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Effects of Propionic Acid and pH on Ethanol Fermentation by *Saccharomyces cerevisiae* in Cassava Mash

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Abstract The effects of propionic acid on ethanol and glycerol production by *Saccharomyces cerevisiae* in cassava mash were examined along with the influence of pH (4.0, 5.0, and 6.0) and of dissolved solids content (22%, 25%, and 27%). Inhibition by propionic acid increased as solids content increased and medium pH declined. Complete inhibition of ethanol fermentation was observed in mashes at pH 4.0 (60 mM propionic acid for 22% solids and 45 mM for 25% and 27%). Glycerol production linearly decreased with increased undissociated propionic acid concentration in all mashes at all pH levels, which partly contributed to increased final ethanol production when propionic acid concentration in mashes was low (≤30 mM).

Keywords Cassava · Ethanol · Glycerol · Propionic acid · *S. cerevisiae*

Introduction

Stillage, the residue after distillation, has been a limiting factor in the further development of cassava-based fuel ethanol production. Stillage recycling processes have been widely investigated to prevent stillage pollution [1]. Plentiful industrial practice has revealed that a process which includes stillage recycling has three major drawbacks. (1) Before reutilization, stillage must be treated by solid–liquid separation to avoid accumulation of solids during long-term operation [2]. During separation, the equipment suffers synergistic chemical corrosion and physical abrasion, due to high temperature (80–90 °C), low pH (3.8–4.0), and sand introduced with the raw material. (2) When recycling acidic stillage, the

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medium pH should first be adjusted to 6.0 to allow liquefaction and then adjusted to 4.2 to allow saccharification. During pH adjustment, quantities of alkali and acid are consumed, introducing substantial amounts of soluble ions, and thus stillage in a closed cycle leads to salt accumulation. (3) Stillage contains many microorganism metabolic end products, some of which cannot be effectively removed by distillation and which, when reutilizing stillage, can also accumulate and eventually inhibit ethanol fermentation [1, 3]. In fact, only 15–30% of thin stillage can be recycled at the industrial scale in long-term operation (e.g., at Henan Tianguan Fuel Ethanol Co., Ltd., China), with the remaining thin stillage needing to be treated by anaerobic–aerobic treatment. The major drawback of such biological treatment is its high investment and operational costs [4, 5].

With these incentives for seeking useful solutions, a coupled ethanol–methane fermentation process is proposed here (Fig. 1). In practice, methane fermentation was applied with a thermophilic–mesophilic digestion system, and the mesophilic anaerobic effluent was fully recycled as cooking water for the next ethanol fermentation batch, such that there was no requirement for ancillary stillage treatment. Equipment corrosion was also avoided because solid–liquid separation occurred after thermophilic digestion. At this point, the temperature of the stillage was decreased from 90–100 to 50–55 °C, and the stillage pH raised from 4.0 to 7.0–8.0. Furthermore, potential inhibitory substances, such as organic acids, appeared to be decomposed during anaerobic digestion. Concurrent acidification of the anaerobic digestion system, however, must be prevented as abundant organic acids will form under acidification conditions [6, 7]; organic acids contained in anaerobic effluent can significantly prolong ethanol fermentation time [8]. Previous experiments in this laboratory confirmed acetic and propionic acid as the two most abundant acids in anaerobic effluent, with concentrations up to 4.44 and 2.96 g/L, respectively [8]. It should be noted that, while small amounts of acetic acid and barely detectable propionic acid are found in traditional ethanol fermentation, both acids were detected in the present coupled ethanol–methane fermentation process. Consequently, it was necessary to systematically evaluate the influence of these two acids on ethanol fermentation under different conditions to support and understand the application of this coupled process at the industrial scale.

