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Bioconversion of glycerol to 1,3-propanediol in thin stillage-based media by engineered *Lactobacillus panis* PM1

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Abstract Thin stillage (TS) is a waste residue that remains after bioethanol production, and its disposal reflects the high costs 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 a NADPH-dependent aldehyde reductase (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 °C 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 an 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 of 1,3-PDO biosynthetic pathway in *L. panis* PM1. Our 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.

Keywords Thin stillage · 1,3-propanediol · Glycerol · Bioethanol · Fermentation

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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 [1]. 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 [10]. The 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. The dry-grind process includes two energy intensive procedures, distillation of ethanol and evaporation of TS, which reflect the 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 [10], 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* [13], butanol by *Clostridium pasteurianum* [2], and lactic acid by *Lactobacillus rhamnosus* [4].

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 [8, 16]. 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 an expensive catalyst along with 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*) [8, 16].

Lactobacillus panis PM1 is a natural 1,3-PDO producing organism originally isolated from TS [9]. 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 microorganisms 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 [11]. 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 [12].

The goal of this study was to test the feasibility of utilization of 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 conditions suggested that *L. panis* PM1 and TS were an ideal host and medium, respectively, for 1,3-PDO bioconversion.

Materials and methods

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 °C) by a Sorvall 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 °C for 20 min.

Bacterial strains and growth conditions

Lactobacillus panis PM1 was cultured at 37 °C under microaerobic conditions using commercial MRS medium (BD, Franklin Lakes, NJ, USA) until late log phase at which time 1 % (v/v) of this pre-culture was transferred to fresh modified MRS (mMRS) medium or LS. The mMRS medium consisted of 10 g glucose, 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 glycerol per liter. The cultures were incubated at 30 °C under microaerobic conditions (described below), 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. Air-tight 15-ml tubes, filled to the two-thirds level, were incubated under static conditions to establish microaerobic conditions. *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, CA, USA) was used for cloning and preparation of the target plasmid, and cultured in LB medium at 37 °C with vigorous shaking. Concentrations of erythromycin (Em) for selective plating were 10 and 300 μg/ml for *L. panis* PM1 and *E. coli*, respectively.

Plasmid construction and bacterial transformation

The coding region of *yqhD* was amplified by PCR from the genomic DNA of *E. coli* JM109. For PCR amplification, a 50-μl PCR mixture was prepared using 0.5 μM each of fYqhD and rYqhD primers (Table 1), 1.25 U *Pfu* DNA Polymerase (Fermentas), 1× *Pfu* buffer with $MgCl_2$, and 0.2 mM of deoxynucleotide triphosphates (dNTPs). The DNA template was denatured for 5 min at 95 °C, and then subjected to 30 PCR cycles, using a thermal cycler (*FTGENE-5D*, *Techgene*, Burlington, NJ, USA), consisting of: 30 s at 95 °C, 30 s at 55 °C, and at 72 °C for 1 min. After the final cycle, the mixtures were further incubated for 5 min at 72 °C. 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 [6] (Table 1). The resultant plasmid was transformed into *L. panis* PM1 by electroporation, as described previously [6].

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

Table 1 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				[6]
pCER-PocR	pCER derivative expressing the PocR gene under the control of its natural promoter, Em^r Cm^r, 6.5 kb				[6]
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	Tm (°C)	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

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 the both batch and fed-batch fermentation using LS. The glucose to glycerol molar ratio in batch fermentation using mMRS was 0.18. The fermentation tests were conducted at 30 °C 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 the 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 mid-log phase culture was used as inoculum. Fermentation temperature was maintained at 30 °C, 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.

Determination of end products

Optical density was measured at 600 nm 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 °C for HPLC analysis. To quantify the concentration of glucose, glycerol, 1,3-PDO, 3-HPA, organic acids and ethanol, samples were analyzed on an organic acid column (HPX-87H; Bio-Rad, Hercules, CA, USA) using an HPLC system equipped with a refractive index detector (RID G1362A, 1100 series; Agilent Technologies, Palo Alto, CA, USA). Operating conditions necessary to optimize peak separation were determined by the method described in the column manual with minor modifications. Filtered culture medium (40 μl) was loaded onto the column and eluted with 5 mmol/L sulfuric acid at a flow rate of 0.6 mL/min at 55 °C for 30 min.

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 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 $P < 0.05$ was considered significant.

Results and discussion

Optimizing 1,3-PDO production conditions

In our previous study, two factors, temperature and pH, contributed to a shift in NADH recycling routes, significantly affecting 1,3-PDO production in *L. panis* PM1 [7]. Thus, in this study with a new recombinant variant of *L. panis* PM1, we examined batch fermentations for 1,3-PDO production using four combinations of temperature (30 or 37 °C) and pH (4.5 or 6.5) for 96 h to confirm the optimal conditions for the recombinant strain (Fig. 1). Temperature was a significant factor affecting 1,3-PDO production ($P < 0.01$). Under low temperature (30 °C) 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 6.5 to 1.80 g/L at 4.5). Therefore, it was determined that pH-4.5/30 °C was the optimal condition for 1,3-PDO fermentation by *L. panis* PM1. This combination was employed for further studies of the recombinant variant.

