Suitability of anaerobic digestion effluent as process water for corn fuel ethanol fermentation

Ke Wang, Jian-Hua Zhang, Pei Liu and Zhong-Gui Mao

ABSTRACT

A corn fuel ethanol plant integrated with anaerobic digestion treatment of thin stillage increases the net energy balance. Furthermore, the anaerobic digestion effluent (ADE) can be reused as a potential substitute for process water in the ethanol fermentation. In this study, the suitability of ADE as process water for corn ethanol fermentation was investigated by analyzing the potential inhibitory components in the ADE. It was found that ammonium influenced the growth and metabolism of *Saccharomyces cerevisiae*. Maximum ethanol production was obtained when the concentration of ammonium nitrogen was 200 mg/L, and ammonium could replace urea as the nitrogen source for *S. cerevisiae* under this concentration. In the ethanol fermentation with a higher concentration of ammonium, more glycerol was produced, thereby resulting in the decrease of ethanol production. In addition, components except ammonium in the ADE caused no inhibition to ethanol production. These results suggest that ADE could be reused as process water for corn ethanol fermentation without negative effect when ammonium concentration is well controlled.

Key words | anaerobic digestion effluent, corn ethanol fermentation, inhibitory components, nitrogen source

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INTRODUCTION

Ethanol has attracted worldwide attention for its nature of being renewable and sustainable, efficient, and safe to the environment (Mussatto *et al.* 2010). Its market has strongly grown in recent decades and is expected to reach 100 billion liters in 2015 (Licht 2006).

Biotechnological processes are responsible for the vast majority of ethanol currently produced. Approximately 95% of ethanol in the world is from agricultural products (Rossillo-Calle & Walter 2006). Corn is the main feedstock used to produce ethanol and most corn ethanol plants are based on dry grinding because of the relatively lower capital costs compared with wet grinding (Bothast & Schlicher 2005). In the traditional dry-grind process, thin stillage is evaporated and dried together with wet distiller's grain to produce distiller's dried grain with solubles for animal feed. This process consumes massive energy, resulting in a small positive net energy balance (NEB) for corn ethanol production (Meredith 2003; Hill *et al.* 2006). Hill *et al.* (2006) calculated through life-cycle assessment that the NEB ratio was only 1.26. Therefore, alternative methods were explored to treat the corn thin stillage. Anaerobic digestion of the thin stillage is an advantageous method because energy is recovered in the form of biogas, which can substantially increase the NEB. In previous work, anaerobic digestion has proven effective in treating corn thin stillage. Lee *et al.* (2011) applied mesophilic continuously stirred tank reactor to treat thin stillage, achieving a total chemical oxygen demand (TCOD) removal efficiency of 84–85%. Energetic assessment showed that a corn-ethanol plant integrated with mesophilic anaerobic digestion could increase the NEB ratio from 1.26 to 1.80. In the study of Agler *et al.* (2008), thin stillage was treated by anaerobic sequencing batch reactor and the removal efficiency of TCOD and volatile solids were 90 and 89%, respectively; this process could improve the NEB ratio from 1.26 to 1.70. It was also reported that the anaerobic digestion effluent (ADE) can be used as a potential substitute for process water in ethanol fermentation (Agler *et al.* 2008; Alkan-Ozkaynak & Karthikeyan 2011). However, the ADE contains various organic compounds and inorganic macronutrients, which may cause potential inhibition or toxicity to the yeast used in ethanol fermentation. Therefore,

doi: 10.2166/wst.2014.094

it is necessary to determine the suitability of ADE as process water for ethanol fermentation by analyzing the potential inhibitory components in the ADE.

Ammonium is a product of anaerobic protein degradation, the minority of which is assimilated by microorganisms while the majority remains in the ADE. When the ADE is reused in the corn ethanol fermentation, ammonium can be used as a nitrogen source for yeast. It was also reported that ammonium affects the metabolism of yeast. This study sought to investigate the effect of ammonium in the ADE on corn ethanol fermentation and the feasibility of replacing urea as the nitrogen source for *Saccharomyces cerevisiae* with ammonium. In addition, because constituents of the ADE are complex and it is difficult to determine all components, all components except ammonium in the ADE were considered as a whole and the influence on corn ethanol fermentation was studied.