Although the effects of organic acids, particularly acetic and lactic acids, on yeast growth and ethanol fermentation have been widely investigated [9–12], the effect of propionic acid on ethanol fermentation has rarely been investigated. In this study, the effects of propionic acid, pH, and dissolved solids content on ethanol and glycerol production by *Saccharomyces cerevisiae* in cassava mash were investigated. Based on the results,

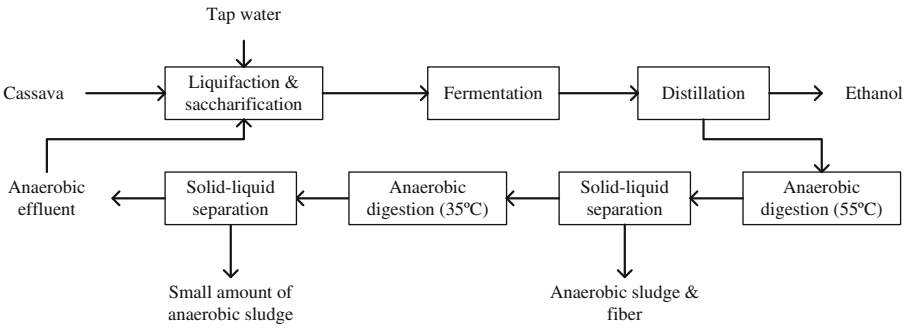

Fig. 1 Flowchart of the coupled ethanol–methane fermentation process

suggestions are provided for avoiding further deterioration of the coupled process with acidification of the anaerobic digestion system.

Materials and Methods

Microorganism and Growth Conditions

Angel strain yeast (strain AG, a commercial strain of *S. cerevisiae* for ethanol production) was obtained from Hubei Angel Yeast Co., Ltd., China, and cultivated in an orbital shaker (100 rpm) at 30 °C for 19 h. Seed medium contained (in grams per liter) glucose 20, yeast extract 8.5, $(NH_4)_2SO_4$ 1.3, $MgSO_4 \cdot 7H_2O$ 0.1, and $CaCl_2 \cdot 2H_2O$ 0.06.

Cassava Mash Preparation

Liquefied mash was prepared using cassava powder (starch content at 65–70%, size of ~0.45 mm), which was kindly provided by the Henan Tianguan Fuel Ethanol Co., Ltd., China. In mash preparation, cassava powder at 22%, 25%, and 27% dissolved solids was mixed with tap water (800/2,400, 914/2,286, and 1,000/2,200 g powder/mL water, respectively), and the slurries' pH was adjusted to 6.2–6.4 with 30% H_2SO_4 (*w*/*w*). During liquefaction, 10 IU of thermostable α-amylase (20,000 IU/mL, optimal temperature range of 95–105 °C; Genencor China Co., Ltd.) per gram of cassava powder was added. Subsequently, the slurries were heated to 100 °C for 60 min and autoclaved at 121 °C for 20 min; water lost during autoclaving was replaced with sterile water.

Ethanol Fermentation

Triplicate fermentations were carried out in 250-mL flasks containing 150 mL of cassava mash and propionic acid added to produce 0, 15, 30, 45, 60, or 75 mM. The resulting slurry pH was adjusted to pH 4.0, 5.0, or 6.0 using 30% H_2SO_4 (*w*/*w*) or 10% NaOH (*w*/*v*). Doses of 130 IU glucosidase (130,000 IU/mL; Genencor China Co., Ltd.) per gram of cassava powder were added to promote saccharification, and 0.3% urea (urea/mash, *w*/*w*) was added as a nitrogen source. Seed broth at 10% of mash volume in each flask (15/150 mL/mL) was inoculated to each flask to produce an inoculation rate of 10 million cells/mL mash. Flask temperature was maintained throughout fermentation at 30 °C in an incubator, and fermentation terminated at 48 h for 22% solids mashes or 60 h for 25% and 27% solids mashes. As the yeast dry weight in these experiments was difficult to measure because of solids in the medium, yeast growth was evaluated through cell counts by hemocytometer. Cell viability was assessed through methylene blue staining.

HPLC Analysis

Ethanol and glycerol concentrations were determined by high performance liquid chromatography (Dionex UltiMate 3000 HPLC, USA) using samples pretreated according to a method described by Graves et al. [11]. A 20-μL aliquot from a suitably diluted sample was analyzed using a Bio-Rad HPX-87 H Aminex ion exclusion column coupled to a refractive index detector (Shodex RI-101, Japan). The column was operated at 65 °C with 0.005 M sulfuric acid as the mobile phase at 0.6 mL/min and data processed using Chromeleon Software (Dionex, USA).

Data Analysis

All final ethanol, glycerol, and biomass data were analyzed using SPSS version 13.0 software (SPSS Inc., IL, USA). Differences among means were analyzed at a significance level of 0.05 by one-way ANOVA. The p values were calculated to estimate significance with $p<0.01$ indicating highly significant, $0.01<p<0.05$ significant, and $p>0.05$ not significant.