The *E. coli*-derived enzyme, YqhD, is a NADPH-dependent aldehyde reductase. According to Nakamura and Whited [14], the recombination of 1,3-PDO producing pathway (*K. pneumoniae* DhaB and DhaT) showed lower 1,3-PDO concentration compared to the utilization of

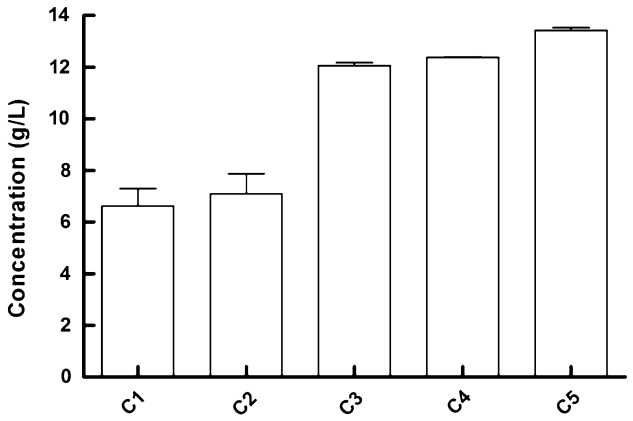

Fig. 1 Concentration of 1,3-PDO resulting from different fermentation conditions and strains. *Lactobacillus panis* PM1 containing empty plasmid, pCER, was cultured in mMRS medium containing 28 g/L glycerol for 96 h under the following conditions: pH 6.5–37 °C (C1), pH 4.5–37 °C (C2), pH 6.5–30 °C (C3),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

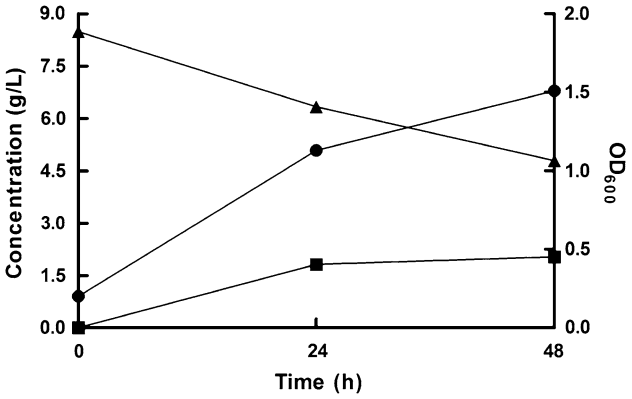

Fig. 2 Growth, glycerol consumption, and 1,3-PDO production in batch fermentation of the liquid stillage by *L. panis* PM1. *Circles* cell density (OD_{600}), *triangles* glycerol concentration, and *squares* 1,3-PDO concentration

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 [5]. *Lactobacillus panis* PM1 has been reported as a natural 1,3-PDO producer via the glycerol reductive pathway [9]. 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 [7]. 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 [6]. 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, $P < 0.01$) (Fig. 1). In the 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.

Growth and 1,3-PDO production in LS

The acid characteristic of TS (in the range of pH 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 [10]. 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 a low 1,3-PDO yield (Fig. 2). During batch fermentation using mMRS (Fig. 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 conditions 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 °C). Figure 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 overexpression 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 the 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 (an additional 96 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