MATERIALS AND METHODS

Preparation of inocula

Angel alcohol yeast (a commercial strain of *S. cerevisiae* for ethanol production obtained from Hubei Angel Yeast Co. Ltd, China) 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. The seed medium contained (g/L): glucose 20, yeast extract 8.5, $(NH_4)_2SO_4$ 1.3, $MgSO_4 \cdot 7H_2O$ 0.1, $CaCl_2 \cdot 2H_2O$ 0.06.

Mashing and ethanol fermentation

Corn meal (size of about 0.45 mm) was mixed with process water to produce slurry (1 g corn meal per 3 mL water). High-temperature α-amylase (10 IU/g corn, Genencor China Co. Ltd) was added after the slurry pH was adjusted to 6.0 using sulfuric acid or sodium hydroxide. The slurry was then heated to 100 °C, held there for 2 hours, and cooled to 60 °C. Then, glucoamylase (130 IU/g corn, Genencor China Co. Ltd) was added, along with sulfuric acid for pH (4.4). When the temperature was cooled to 30 °C, the slurry was divided and put into 250-mL flasks. The nitrogen source (the type and amount of the nitrogen source were determined by experimental design) and 10% (v/v) inocula was added to start the fermentation. Temperature was maintained at 30 °C. Total retention time in the flasks was 48 hours.

ADE collection and treatment

ADE collected from the two-stage anaerobic digestion treated corn thin stillage in our laboratory was centrifuged at 4,000 *g* for 15 min and stored at −20 °C before being used as process water for corn ethanol fermentation.

Analytical methods

Ethanol, acetic acid, lactic acid and glycerol were determined by Dionex U3000 high-performance liquid chromatography. The samples collected were centrifuged (10,000 *g* for 10 min) and filtered (0.20-μm filter) prior to analysis. A 20-μL portion or a standard solution was injected into a Bio-Rad HPX-87H Aminex ion exclusion column. The column was operated at 65 °C and 0.005 mol/L sulfuric acid was used for the mobile phase at a flow rate of 0.6 mL/min. A refractive index detector (Shodex RI-101, Japan) was used for detection. Data were processed using Chromeleon software (Dionex, USA). Elemental analysis (K, Ca, Na, Mg, Fe, Cu, Mn and Zn) was conducted by a SpectrAA-220 atomic absorption spectrometer (VARIAN, Australia). The amino acid profile of ADE was determined by an amino acid analyzer (Hitachi 835-50, Japan). NH_3-N and volatile fatty acids (VFAs) were determined by *Standard Methods* (APHA 1998). All the experiments were repeated at least three times, and the statistical analysis (Fisher's least significant difference, LSD) was carried out using SPSS Statistics 19 (IBM, USA). Differences were considered significant when p-value < 0.05.

RESULTS AND DISCUSSION

Effects of components except ammonium in the ADE on ethanol fermentation

To investigate the effect of components except ammonium in the ADE on corn ethanol fermentation, original (1.0×) and two-times concentrated (2.0×) ADE was used as process water. Meanwhile, deionized water (DI water), added with low and high concentrations of ammonium nitrogen, was also used for ethanol fermentation, as control, respectively. Results are shown in Figure 1(a) and Table 1.

When the concentration of the ammonium nitrogen in the process water was equal, ethanol production and the fermentation rate of the fermentation with ADE were slightly higher compared with the control. This phenomenon indicated that components except ammonium in ADE could

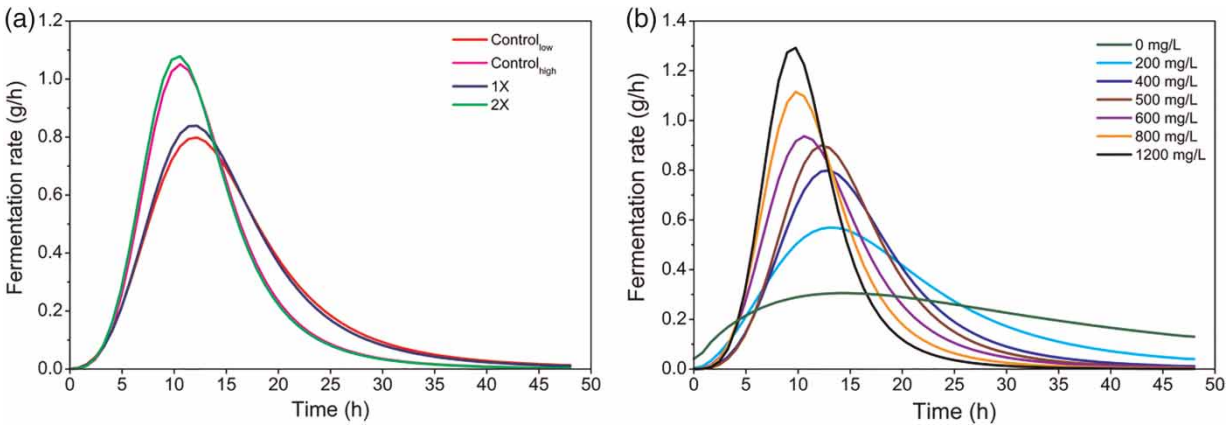

Figure 1 | Fermentation rate of ethanol fermentation with different process water (a) and different ammonium concentrations (b).

