group of lactobacilli cannot use glycerol as the sole carbon source, and their ability to utilize glycerol (producing 1,3-PDO from glycerol) is restricted to the glycerol reductive pathway. Most of the previous studies for 1,3-PDO production have focused on the optimization of feeding strategy (the ratio of glucose to glycerol), medium composition, and fermentation method (two-step fermentation and co-fermentation) (Grahame et al. 2013; Lüthi-Peng et al. 2002; Pflügl et al. 2012; Tobajas et al. 2009). To further expand applications of these bacteria, in-depth studies including physiology, metabolism, and genetics are needed. In addition, the utilization of renewable feedstocks enables the applications of these strains to become more competitive from an industrial point of view. The objectives of this thesis are: (i) to understand how *L. panis* PM1 harmoniously utilizes central and auxiliary metabolic pathways in order to adapt a wide spectrum of environmental and nutritive conditions, (ii) to metabolically manipulate *L. panis* PM1 on the basis of this improved understanding of metabolic pathways, and (iii) to produce value-added chemicals (e.g., 1,3-PDO, lactate, and ethanol) from glycerol in TS using the engineered PM1 strains.

In the first phase of this thesis (Chapters 3 and 4), basic metabolic characteristics of *L. panis* PM1 were investigated. This strain belonged to obligatory heterofermentative (group III) lactobacilli that ferment glucose to lactate, ethanol and/or acetate, and carbon dioxide via the 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway (Chapter 3). Only a few group III lactobacilli, including *L. reuteri* and *L. brevis*, are evidenced to utilize the Embden-Meyerhof (EM) pathway (Årsköld et al. 2008; Saier et al. 1996), which is a central

metabolic pathway for group I and II lactobacilli. In this thesis research, it was found that *L. panis* PM1 possessed the EM pathway and fermented fructose via this pathway in conjunction with the 6-PG/PK pathway. In Chapter 4, how *L. panis* PM1 utilizes the dual-glycolytic pathways for its central metabolism was first reported. The two intermediates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, of the EM pathway regulated the utilization of the two pathways, and triosephosphate isomerase (TPI) activity governed the full utilization of the EM pathway. The expressing of exogenous TPI resulted in a shift in the central metabolic pathways from the 6-PG/PK to EM pathway. In general, the EM pathway is considered more efficient, in terms of ATP yield, than the 6-PG/PK pathway (Heinrich et al. 1997). The lower ATP yield should be a disadvantage for group III lactobacilli. To compensate for this disadvantage, group III lactobacilli have developed a variety of auxiliary systems to shift metabolism between the glycolytic pathways or to use alternate routes for NAD^+ regeneration, yielding different end-products and extra ATP. Under given environmental and nutritive conditions, these auxiliary systems can enable group III lactobacilli to inhabit more diverse environmental niches than group I lactobacilli. Genome sequence analysis of group I lactobacilli, especially *L. johnsonii* and *L. acidophilus*, revealed a lack of genes for *de novo* synthesis of amino acids, cofactors, and vitamins; thus, these biosynthetic limitations exclusively restrict their habitats to specific ecological niches (e.g., gastrointestinal tract where amino acids and peptides levels are sufficient for growth) (Claesson et al. 2007). All *L. salivarius* strains (group I) contain megaplasmids (from 100 to 380 kb in size) that encode genes for the pentose phosphate pathway, allowing adaptation to various environments within the gastrointestinal tract (Claesson et al. 2006). Furthermore, the genome sequence of *L. bulgaricus* (group I) shows a significantly-higher GC content at codon position 3 than its overall GC content, indicating an evolution toward a higher GC content. This high GC content suggests the adaptation of *L. bulgaricus* from a plant-associated habitat to the environment of protein and lactose-rich milk (van de Guchte et al. 2006). Findings in Chapters 3 and 4 suggested that *L. panis* PM1 evolutionarily diverged from the group I lactobacilli wherein the function of the TPI enzyme was lost, leading to the distinct characteristics of this strain discussed in Chapter 4.