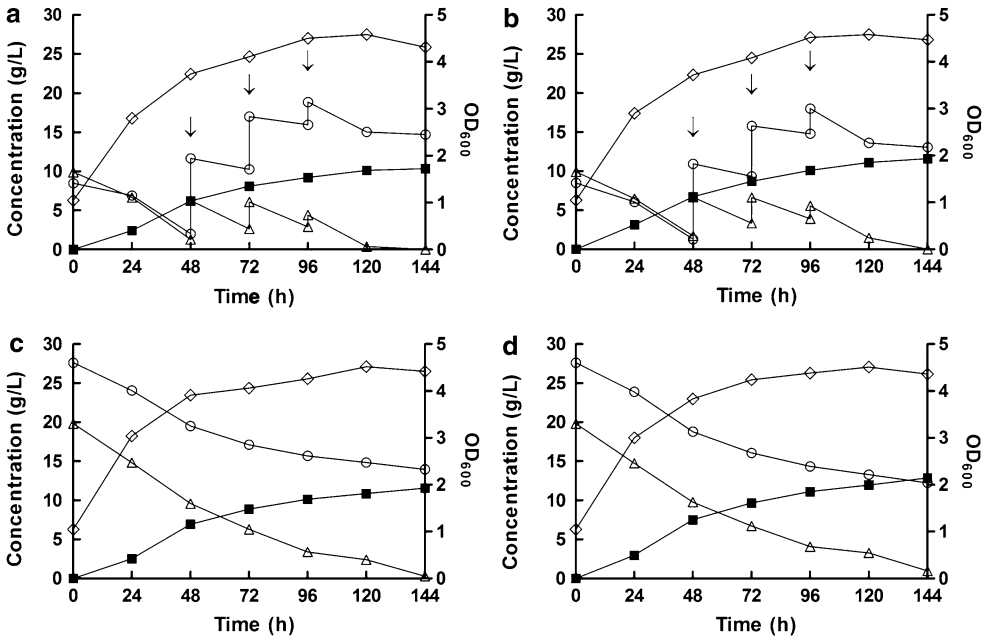

Fig. 3 Production of 1,3-PDO by PM1-pCER (**a**, **c**) and PM1-pCER-YqhD (**b**, **d**) using different fermentation methods. **a**, **b** Fed-batch fermentation and **c**, **d** Batch fermentation. The concentrations of glycerol (*circles*), glucose (*up-pointing triangles*), and 1,3-PDO (*black squares*) are provided on the left-hand *y*-axis, and the cell density (*diamonds*) is provided on the right-hand *y*-axis. *Arrows* represent time of additional glucose and glycerol supplementation

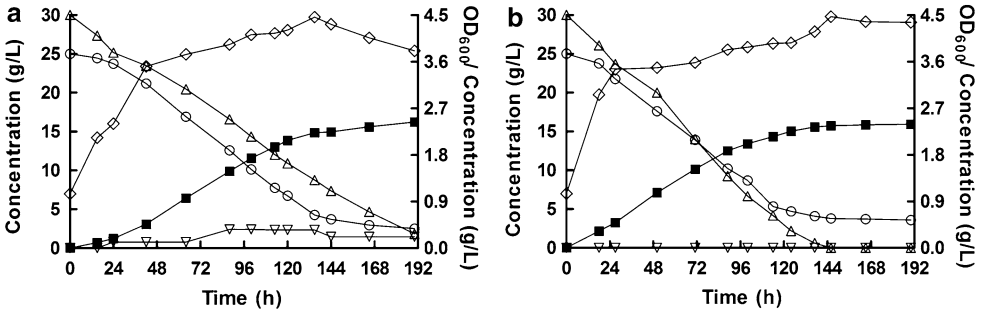

Fig. 4 Production of 1,3-PDO in pH-controlled batch fermentation of the liquid stillage by PM1-pCER-YqhD. LS, containing 30 g/L glucose and 25 g/L glycerol, supplemented without (**a**) or with (**b**) 100 mg/L $MgSO_4 \cdot 7H_2O$ and 50 mg/L $MnSO_4$ was used as fermentation media. The concentrations 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

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.

pH-controlled batch fermentation

Based on the results shown in Fig. 3, the molar ratio of glucose to glycerol was adjusted at 0.61 in LS with the addition of glucose, and the PM1-pCER-YqhD strain was further used for the 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 Sect. 3.2 (0.2 g L^{-1} h^{-1} vs. 0.14 g L^{-1} h^{-1}), and maximal 1,3-PDO production (16.23 g/L) was achieved along with low ethanol concentration (below 1 g/L) (Fig. 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 DhaB, and the glycerol-inactivated enzyme is rapidly reactivated by its reactivating factor in the presence of ATP and Mg^{2+} or Mn^{2+} [3]. 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 a causative factor for reduced 1,3-PDO yield and prolonged fermentation time. To accelerate 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 conducted under the same pH-controlled condition. The presence of the two trace elements increased the glucose consumption rate, and 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 (Fig. 4b). After 120-h culture, 1,3-PDO production was also significantly reduced; however, this reduction was mostly related with glucose depletion. In our previous paper, glucose depletion resulted 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 [7]. 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.

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 L^{-1} h^{-1}) of 1,3-PDO in batch fermentation are superior to those of *L. panis* reported in this paper [16]. 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. [15] 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) to *L. panis* PM1, in fed-batch culture using MRS as a medium [15]. However, this attempt also required expensive culture media, supplementation of high concentrations of glucose (approximately 60 g/L), and the addition of vitamin B_{12} (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 our result (16.2 g/L) obtained from the pH-controlled batch fermentation on LS supplemented with 3 % glucose and 2.5 % glycerol (Fig. 4a). Thus, this suggests that LS is a good culture medium for 1,3-PDO fermentation by *L. panis* PM1 compared with the 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, our findings demonstrate the feasibility of a cost-effective way to produce 1,3-PDO using bioethanol waste material, TS.

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. Our findings offer a new platform for the production of value-added chemicals from waste residue, such as TS, from ethanol plants.

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