Table 1 | Ethanol and by-product production of ethanol fermentation with different water

	1.0X	2.0X	Control[a]$_{low}$	Control[a]$_{high}$
NH_3-N (mg/L)[b]	337	674	337	674
Ethanol (% v/v)	9.72 ± 0.12*	9.46 ± 0.08	9.65 ± 0.05*	9.45 ± 0.07
Glycerol (g/L)	10.59 ± 0.04*	12.19 ± 0.03***	10.28 ± 0.05	11.70 ± 0.13**

Values are the mean of three experiments, S.E.M. (±), *, ** and *** were used to describe the statistical result and different symbols meant significant difference.
[a]Control experiment using DI water with a low or high level of ammonium concentration as the process water.
[b]Ammonium nitrogen concentration in the process water.

slightly promote *S. cerevisiae* growth. In the fermentation with concentrated ADE, the fermentation rate was significantly higher and only about 40 hours were required for *S. cerevisiae* to consume all of the available sugar, while ethanol production decreased compared to fermentation with original ADE. Similar results were also found in the control experiments. These results suggest that a high concentration of ammonium nitrogen in the ADE promoted the growth of *S. cerevisiae*, whereas it reduced ethanol production. It should be noted that more glycerol was produced in the fermentation with concentrated ADE than in the control experiment with a high concentration of ammonium nitrogen because of the higher osmotic pressure of the concentrated ADE. The higher production of glycerol could be attributed to the osmotic regulation of *S. cerevisiae* under osmotic stress conditions (Blomberg & Adler 1992). Effluent from anaerobic digestion systems contains a variety of organic and inorganic compounds (Table 2). Inorganic ions are required by *S. cerevisiae* for optimum growth and fermentation and this requirement varies with the yeast strain and growth media differences (Inge 2003). Magnesium, zinc and copper are known to function as cofactors for a number of enzymes in biological and biochemical reactions. Calcium protects membrane structure and helps maintain membrane permeability growth under adverse conditions. Manganese acts as an intracellular regulator of key enzymes. Potassium is a potent stimulator of glycolytic flux. Grain and corn based fermentation media are not usually sufficient in these ions, with the exception of zinc; therefore, ions in the ADE played a

Table 2 | Composition of the ADE

Parameter	Concentration
Na (g/L)	0.296
K (g/L)	1.062
Ca (g/L)	0.197
Mg (mg/L)	16.4
Fe (mg/L)	1.06
Zn (mg/L)	0.5
Mn (mg/L)	0.02
Cu (mg/L)	0.02
Amino acids (mg/L)	106
VFAs (mg/L)	0.19

critical role in promoting ethanol fermentation. Meanwhile, amino acids were also reported to have a positive effect on ethanol fermentation (Thomas & Ingledew 1990; Albers *et al*. 1996). VFAs, mainly including acetic and propionic acid, are very important intermediates in the anaerobic digestion system and small number of VFAs were maintained in the effluent the anaerobic digester performed efficiently and stably (Speece 1996). In this study, the concentration of the VFAs contained in the ADE used was only 0.19 mg/L. It was reported that acetic and propionic acid inhibited the growth of yeast and prolonged fermentation time. However, a low concentration of acetic acid and propionic acid could increase ethanol production (Taherzadeh *et al*. 1997; Thomas *et al*. 2002; Abbott & Ingledew 2004; Zhang *et al*. 2011). Therefore, the concentration of VFAs in the ADE should be controlled at a low level when ADE is reused for ethanol fermentation.