Inefficiencies in the 6-PG/PK pathway suggest that auxiliary metabolic pathways in group III lactobacilli are important as much as their central metabolic pathway for their efficient energy metabolism and survival under various conditions. In the second stage of this thesis

(Chapters 5, 6, 7, and 8), the synergic roles of such auxiliary routes were studied to elucidate total redox balance maintenance and additional energy generation. Aerobic conditions greatly affected the physiology of *L. panis* PM1 and limited the growth of this strain. A coupled NADH oxidase-NADH peroxidase system processed molecular oxygen, where the NADH oxidase mainly recycled NADH, producing additional ATP. However, this route accumulated hydrogen peroxide as a major end-product, leading to oxidative stress (Chapter 5). Under aerobic conditions, molecular oxygen became a preferred electron acceptor in the NADH recycling system of *L. panis* PM1. This finding adequately explained why this strain could not produce 1,3-PDO under aerobic conditions, and suggested that optimized (microaerobic or anaerobic) culture conditions would be needed for the production value-added chemicals from glycerol using this strain. *Lactobacillus panis* PM1 possesses the reductive tricarboxylic acid (TCA) cycle that ferments citrate to succinate and lactate (Chapter 6). Recently, Tsuji et al. (2013) engineered a *L. plantarum* strain that possessed the reductive TCA cycle and produced significantly-increased amount of succinate from the engineered strain. This suggests that an improved understanding of citrate metabolism in *L. panis* PM1 can warrant manipulation of this strain for the production of succinate. The citrate metabolism of the PM1 strain was regulated by the presence of citrate, its end-products (succinate and acetate), and the transcriptional repressor (PocR). This regulatory information could be applied to engineering work of *L. panis* PM1, enabling the engineered strain to produce succinate for food, cosmetic, and biomedical processes. The fact that *L. panis* PM1 can convert glycerol to 1,3-PDO was a starting point of this thesis, as well as an interesting topic in the industrial applications of this strain. The glycerol reductive pathway is one of the auxiliary pathways, like oxygen and citrate, that results in the shifting of the main NAD^+ regeneration route from ethanol to 1,3-PDO (Chapters 7 and 8). Various environmental factors, including temperature, pH, and the presence of external electron acceptors, influence 1,3-PDO production by *L. panis* PM1. Among them, two factors, the molar ratio of glucose to glycerol and the presence of the transcriptional repressor (PocR), significantly reduced 1,3-PDO production. The ratio of glucose to glycerol has been considered a key factor for the production of 1,3-PDO by microbial fermentation in many studies (Lüthi-Peng et al. 2002; Tobajas et al. 2009). In this thesis, the regulatory role of this ratio on 1,3-PDO production was elucidated. Under lower ratio of glucose to glycerol (0.18 vs. 0.37 mol/mol), glucose could not provide sufficient NADH for the conversion of 3-hydroxypropionaldehyde (3-HPA) to 1,3-

PDO, which resulted in the accumulation of 3-HPA. The accumulated 3-HPA repressed the expression and specific activity of 1,3-PDO dehydratase (DhaT), leading to the shutdown of the glycerol reductive pathway. Thus, sufficient provision of NADH by glucose metabolism is a key factor for 1,3-PDO production. Interestingly, PocR repressed both the citrate and glycerol reductive pathways of *L. panis PM1* (Chapter 8); whereas PocR of *L. reuteri* was reported as a transcriptional activator of the glycerol reductive pathway and over-expression of this gene increased 1,3-PDO production by *L. reuteri* (Santos et al. 2011). These observations indicated species-specific regulatory systems of the glycerol reductive pathway and suggested that PocR is a good target gene to be deleted as part of future engineering works.

Glycerol has become an abundant and cheap C3 carbon source due to dramatic demand for bioethanol and biodiesel, and TS is the largest source of waste glycerol. The final goal of this thesis was the cost-effective utilization of glycerol in TS by *L. panis* PM1, or its engineered derivative strains, on the basis of the improved metabolic knowledge of this strain. In Chapter 9, *L. panis* PM1 was genetically-manipulated for the conversion of glycerol in TS to 1,3-PDO. TS was a good culture medium for 1,3-PDO fermentation, and a recombinant PM1 strain expressing an *E. coli* YqhD gene produced 16.23 g/L 1,3-PDO from TS supplemented with 30 g/L glucose and 16.5 g/L glycerol. In a study by Djukic-Vukovic et al. (2013), a similar approach, using TS as a medium, was applied to a wild-type *L. rhamnosus* strain, resulting in the successful production of increased amounts of lactate (42 g/L). However, these attempts required the supplementation of high amounts of fermentable sugar due to the absence of glycerol oxidative pathways, i.e., inability to use glycerol as the sole carbon source. This fact obstructs further cost-effective bioconversion processes. Therefore, in Chapter 10, an artificial glycerol oxidative pathway, comprised of five genes (*glpF*, *glpK*, *glpD*, *tpi*, and *yqhD*) from *E. coli*, was constructed based on the metabolic knowledge obtained from Chapters 3 to 9. *Escherichia coli* possesses two glycerol oxidative pathways, GlpKD or DhaDK, and mostly uses the DhaDK route under anaerobic conditions as described in Chapter 2 and Figure 3.1; however, the DhaDK route did not work properly in *L. panis* PM1 since DhaK is a PTS-dependent kinase (Bächler et al. 2005), and group III lactobacilli generally tend to lack PTS activity (Wisselink et al. 2002). Alternatively, the GlpKD route, which contains an ATP-dependent kinase, was chosen as the backbone of the artificial glycerol oxidative pathway, and two more genes, *tpi* and *yqhD*, were added to the artificial operon due to the absence of TPI activity (Chapter 4) and the insufficient

