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Effect of Acetic Acid and Propanoic Acid on Ethanol Fermentation by *Saccharomyces cerevisiae* in an Ethanol-Methane Coupled Fermentation Process

C.M. ZHANG[1,2], J.H. ZHANG[1,2], L. TANG[1,2], Z.G. MAO[1,2,*], R.S. ZHU[1,2] and H.J. ZHANG[1,2]

[1]The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, P.R. China
[2]Fermentation and Ecological Engineering Laboratory, School of Biotechnology, Jiangnan University, Wuxi 214122, P.R. China

*Corresponding author: Tel/Fax:+ 86 510 85918296; E-mail: zhangchengming01@163.com; feelingmao@hotmail.com

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Waste distillage can be fully recycled in an ethanol-methane coupled fermentation process. In this process, waste distillage from ethanol fermentation was first treated in an anaerobic digestion and then the effluent used for the next ethanol fermentation batch. Organic acids, mainly acetic acid and propanoic acid, contained in the effluent could potentially inhibit the ethanol fermentation. The effects of organic acids on ethanol fermentation were investigated in this study. Results indicated that, to avoid ethanol fermentation inhibition, aceticacetic acid and propanoic acid in the medium should be < 80 and < 30 mM, respectively. Interestingly, ethanol production increased 20 and 13 % in the presence of 20 mM of aceticacetic acid and propanoic acid, respectively. Decreased by-product production, biomass and glycerol could have contributed to the increased ethanol production.

Key Words: Acetic acid, Propanoic acid, *Saccharomyces cerevisiae*, Ethanol, Glycerol.

INTRODUCTION

In China, ethanol production from cassava has been attracting widespread interest, because cassava is not a staple food of the chinese people. However, waste distillage, the residue from distillation, has been a limiting factor in further development of this variety of ethanol production and stillage recycling methods have been widely investigated to prevent pollution[1-4]. Industrial experience has confirmed that a continuous full stillage recycling process is difficult to operate. Stillage contains many yeast and contaminating bacterial metabolic end products, such as low-molecular-weight organic acids, glycerol and ethanol homologues, which cannot be effectively removed by distillation. These substances accumulate when reutilizing stillage and eventually inhibit ethanol fermentation[3,4], such that only 15-30 % of thin stillage can be recycled at the industrial scale in long-term operation[5], with the remaining thin stillage need to be subjected to anaerobic-aerobic treatment[6-8]. Although anaerobic-aerobic treatment has several advantages, such as easy access and energy recovery[9-11], major drawbacks include high investment and operational costs and the aerobic effluent of this more complicated treatment has a chemical oxygen demand usually > 300 mg/L, which severely threatens the environment when released. Consequently, the resulting aerobic effluent requires even further treatment before discharge.

To avoid wastewater pollution, an ethanol-methane coupled fermentation process is proposed here, that allows full stillage recycling in cassava-based ethanol production (Fig.1)[12]. In this process, ethanol fermentation stillage was first treated by a thermophilic-mesophilic digestion and, then, the mesophilic anaerobic effluent fully recycled for the next ethanol fermentation batch. As a result, this coupled process generated no wastewater and the ethanol fermentation inhibition caused by metabolic end products was avoided as these potential inhibitors were effectively degraded during anaerobic digestion. However, remaining organic acids in the anaerobic effluent could potentially inhibit ethanol fermentation.

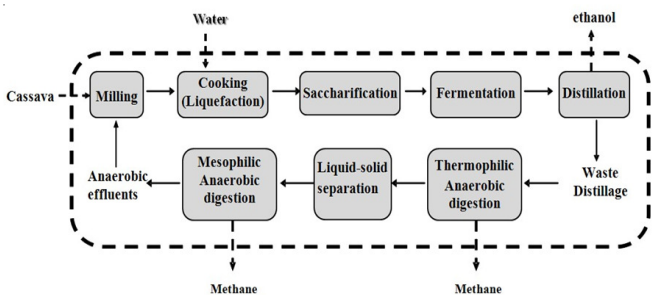

Fig. 1. Flowchart of the coupled ethanol-methane fermentation process.