Results

Effect of Propionic Acid on Ethanol Fermentation by *S. cerevisiae*

Propionic acid inhibition of ethanol fermentation increased as mash solids content increased and medium pH decreased (both $p<0.01$). At pH 6.0, propionic acid addition did not decrease ethanol production in all solids content mashes (Fig. 2). At pH 5.0, addition of propionic acid did not inhibit ethanol production in 22% solids mashes, whereas reductions in final ethanol quantities were observed in mashes containing 25% and 27% solids with 75 and 60 mM propionic acid, respectively (Fig. 2). At pH 4.0, 60 mM propionic acid completely inhibited ethanol fermentation in 22% solids mash, whereas 45 mM propionic acid completely inhibited ethanol fermentation with 25% and 27% solids. Increased final

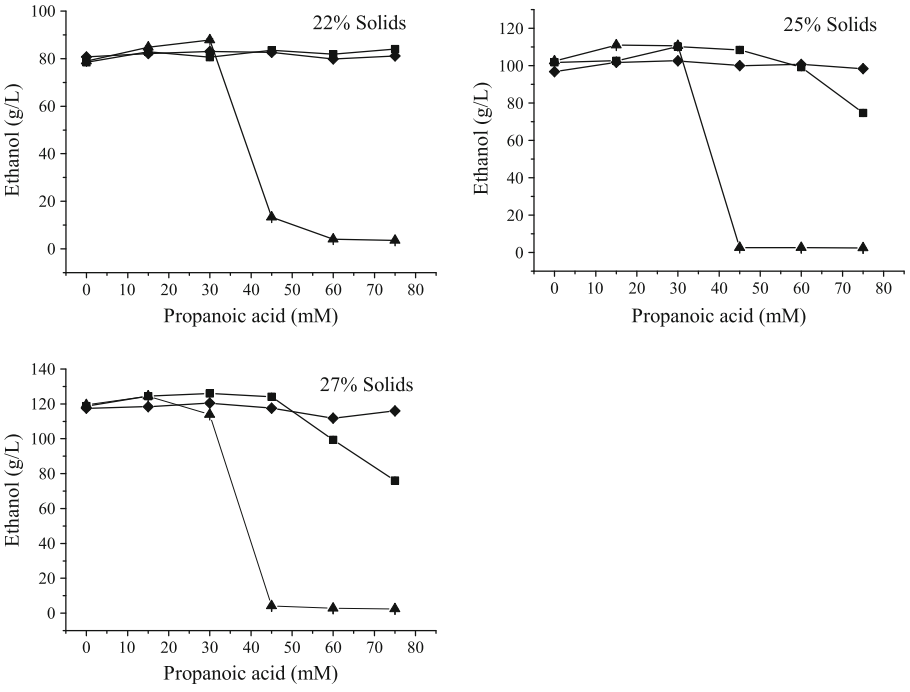

Fig. 2 Final ethanol concentrations produced by *S. cerevisiae* from cassava mash fermentation at 30 °C with various solids contents and propionic acid concentrations and adjusted to different pH values. Symbols: *triangle* pH 4.0, *square* 5.0, and *diamond* 6.0. *Error bars*, too small to show; standard deviation, <2.5%

ethanol concentrations were observed in all solids content mashes compared with controls (no added propionic acid) at all pH values ($p<0.01$). Without propionic acid addition, final ethanol increased as the mash solids content increased ($p<0.01$) but was not affected by medium pH ($p>0.05$).

Effect of Propionic Acid on Glycerol Production by *S. cerevisiae*

Glycerol production decreased as propionic acid concentrations increased in all solid contents mashes and at all pH levels ($p<0.01$, Fig. 3). When ethanol fermentation was complete, the highest reduction of glycerol production (54.0%) was observed at pH 5 with 27% solids and 75 mM propionic acid. It should be noted that glycerol production was compared only when ethanol fermentation was complete as glycerol and ethanol production in the ethanol fermentation are simultaneous. Ethanol fermentation was intensively inhibited and could not finish in the same time when propionic acid in the medium was ≥45 mM and at pH 4.0. As a result, the glycerol production obtained under these conditions was not compared. The higher glycerol obtained at 75 mM propionic acid compared to 60 mM propionic acid (pH 4.0 and 27% solids) could be attributed to experimental error. Furthermore, glycerol production linearly decreased as undissociated propionic acid in the mash increased (Fig. 4). Without propionic acid addition, glycerol production was also affected by solids content and medium pH but to a much smaller extent ($p<0.01$).

