The effect of sulfide and ammonia on cassava fermentation for ethanol production in an ethanol–methane coupled system

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ABSTRACT

An ethanol–methane coupled system was proposed to resolve wastewater pollution in cassava ethanol production. The wastewater originated from ethanol distillation is treated with two-stage anaerobic digestion and then recycled for medium preparation for the next batch ethanol fermentation, thus eliminating wastewater discharge and saving fresh water. The constituents of the two-stage anaerobic digestion effluent were complex which influenced the ethanol fermentation performance. This paper aimed to study the effect of two constituents in the effluent, i.e. sulfide and ammonia, on cassava-based ethanol fermentation performance. It was found that sulfide reduced the consumption rate of total sugar by significantly inhibiting the growth of *Saccharomyces cerevisiae*, but the total consumption amount of total sugar at the end of fermentation was not influenced. *S. cerevisiae* produced more glycerol and less ethanol at the end of fermentation containing higher concentration of sodium sulfide. Ethanol fermentation performance could be hardly influenced by the sulfide in the two-stage effluent because of the very low concentration. More glycerol was produced while final ethanol concentration was reduced when the level of ammonia in the two-stage effluent was higher.

**Key words** | ammonia, cassava-based ethanol fermentation, ethanol–methane fermentation coupled system, *S. cerevisiae*, sulfide

INTRODUCTION

Ethanol as an alternative to fossil fuel has been attracting worldwide interest. Among the fuel alcohol feedstock available, apart from sugarcane (in Brazil) and corn grain (in USA), cassava has been of particular interest for its nature of being cheap, high productivity, and no competition for arable land (Sanchez & Cardona 2008). However, ethanol fermentation uses a great deal of water and 8–15 L of distillery waste (with very high chemical oxygen demand (COD), suspended substance, and low pH) is generated on average per litre of ethanol produced (Saha et al. 2005). Physical, chemical or biological methods are being used to treat the distillery waste because its direct disposal to surrounding environments is strictly prohibited in China. The distillery waste from the fermentative fuel alcohol industry is generally treated by anaerobic digestion followed by an aerobic processing (Jördening & Winter 2005). However, capital investment and operating costs of this process are very high while our national discharge standard requirements are difficult to meet. Therefore this phenomenon has greatly limited further development of the cassava-based ethanol industry.

Many studies regarding the direct recycling of distillery waste have been reported (Kim et al. 1997; Ding et al. 2009; Bialas et al. 2010). But two problems exist: (1) because the waste distillate contains sand, the liquid–solid separation at high temperature and low pH causes very serious physical wear and chemical corrosion in the separation equipment and consumes lots of energy. (2) Many by-products of *Saccharomyces cerevisiae* remain in ethanol fermentation liquor, such as low-carbon organic acids, glycerol, ethanol homologues (butanol, amyl alcohol, isoamyl alcohol) and other organic compounds. These compounds...
are difficult to remove by distillation and are bound to accumulate when the wastewater is directly recycled.

Our laboratory proposed the ‘ethanol-methane fermentation coupled system’ to explore the technology of full wastewater reutilization, and the feasibility of the technology has been proved (Zhang, C. M. et al. 2010; Zhang, Q. H. et al. 2010). Figure 1 summarizes the proposed integrated process, during which cassava starch is transformed into ethanol by fermentation while fiber, pectin, and the end metabolites of S. cerevisiae are converted to biogas by anaerobic digestion. The biogas can be used to produce electricity and the digestion effluent reused in ethanol fermentation. Besides, the solid materials withdrawn from liquid-solid separation can be used as fertilizer. Such a process can result in zero wastewater discharge and low energy consumption. Meanwhile the accumulation of materials in the coupled system is eliminated. Furthermore, lipid-solid separation at high temperate and acidic condition can be avoided by the use of two-stage anaerobic digestion effluent as cooking water (i.e. water used for making fermentation medium).

The system we proposed couples the ethanol fermentation with the methane fermentation, thus making the system more complicated. Besides, the quality of two-stage anaerobic digestion effluent can influence ethanol fermentation performance (Zhang, C. M. et al. 2010) because the constituents of anaerobic digestion effluent are very complex, which include suspended substances, organic substances, sulfide, ammonia and other components. It has been reported that ammonia affects the yeast growth and product formation. Maria et al. (2003) showed that ammonia consumption by yeasts produced a great acidification of the media, thus causing an enormous impact on yeast metabolism during alcoholic fermentation. ter Schure et al. (2000) found that yeast growth on ammonia as a nitrogen source seems to yield relatively higher growth rates than that for urea-grown culture. The effects of different nitrogen sources on metabolite formation, growth, and cell composition of S. cerevisiae were studied by Albers et al. (1996). They found that glycerol yields obtained with ammonia as a nitrogen source were clearly lower than those for glutamic acid or a mixture of amino acids-grown cultures. In addition, the ethanol yield increased for growth on both glutamic acid and the mixture of amino acids. Few studies have investigated the influence of sulfide on yeast growth. The objective of this paper was to study the effects of two ingredients in the effluent, i.e. sulfide and ammonia, on cassava-based ethanol fermentation performance.