Anaerobic digestion is a complex mini-ecosystem, with a microbial food chain consisting mainly of three groups of

organisms-hydrolytic fermentative, syntrophic acetogenic and methanogenic bacteria, which perform hydrolysis, acidification (small organic acids formation) and methane conversion, respectively. In such anaerobic digestion of stillage, the speed of hydrolysis and acidification was faster than methane formation and, thus, smaller organic acids gradually accumulated in the anaerobic effluent, with acetic acid and propanoic acid the most abundant, reaching up to 4.44 and 2.96 g/L, respectively[12]. Although these acids were decreased with extended retention times in anaerobic digestion, they were always detected in the effluent. In applying this coupled process at the industrial scale, it would be necessary to evaluate the influences of acetic acid and propanoic acid on ethanol fermentation.

In this study, the effects of acetic acid and propanoic acid concentrations on growth of *S. cerevisiae* growth, ethanol fermentation and glycerol production have been investigated.

EXPERIMENTAL

Organism and Medium: Angel alcohol active dry yeast (ADY, a commercial strain of *S. cerevisiae* for ethanol production) was obtained from Hubei Angel Yeast Co. Ltd., China. The seed medium contained (g/L): glucose 20, yeast extract 8.5 $(NH_4)_2SO_4$ 1.3, $MgSO_4$ 0.1, $CaCl_2$ 0.06. The fermentation medium contained (g/L): glucose 50, yeast extract 5, NH_4Cl 1.5, $MgSO_4$ 0.65, KH_2PO_4 1.5 and $CaCl_2$ 0.06.

Growth and ethanol conditions: *S. cerevisiae* was first grown in a 500 mL shake flask containing 200 mL seed medium for 19 h before inoculation. All flasks were incubated at 30 ºC and 100 rpm. 10 % seed broth (v/v) was inoculated to start the fermentation, the temperature for fermentation was 30 ºC and the initial pH was adjusted to 4 after seed and acids addition. Initial pH was set at 4, because this pH value was the optimum pH for the glucoamylase in the industrial scale production. The lower pH also inhibited the growth of contamination bacteria in the ethanol fermentation. The concentrations of acetic acid and propanoic acid tested were 0, 20, 40, 60, 80 and 100 mM and 0, 10, 20, 30 and 40 mM, respectively. At pH 4.0, 85 % of acetic acid is in the undissociated form (pKa = 4.76) and 88 % of propanoic acid is in the undissociated form (pKa = 4.87).

Analysis methods: Concentrations of ethanol, acetic acid, propanoic acid and glycerol were determined by high performance liquid chromatography (Dionex UltiMate 3000 HPLC, USA). Samples were pretreated as described by Graves *et al.*[13]. A 20-µL aliquot from a suitably diluted sample was analyzed using a Bio-Rad HPX-87H Aminex ion exclusion column coupled to a refractive index detector (Shodex RI-101, Japan). The column was operated at 65 ºC, 0.005 M sulfuric acid was the mobile phase at 0.6 mL/min and the data was processed using the Chromeleon Software (Dionex, USA). Growth was estimated by optical density (OD) at 600 nm and dry weight[14].

All final ethanol, glycerol and biomass data were analyzed using software SPSS.

RESULTS AND DISCUSSION

Inhibitions of *S. cerevisiae* growth by acetic acid and propanoic acid: The lag times increased as the concentrations of these acids in the medium were increased (Fig. 2 and 3). No growth of yeast was detected for 24 h after inoculation when the acetic acid and propanoic acid in the medium was 100 and 40 mM, respectively. The undissociated forms of these acids (because of their lipophilic nature) diffuse into yeast cells through the cell membrane and dissociate at higher intracellular pH, producing hydrogen ions and thereby causing cytoplasm acidification[15]. As a result, yeast metabolic activity and growth are inhibited. Some authors suggested that the increased time of the lag phase reflects the time taken by yeast to pump out excess protons to achieve the required intracellular pH for growth[16]. The present results revealed that propanoic acid was more lethal to *S. cerevisiae* than acetic acid, because the former acid's longer aliphatic group makes it more lipophilic than the latter and can more easily enter cells. Besides causing cytoplasm acidification, propanoic acid could also cause disordering of membrane structure[17], which could also contribute to its greater inhibitory effect.