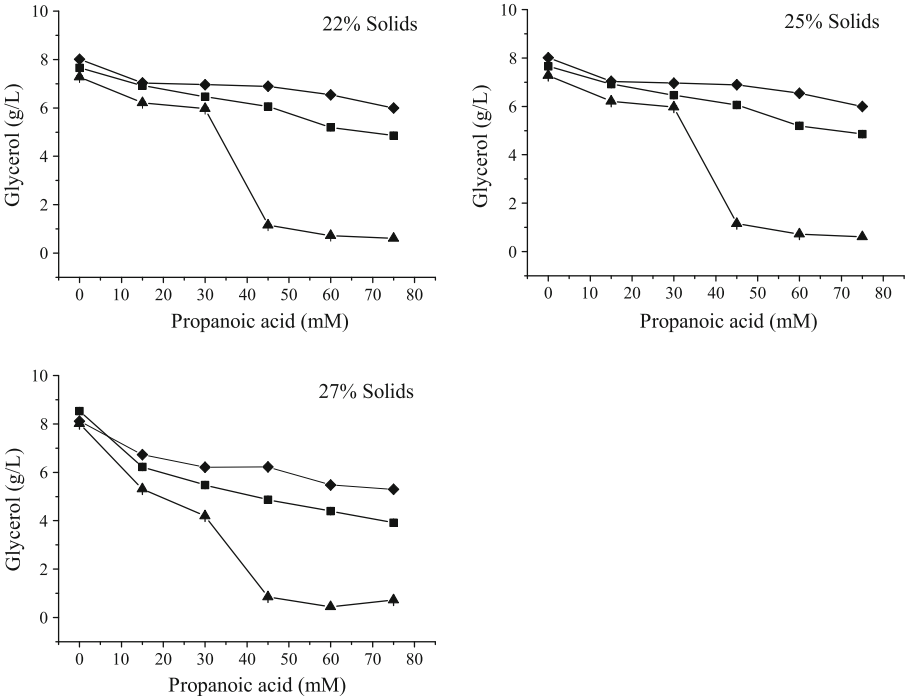

Fig. 3 Final glycerol concentrations produced by *S. cerevisiae* from cassava mash fermentation at 30 °C with various solids contents and propionic acid concentrations and adjusted to different pH values. Symbols: *triangle* pH 4.0, *square* 5.0, and *diamond* 6.0. *Error bars*, too small to show; standard deviation, <10%

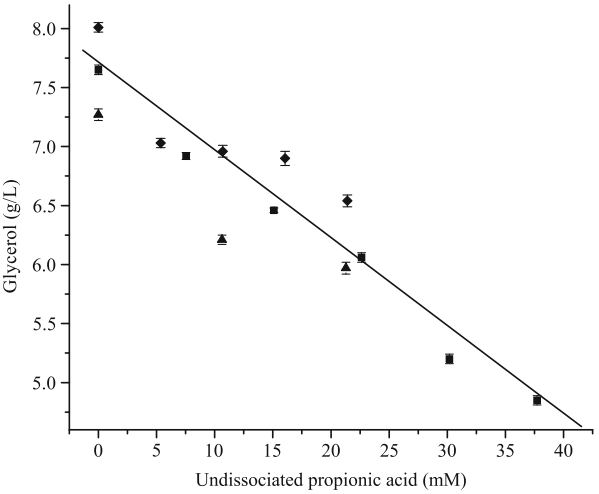

Fig. 4 Relationship between glycerol production and undissociated propionic acid during ethanol fermentation by *S. cerevisiae* from cassava mash at 30 °C with 22% solids content and at different pH values. Symbols: *triangle* pH 4.0, *square* 5.0, and *diamond* 6.0. Undissociated propionate was calculated using the Henderson–Hasselbalch equation, $pH = pKa + \log([A-]/[HA])$, and propionic acid p*K*a of 4.88

Effect of Propionic Acid on the Growth of *S. cerevisiae*

Cell numbers in the fermentations were not significantly affected by medium pH and solids content when also in the absence of propionic acid ($p>0.05$ and $p>0.05$, respectively; Fig. 5),