capabilities of DhaT for NADH recycling (Chapters 7 and 9). The engineered strain mostly produced lactate (85.43 mM) at pH 6.5 or 1,3-PDO (59.96 mM) at pH 7.5, as well as similar amounts of ethanol (82.16 and 83.22 mM at pH 6.5 and 7.5, respectively) from TS without requiring expensive fermentable sugar and nutrient source supplementation. The results in this thesis research showed the first example of the production of platform chemicals solely from TS, suggesting that significant feasibility of cost-efficient TS treatment by *L. panis* PM1 for bioethanol plants exists.

12. GENERAL CONCLUSIONS

Lactobacillus panis PM1 was selected as the organism of focus for this thesis due to its ability to produce 1,3-propanediol (1,3-PDO) from glycerol. For the further utilization of this strain, the current thesis: (i) developed analytical methods and genetic tools, (ii) investigated central and auxiliary metabolic pathways, (iii) metabolically-engineered the PM1 strain on the basis of the improved understanding of metabolic pathways, and finally (iv) tested the ability of engineered strains to produce the value-added chemicals, ethanol, lactate, and 1,3-PDO, from thin stillage (TS).

Key findings and highlights of this thesis include are as follows:

1. *Lactobacillus panis* PM1 belongs to group III lactobacilli, and uses the dual-pathways, 6-phosphogluconate/phosphoketolase (6-PG/PK) and Embden-Meyerhof (EM), for central metabolic processing.

2. The dual-metabolic system is regulated by balancing the accumulation of the EM pathway intermediates, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate; whereas the 6-PG/PK pathway is the major central metabolic route due to the absence of triosephosphate isomerase activity in the EM pathway.

3. Under aerobic conditions, the coupled NADH oxidase-NADH peroxidase system is the main oxidative stress resistance mechanism and NADH recycling route in *L. panis* PM1, producing hydrogen peroxide as a major end-product.

4. The citrate metabolic pathway provides the PM1 strain with an alternate NAD^+ regeneration route and additional ATP for stationary phase survival. This pathway is regulated by the presence of citrate, the two end-products (succinate and acetate), and PocR.

5. The glycerol reductive pathway is a main route for NADH recycling in the presence of glycerol. The low ratio of glucose to glycerol negatively affects 1,3-PDO dehydrogenase activity, resulting in less 1,3-PDO production; whereas temperature and

pH indirectly affect 1,3-PDO production by changing the NADH recycling route (e.g., erythritol pathway).

6. The glycerol reductive pathway is regulated by PocR that represses the transcriptional level and specific activity of glycerol dehydratase.

7. Auxiliary pathways and fine-regulation system provide *L. panis* PM1 with a more competent ability to adapt to a wide spectrum of environmental and nutritive conditions.

8. The improved metabolic knowledge enables the genetic engineering of *L. panis* PM1, with the engineered strain expressing an *E. coli* YqhD gene to successfully produce 1,3-PDO from TS supplemented with only glucose.

9. An artificial glycerol oxidative pathway and NADH recycling system are constructed in *L. panis* PM1, and this engineered strain produces lactate or 1,3-PDO along with ethanol, solely from TS.

10. Through the strategy used in this thesis, i.e., an improved understanding of basic characteristics of the host strain, metabolic engineering and testing of the engineered strains at the pilot scale, a significant feasibility for industrial applications of *L. panis* PM1 is successfully demonstrated.

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