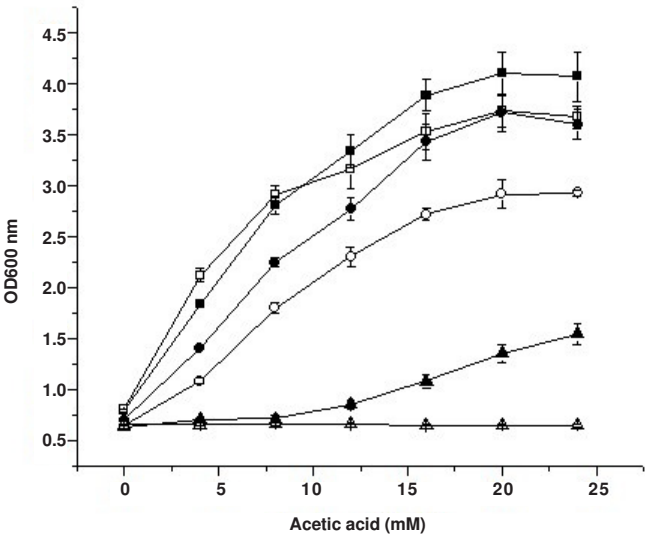

Fig. 2. Effect of acetic acid on growth of *S. cerevisiae* at 30°C and pH 4.0 Symbols: 0 mM (■); 20 mM (□); 40 mM (●); 60 mM (o); 80 mM (▲); and 100 mM (Δ)

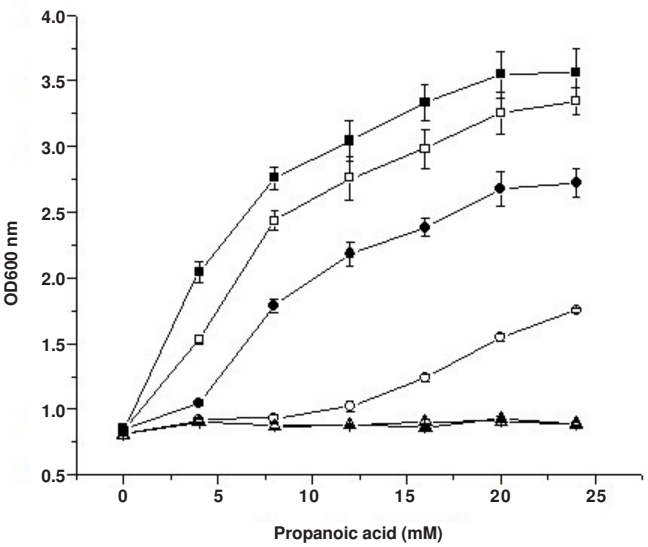

Fig. 3. Effect of propanoic acid on growth of S. cerevisiae at 30 ºC and pH 4.0, Symbols: 0 mM (■); 10 mM (□); 20 mM (●); 30 mM (o); 40 mM (▲); and 50 mM (Δ)

Biomass decreased ($P < 0.01$) as the concentrations of the acids in the medium were increased (Fig. 4). In the presence of organic acids, in order to maintain the intracellular pH homeostasis, cells pump the excess protons out at the expense of metabolic energy, in the ratio of 1 mol of ATP per mol of H^+ transported[14]. In order words, the ATP required for production of cell mass is channeled for maintenance of pH homeostasis inside the cell rather than growth. This causes a reduction in the biomass production.

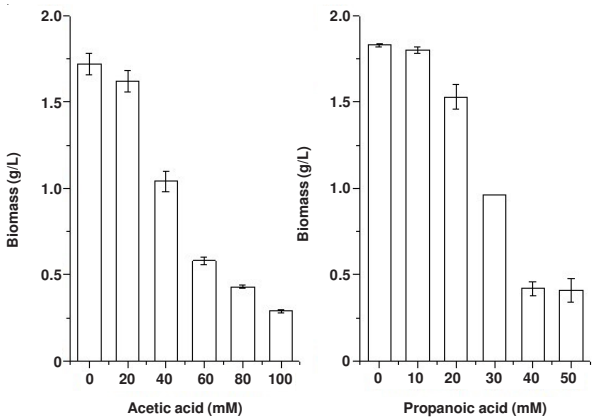

Fig. 4. Effect of acetic acid and propanoic acid on biomass production of *S. cerevisiae* at 30°C and pH 4.0

Effect of acetic acid and propanoic acid on ethanol production by *S. cerevisiae:* The ethanol production was significantly affected ($P < 0.01$) by the organic acids (Fig. 5). For example, a 20 and 13 % increased ethanol production were obtained with the addition of 20 mM of acetic acid and propanoic acid, respectively. Similar stimulating effects by low concentrations of acetic acid have been reported[18,19]. On one hand, in some cases, the organic acids can be used as carbon source by the yeast and thereby the ethanol production increased[18]. On the other hand, addition of organic acid decreases the by-product, biomass and glycerol, production, which result in an increased ethanol production[14]. Other researchers have also reported that the presence of acid may modify the control of glycolysis and enolase and/or phosphorylating enzymes (hexokinase and phosphofructokinase) are presumably involved in the process[20].