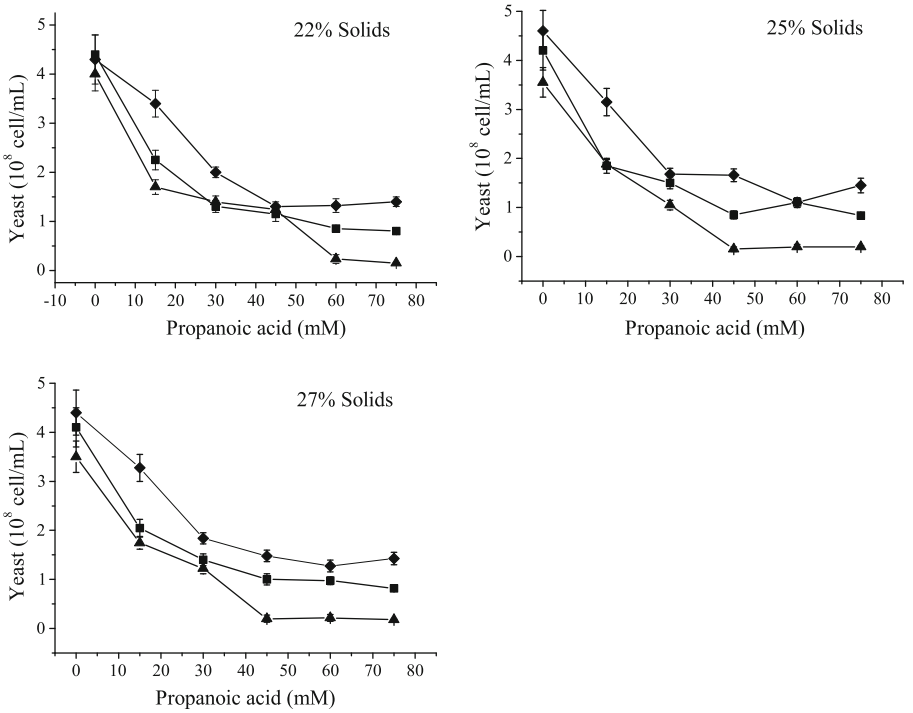

Fig. 5 Biomass during ethanol fermentation of cassava mash at 30 °C with various solids contents and propionic acid concentrations and adjusted to different pH values. Symbols: *triangle* pH 4.0, *square* 5.0, and *diamond* 6.0

but biomass clearly decreased as propionic acid increased ($p<0.01$). Moreover, this decreasing trend was less obvious when mash propionic acid was >30 mM. This observation also suggested that biomass was determined by the total propionic acid concentration. In addition, the cells were verified to be almost viable through methylene blue staining (data not shown).

Discussion

In the present work, examination of the effects of propionic acid on ethanol and glycerol production by *S. cerevisiae* in cassava mash showed that the inhibition of ethanol fermentation by propionic acid grew stronger as medium pH decreased, undissociated acid being the effective inhibitory form. Undissociated acid can diffuse through the plasma membrane, dissociate intracellularly, and thus acidify the cytoplasm. Concurrently, to maintain a constant intracellular pH, protons must be transported across the membrane by plasma membrane ATPase, resulting in increased ATP consumption and decreased biomass yield [13]. The inhibition of fermentation by acetic acid can also be explained by decreased hexokinase, phosphofructokinase, and enolase activities in yeast cells [14]; the effect of propionic acid on these enzymes has not been reported. Interestingly here, low propionic acid concentrations increased final ethanol concentrations (Fig. 2), which was similar to widely reported results when acetic acid is added to the medium [9, 12, 15]. Thomas et al. have reported that acetic acid stimulates ethanol production as much as 20% with 167 mM acetic acid in pH 4.5 medium [12]. Taherzadeh et al. have reported a 21% increase in ethanol yield, 50% decrease in glycerol yield, and 35% decrease in biomass resulting from the addition of 3.5 g/L of acetic acid at pH 3.5; it has been suggested that acetic acid increased ethanol production at the expense of biomass and glycerol production [15]. The present results confirmed this deduction, such that, at pH 4, 22% mash solids, and 30 mM propionic acid, the highest increase in ethanol production (11.1%) was obtained, while glycerol and biomass production decreased by 17.9% and 65.0%, respectively. These results revealed that low propionic acid concentration could enhance ethanol fermentation by *S. cerevisiae*, rendering both higher ethanol production and lower by-product formation, and also suggested that, in a coupled ethanol–methane process, organic acids in the anaerobic effluent were not always harmful for ethanol fermentation.