MATERIALS AND METHODS

Yeast strain

An Angel alcohol yeast (ADY, a commercial strain of S. cerevisiae for ethanol production) was used in the study. This strain was obtained from Hubei Angel Yeast Co. Ltd., China.

Seed medium

The seed medium contained (g/L): glucose 20, yeast extract 8.5, (NH4)2SO4 1.3, MgSO4·7H2O 0.1, CaCl2·2H2O 0.06.

Preparation of inoculums

Angel alcohol yeast was inoculated into a 500 ml Erlenmeyer flask containing seed medium. The flask was incubated on a rotating shaker at 100 rpm, 30 °C for 19 hours before the cells were used as an inoculum for ethanol production.

Mashing of cassava and fermentation

Liquefied mash was prepared using cassava powder (starch content 65–70% (w/w), size is about 0.45 mm), which was kindly provided by the Henan Tianguan Fuel Ethanol Co.
Ltd., China. To prepare the mash, 100 g cassava powder was added per 220 ml cooking water and thus produced slurry. The slurry pH was adjusted to 6.0 with 300 g/l H₂SO₄. Following the adjustment of pH, 10 IU thermostable α-amylase (20,000 IU/ml, optimal temperature range 95–105 °C, Genencor China Co. Ltd.) per gram cassava powder was added to reduce viscosity and prevent starch degradation. The slurry was heated to 100 °C and held at this temperature for 2 hours and then autoclaved at 121 °C for 20 min. Water loss during mashing was made up with sterile water. After being autoclaved, the mash was cooled to room temperature and then divided into 270 ml batches (one batch per treatment, each treatment run in triplicate). The mash pH was adjusted to 5.5, using 300 g/L H₂SO₄ or 100 g/L NaOH. 130 IU Amyloglucosidase (130,000 IU/ml, Genencor China Co. Ltd.) per gram of cassava powder was also added for saccharification of the dextrins. 1 g/L of urea (urea/mash, w/v) was added as nitrogen source (when the ammonium sulfate was added, urea was not added). Seed broth at 10% the volume of mash in each flask (10 mL/100 mL), was inoculated to the mash to achieve an inoculation rate of 10 millions cells/mL mash. Fermentation was carried out in batch mode at 30 °C in an incubator and considered to be over when the residual total sugar was below 20 g/L.

Effect of sulfide on the ethanol performance: sodium sulfide significantly inhibited the growth of *S. cerevisiae* and reduced the consumption rate of total sugar in our experiments. The dry weight of yeast is hard to measure because of the solids contained in the medium, so a hemocytometer was used to count the yeast cells to evaluate the growth of *S. cerevisiae*. The cell number of *S. cerevisiae* decreased by 96% as the sodium sulfide increased from 0 to 54.00 mmol/L (Figure 2). Meanwhile, carbon dioxide produced by *S. cerevisiae* at 48 hour slightly declined as the sodium sulfide increased from 0 to 27.00 mmol/L, and then sharply declined as sodium sulfide concentration increased from 33.75 to 54.00 mmol/L (Figure 3). Ethanol concentrations produced by *S. cerevisiae* at 48 hour had the same decreasing trend with carbon dioxide production as the sodium sulfide concentration increased (Figure 2). This phenomenon indicates that the sulfide has an inhibitory threshold concentration on the ethanol fermentation. So the concentration of sulfide in the effluent should be controlled under this threshold. Sulfide readily permeates the

### Results and Discussions

**Effect of sulfide on the ethanol performance**

Sodium sulfide significantly inhibited the growth of *S. cerevisiae* and reduced the consumption rate of total sugar in our experiments. The dry weight of yeast is hard to measure because of the solids contained in the medium, so a hemocytometer was used to count the yeast cells to evaluate the growth of *S. cerevisiae*. The cell number of *S. cerevisiae* decreased by 96% as the sodium sulfide increased from 0 to 54.00 mmol/L (Figure 2). Meanwhile, carbon dioxide produced by *S. cerevisiae* at 48 hour slightly declined as the sodium sulfide increased from 0 to 27.00 mmol/L, and then sharply declined as sodium sulfide concentration increased from 33.75 to 54.00 mmol/L (Figure 3). Ethanol concentrations produced by *S. cerevisiae* at 48 hour had the same decreasing trend with carbon dioxide production as the sodium sulfide concentration increased (Figure 2). This phenomenon indicates that the sulfide has an inhibitory threshold concentration on the ethanol fermentation. So the concentration of sulfide in the effluent should be controlled under this threshold. Sulfide readily permeates the
cell membrane of *S. cerevisiae* and denatures native proteins inside the cytoplasm producing sulfide and disulfide cross-links between polypeptide chains, thus affecting the ethanol fermentation performance.

Figure 4 shows that residual total sugar at the end of the fermentation with increasing concentrations of sodium sulfide is slightly different, indicating that sulfide reduced the consumption rate of total sugar by inhibiting the growth of *S. cerevisiae* but remained the total consumption amount of total sugar. However, *S. cerevisiae* produced more glycerol and less ethanol at the end of fermentation containing higher concentrations of sodium sulfide.