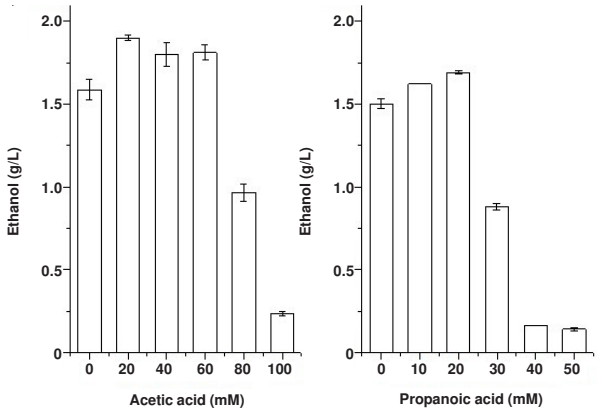

Fig. 5. Ethanol concentration produced by *S. cerevisiae* from glucose medium fermentation at 30 ºC with various acetic acid and propanoic acid concentrations at 30 ºC and pH 4

In contrast, an 85 and 91 % decreased ($P < 0.01$) in ethanol production were observed by the addition of 100 mM of acetic acid or 50 mM of propanoic acid, respectively. Addition of high concentration of organic acid just prolonged the fermentation time, the final ethanol concentration was not decreased when the ethanol fermentation completed (data not shown). The prolonged fermentation time could attribute to the increased lag phase of yeast growth.

Effect of acetic acid and propanoic acid on glycerol production by *S. cerevisiae:* As an important by-product in ethanol fermentation, glycerol production was measured in the present experiments. Compared to the control, glycerol production was found to decease ($P < 0.01$) with acid addition (Fig. 6). For example, glycerol production both decreased 30 % when 20 mM of acetic acid or propanoic acid was added, which could partly contribute to the increased ethanol production. Under anaerobic conditions, glycerol is formed for reoxidation of NADH. A net production of NADH results from the formation and protein and RNA and in the formation of some organic acids, of which acetic acid is the quantitatively most important one [18,19]. Consequently, decreased biomass caused by addition of acids should lead to decreased glycerol production. On the other hand, the formation of NADH directly connected to the formation of acetic acid will decrease when the acetic acid added is used by the yeast and thereby decreasing the glycerol production.

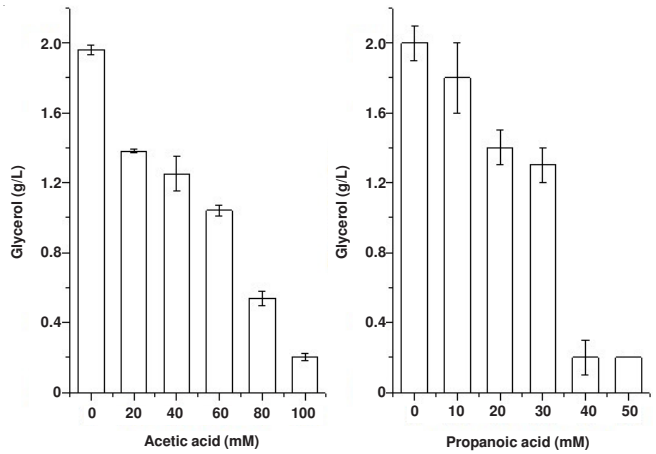

Fig. 6. Glycerol concentration produced by *S. cerevisiae* from glucose medium fermentation at 30 ºC with various acetic acid and propanoic acid concentrations at 30 ºC and pH 4

Conclusion

Ethanol fermentation was significantly affected ($P < 0.01$) by the addition of acetic acid or propanoic acid. The ethanol concentration was increased 20 % with 20 mM of acetic acid added and 13 % with 20 mM propanoic acid added, respectively. In the same conditions, biomass and glycerol production was decreased 6 and 30 % when acetic acid was added and 16 and 30 % when propanoic acid was added, respectively.

Data present here suggested that, in the ethanol-methane coupled fermentation process, the acetic acid and propanoic acid contained in the anaerobic effluent should be < 80 and < 30 mM, respectively, when they existed individually. Otherwise, the ethanol fermentation time would be prolonged. However,

sometimes acetic acid and propanoic acid are simultaneously detected in the anaerobic effluent. They could have a synergistic inhibitory effect on the ethanol fermentation by *S. cerevisiae*.

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