Glycerol helps *S. cerevisiae* maintain its cellular oxidation–reduction balance under anaerobic conditions [16], and glycerol can also improve a yeast strain's tolerance to osmotic stress [17]. Here, propionic acid addition decreased glycerol production, which could be mainly explained by concurrent decreased biomass. A net production of NADH results from the formation of protein, RNA, and some organic acids [16, 18]; consequently, decreased biomass yield should lead to decreased glycerol yield. However, the present results showed that reduced glycerol production was related to undissociated propionic acid concentration (Fig. 4), whereas biomass was determined by total propionic acid concentration (Fig. 5), which suggested that decreased biomass was not the only reason for decreased glycerol production. The effect of propionate ions on related enzymes should be investigated to understand the observed decreased glycerol production.

Although the results obtained here with propionic acid addition were similar to those reported with acetic acid, the mode of effect of these two acids on yeast may differ. First, propionic acid exhibits a stronger inhibitory effect on yeast than acetic acid. Moon reported that the growth inhibition trend was linear in relation to acetic acid concentration but quadratic for propionic acid, and also deduced that these two acids may not act in cells in

the same manner [19]. Freese et al. demonstrated that low propionate concentrations inhibited amino acid transport across bacterial membrane vesicles and were thus more toxic than other acids [20]. Second, these acids enter the cell in different ways, with acetic acid small enough to diffuse through open Fps1p channels and propionic acid entering mainly by non-facilitated diffusion across cell membranes [21]. Furthermore, acetic acid can be used by yeast when glucose is at a low concentration [15], while propionic acid does not appear to be utilized by yeast in ethanol fermentation [22].

In the normal methane fermentation, organic acids are produced by acidogenic bacteria and ultimately transformed to methane and carbon dioxide by methanogens, and the propionic acid in the anaerobic effluent is usually below 0.05 g/L (about 0.7 mM, unpublished data). In fact, organic acids accumulation in the ethanol–methane coupled process would not happen when the anaerobic digestion is normally operated. However, acidification of methane fermentation is the biggest risk for both our coupled process and traditional stillage biological treatment processes (anaerobic–aerobic treatment), which usually results in increased organic acid concentrations in the anaerobic effluent; acetate and propionate simultaneously accumulate in anaerobic effluent. Our unpublished data verified a synergistic inhibitory effect on ethanol fermentation by these two acids when they coexisted in the medium; this synergistic inhibitory effect must be considered in industrial production. It should be stressed that these acids were not effectively degraded during the ethanol fermentation, which could cause potential organic acid accumulation. Under the circumstances, measures should be adopted to regulate the abnormal ethanol–methane coupled fermentation system. On one hand, for ethanol fermentation, elevating the initial fermentation pH could avoid or alleviate the potential inhibitory effect by organic acids. On the other hand, for methane fermentation, increasing the influent pH to 7.0 and/or decreasing the influent organic loading for the anaerobic digestion may allow the regaining of normal methane fermentation as soon as possible. In this case, organic acids, from ethanol fermentation, contained in the stillage could be effectively degraded during subsequent methane fermentation, thus avoiding organic acid accumulation during long-term operation.

Conclusion

Although high propionic acid concentrations exhibited great inhibitory effects on ethanol fermentation by *S. cerevisiae*, low propionic acid concentrations stimulated ethanol production. Overall, the present results suggested that ethanol fermentation performance was not inhibited when the medium propionic acid was <30.0 mM. This improved fermentation could be partly explained by the observed decreased biomass and glycerol production resulting from propionic acid addition. However, decreased glycerol production could not be fully explained by decreased biomass. The effect of propionate on NAD^+-dependent glycerol 3-phosphate dehydrogenase (GPD) should be studied to elucidate the effect of propionate on glycerol production because GPD is the key enzyme for *S. cerevisiae* in producing glycerol. Furthermore, as the effect of propionic acid on ethanol fermentation by this yeast was also influenced by temperature, related research is under way.

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