As mentioned above, the pH of liquefied mash was adjusted to 6.0 with H₂SO₄ which introduced sulfate to the fermentation medium, resulting in sulfate in the waste distillate. When the distillery waste is treated by anaerobic digestion, the sulfate is reduced by sulfate reducing bacteria (SRB) and further stimulates the growth of sulfate reducing bacteria which out-compete methane producing bacteria for substrates (H₂ and acetate). Moreover, H₂S is also produced during the reducing process, which will cause problems of corrosion, malodor and toxicity. Sulfide is also involved in the precipitation of non-alkali metals in digesters, thus reducing their availability for methane producing bacteria (Elferink 1994). However, sulfide concentration in our two-stage effluent was far below the threshold concentration (about 0.6 ± 0.03 mg/l) which hardly influenced the ethanol performance.

**Effect of ammonia on the ethanol fermentation performance**

Figure 5 showed that final ethanol concentration decreased, but glycerol concentration at the end of the fermentation increased as the ammonia nitrogen increases, which implied that ammonium sulfate significantly affects glycerol and ethanol production in the ethanol fermentation. Other researchers have also found that more glycerol is produced during *S. cerevisiae* anaerobic growth when the ammonium sulfate is used as the only nitrogen source (Albers *et al.* 1996, Sahoo & Agarwal 2001, Yalçın & Özbaş 2008). Glycerol formation has two physiological roles in *S. cerevisiae*. During *S. cerevisiae* growth under osmotic stress conditions, glycerol is produced to play a role in the osmotic regulation of the cell (Blomberg & Adler 1992). On the other hand, synthesis of amino acids, RNA, and extracellular metabolites results
in the formation of intracellular NADH. NADH is reoxidized to NAD$^+$ by the formation of glycerol (Nordström 1966) to maintain an intracellular redox balance. When the ammonium sulfate is used as the only nitrogen source, it is used by S. cerevisiae to form amino acids. A net of NADH is produced during the formation of amino acids. The production of NADH must be balanced by a mechanism in which NADH is reoxidized to NAD$^+$ in order to avoid a serious imbalance in the NAD$^+$ / NADH ratio. As mentioned above, when glycerol is produced to maintain the intracellular redox balance, more glycerol is produced when the ammonium sulfate is higher, therefore the ethanol concentration decreases. In our ethanol–methane fermentation coupled system, two-stage effluent contained about 0.72 ± 0.03 g/l of ammonia nitrogen, so ethanol fermentation performance could be influenced when the effluent was used as cooking water.

In the ethanol–methane fermentation coupled system, the nitrogen source is used by S. cerevisiae to synthesize the substances which are necessary for the growth of yeast, such as amino acids, protein. When the distillery waste is treated by anaerobic digestion, proteins are hydrolyzed to amino acids by the exocellular proteinase of anaerobic microorganisms, and then these amino acids are taken up by microorganisms for intracellular metabolism. Ammonia is then produced by deamination of amino acids. A small part of ammonia is for the growth of anaerobic microorganism while most of it is kept in the effluent. When the effluent is used for ethanol fermentation, ammonia can be used as the nitrogen source of S. cerevisiae. Other studies have detected that high or low levels of ammonia in the effluent have a negative effect on the ethanol fermentation. Furthermore, production of ammonia in the process of anaerobic digestion results in a high effluent alkalinity. High alkalinity in turn gives the effluent a strong acid buffer action. Therefore, when the effluent is used for ethanol fermentation, more sulfuric acid is needed to adjust the pH of the slurry before liquefaction of cassava powder. Besides, as mentioned above, sulfate is bad for the whole system. So the amount of ammonia in the whole system should be controlled at an appropriate level so that the effluent cannot negatively affect ethanol fermentation and the alkalinity of effluent will be kept not so high.

CONCLUSIONS

An ethanol–methane fermentation coupled system was proposed by our laboratory to solve the problem of distillery waste, and the feasibility of the technology has been proved (Zhang, C. M. et al. 2010; Zhang, Q. H. et al. 2010). This system associates the ethanol fermentation with the methane fermentation, thus making the system more complicated. Besides, the quality of mesophilic anaerobic digestion effluent can influence ethanol fermentation performance. The effects of two constituents of anaerobic digestion effluent, i.e. sulfide and ammonia on the ethanol fermentation performance were investigated. The results indicated the following: (1) sulfide reduced the consumption rate of total sugar by significantly inhibiting the growth of S. cerevisiae, but remained the total consumption amount of total sugar. Saccharomyces cerevisiae produced more glycerol and less ethanol at the end of fermentation containing higher concentration of sodium sulfide. Ethanol fermentation performance could be hardly influenced by the sulfide in the two-stage effluent because of the very low concentration; (2) more glycerol was produced, and the final ethanol concentration was reduced when the level of ammonia nitrogen in the two-stage effluent was higher. Besides, the amount of ammonia in the whole system should be controlled at an appropriate level so that the effluent cannot negatively affect ethanol fermentation.

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