Effect of ammonium on ethanol fermentation

As mentioned above, a high concentration of ammonium in the culture medium could influence ethanol fermentation. Therefore, it is necessary to further investigate the effect. In this experiment, DI water added with different concentrations of ammonium nitrogen was used to make mash for ethanol fermentation. Results showed that without adding ammonium, ethanol fermentation did not even finish (Figure 1(b)). A low level of yeast assimilable nitrogen has been related to lower fermentation rates and longer fermentative period by reducing yeast cell multiplication and the rate of glycolysis (Bely *et al*. 1990). Ethanol concentration was highest at 200 mg/L of ammonium nitrogen, while lower or higher concentrations of ammonium nitrogen reduced ethanol production (Figure 2(a)).

By-products were also detected at the end of fermentation (Figure 2(b)). More glycerol was produced as the concentration of ammonium nitrogen increased. Albers *et al*. (1996) and Bely *et al*. (1990) also found that more glycerol is produced by *S. cerevisiae* under anaerobic conditions when ammonium is the only nitrogen source. In addition to osmotic regulation, glycerol is produced to maintain an intracellular redox balance. Ammonium is absorbed as a nitrogen source by *S. cerevisiae* to produce amino acids accompanying the formation of intracellular NADH. However, the NADH must be reoxidized to NAD^+ by the formation of glycerol in order to avoid a serious imbalance in the NAD^+/NADH ratio (Yalçın & Özbaş 2008). More sugar converted to glycerol, which decreased ethanol production. Furthermore, the Maillard reaction between reduced sugar and ammonium during the process of mashing may be another reason causing reduction of ethanol production by decreasing the total sugar concentration in the fermentation medium. The effect of the Maillard reaction on ethanol fermentation was investigated by adding 200, 300 and 400 mg/L of ammonium nitrogen before and after the mashing process; ethanol production was not influenced in the two tested conditions containing the same concentration of ammonium nitrogen (Figure 3(c)). Therefore, the decrease of ethanol production at a higher concentration of ammonium nitrogen was caused by greater glycerol synthesis, rather than the Maillard reaction from reduced sugar and ammonium during mashing.

In current ethanol production processes, urea is always used as the nitrogen source for *S. cerevisiae* growth and the

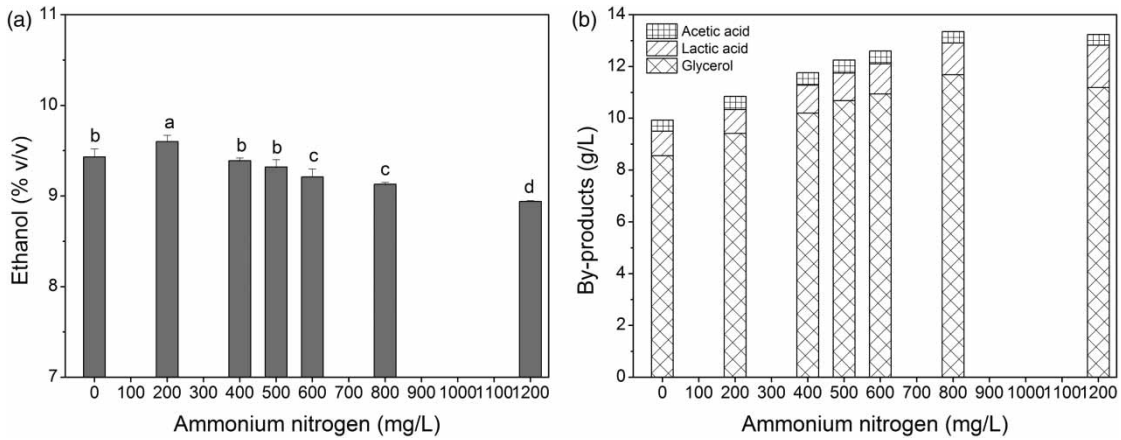

Figure 2 | Ethanol (a) and by-products (b) production of ethanol fermentations with different concentrations of ammonium nitrogen. Different letters in (a) mean significant difference.

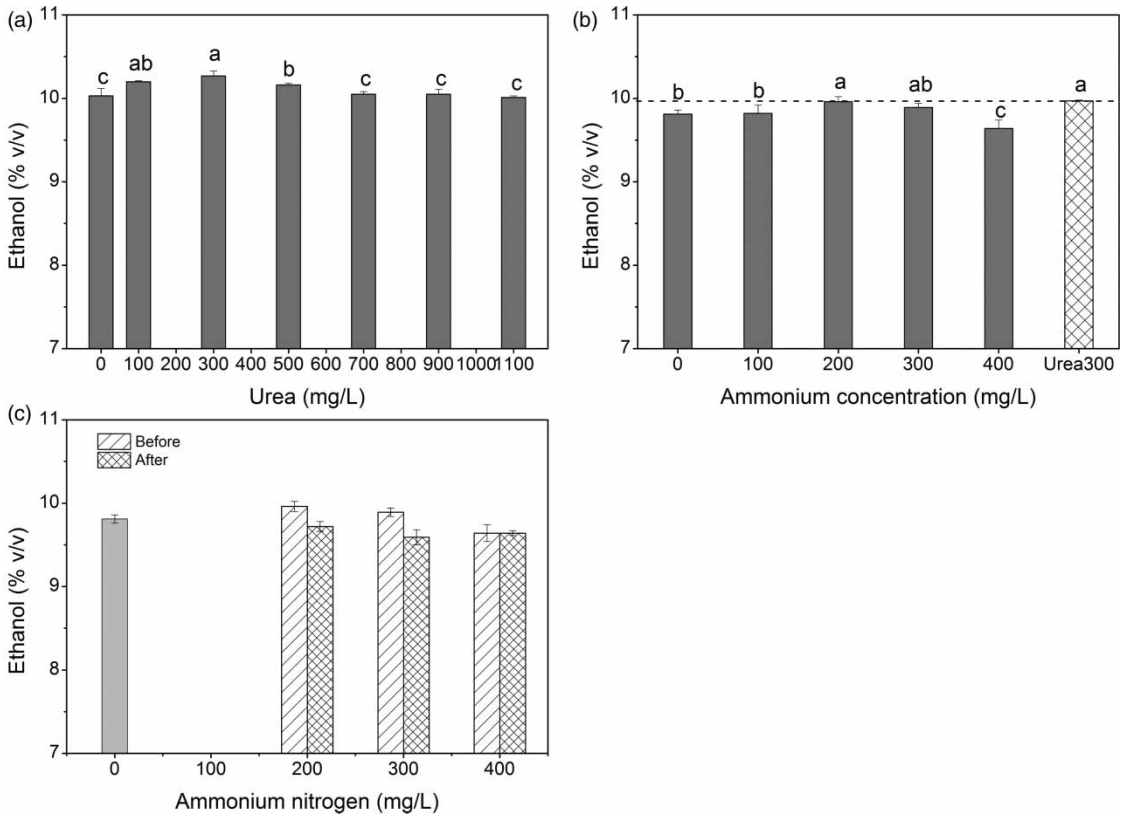

Figure 3 | Ethanol production of ethanol fermentations with different concentrations of urea (a). Comparison of ethanol production between fermentation with ammonium and urea as nitrogen source (b). Ethanol production from ethanol fermentation with ammonium added before and after mashing process at different concentrations (c). Different letters in (a) and (b) mean significant difference.

dosage varied with different yeast strains and feedstock. Ammonium can also be used as a nitrogen source. Therefore, the feasibility of replacing urea with ammonium in the ADE was investigated in this study. Results showed that the maximum ethanol concentration was obtained with 300 mg/L of urea (Figure 3(a)), while a similar ethanol concentration was also produced with 200–300 mg/L of ammonium nitrogen (Figure 3(b)). Therefore, when the concentration of ammonium nitrogen in the ADE was a in this range, urea could be replaced by ADE as a nitrogen source for *S. cerevisiae* in corn ethanol fermentation. However, an ammonium removal process is required when its concentration exceeds 300 mg/L.

CONCLUSIONS

The suitability of ADE as process water for corn ethanol fermentation was studied by analyzing potential inhibitory components in the ADE in this study. Results showed that ammonium in the ADE affected corn ethanol fermentation in terms of *S. cerevisiae* growth and metabolism. When ammonium concentration in the process water was 200–300 mg/L, maximum ethanol production was obtained and urea could be replaced by the ADE as a nitrogen source for *S. cerevisiae*. In addition, components except ammonium in the ADE caused no inhibition to corn ethanol production. Therefore, the ADE was suitable for recycling as process water in corn ethanol fermentation when the concentration of ammonium nitrogen was well controlled.

ACKNOWLEDGEMENTS

This work was supported by the Science and Technology Program of Jiangsu Province (No. BE2011623) and the Scientific Research Project of the Provincial Environmental Protection Bureau of Jiangsu Province (No. 2012047). We thank the Tianguan Fuel Ethanol Co. Ltd for their support, and anonymous reviewers for critically evaluating the manuscript.

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First received 12 November 2013; accepted in revised form 10 February 2014. Available online 26 February 2014