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Effect of nitric acid on pretreatment and fermentation for enhancing ethanol production of rice straw

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ABSTRACT

In this study, nitric acid (HNO_3) was evaluated as an acid catalyst for rice straw pretreatment, and, after neutralization, as a sole nitrogen source for subsequent fermentation. Response surface methodology was used to obtain optimal pretreatment condition with respect to HNO_3 concentration (0.2–1.0%), temperature (120–160 °C) and reaction time (1–20 min). In a condition of 0.65% HNO_3, 158.8 °C and 5.86 min, a maximum xylose yield of 86.5% and an enzymatic digestibility of 83.0% were achieved. The sugar solution that contained nitrate derived from the acid catalyst supported the enhancement of ethanol yield by *Pichia stipitis* from 10.92 g/L to 14.50 g/L. The results clearly reveal that nitric acid could be used not only as a pretreatment catalyst, but also as a nitrogen source in the fermentation process for bioethanol production. It is anticipated that the HNO_3-based pretreatment can reduce financial burden on the cellulosic bioethanol industry by simplifying after-pretreatment-steps as well as providing a nitrogen source.

1. Introduction

Due to the depletion of the world petroleum supply and increasing concerns about climate change, biomass-based renewable fuels such as lignocellulosic ethanol is generally counted as a potential solution to the energy crisis and global warming (Alvira, Tomás-Pejó, Ballesteros, & Nergo, 2010). Lignocellulosic materials including agricultural residues, hardwood, and softwood are plentiful and generate low net greenhouse gas emissions (Galbe & Zacchi, 2007). However, its complex and heterogeneous cell walls make it hard downstream steps, such as enzymatic hydrolysis of sugar polymers. Therefore, pretreatment is absolutely required to increase the yield of fermentable sugars.

Chemical pretreatment with diluted acid combined with enzymatic hydrolysis has been considered as a promising procedure for the conversion of lignocellulose to mono-sugars for ethanol-making process (Hendriks & Zeeman, 2009). This acid-catalyzed pretreatment causes the hydrolysis of hemicellulose and increases portion of amorphous cellulose, resulting in high recovery of hemicelluloses as monomers in the liquid fraction and high digestible cellulose in the solid fraction (Linde, Jakobsson, Galbe, & Zachhi, 2008; Sassner, Martensson, Galbe, & Zacchi, 2008; Yang & Wyman, 2008). Literally every strong acid has been investigated as a catalyst, e.g., sulfuric acid (Hsu, Guo, Chen, & Hwang, 2010), hydrochloric acid (Marcotullio, Krisanti, Giuntoli, & de Jong, 2011), phosphoric acid (Geddes et al., 2010), and also nitric acid (Zhang, Lu, Sun, Wang, & Zhang, 2011). Among these, sulfuric acid, the cheapest agent, is most commonly used. On the other hand, nitric acid can be massively produced via a NO_x capture process from flue gases (Kim & Han, 2013), displaying a much shorter reaction time (Zhang et al., 2011) and higher efficiency for saccharification (Rodríguez-Chong, Ramírez, Garrote, & Vázquez, 2004). Furthermore, nitrate, formed upon being neutralized, could be used as a nitrogen source for fermentation process, as a variety of nitrogen sources including yeast extract and peptone (Laopaiboon, Nuanpeng, Srinophakun, Klanrit, & Laopaiboon, 2009), ammonium (Yue, Yu, Zhang, & Tan, 2012), amino acid (Albers, Larsson, Liden, Niklasson, & Gustafsson, 1996) and urea (Jones & Ingledew, 1994) have been reported to be attempted for ethanol production.

In this work, rice straw, the most abundant biomass feedstock in Korea, was chosen as a raw lignocellulosic material for ethanol production. Response surface methodology (RSM) was used to optimize pretreatment condition of rice straw with nitric acid (HNO_3), particularly aiming at maximum xylose yield. Nitrate was also tested as the nitrogen source for the fermentation of *Pichia stipitis*.

2. Materials and methods

2.1. Feedstock materials

Rice straw was collected from Nonsan field near the city of Daejeon, South Korea. The air-dried rice straw was milled to small

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particles by a grinding machine, screened to obtain a size of less than 2 mm, and stored in a drying oven prior to use. The rice straw was composed of 41.7% glucan, 18.3% xylan, and 16.6% lignin based on its dry weight.

2.2. HNO_3 pretreatment

Pretreatment was carried out in a stainless steel cylindrical reactor with a total volume of 70 mL. Before pretreatment, 5 g rice straw and 50 mL HNO_3 solution were mixed together into the reactor. The reactor was then heated to set temperatures in an oil bath. The average heating rate was about 15 °C/min. Once the targeted temperature inside the reactor was reached, reaction time was counted. At the end of the pretreatment, solid residue was separated by filtration and then washed with deionized water for subsequent enzymatic hydrolysis, and liquid fraction was gathered for the analyses of sugar and inhibitor.

2.3. Enzymatic hydrolysis

After pretreatment, an enzymatic hydrolysis experiment was conducted in a 150-mL Erlenmeyer flask containing 50 mM sodium citrate buffer (pH 4.8) at a solid loading of 10% (w/v). A novel commercial enzyme blend Cellic C-Tec (Novozymes, Denmark) derived from *Trichoderma reesei* with an activity loading of 30 FPU/g cellulose was added to the pretreated rice straw. The flask was incubated at 50 °C and 170 rpm for 72 h in a shaking incubator and the hydrolysate was sampled periodically for sugar analysis.

2.4. Fermentation

P. stipitis KCTC 7222 was obtained from Korean Collection for Type Culture (Daejeon, Korea). The yeast was maintained on YM agar plates at 4 °C and transferred monthly. YM agar contained yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, glucose 10 g/L, and agar 20 g/L. In order to increase cell density, the yeast was transferred into a 250-mL Erlenmeyer flask with 100 mL of the inoculation medium containing yeast extract 3 g/L, peptone 5 g/L, glucose 35 g/L, and xylose 15 g/L. After incubation for 16 h, cells were harvested and applied as an inoculum for fermentation test. The yeast cell concentration in the inoculum was determined to 5 g dry weight of cells/L.

Fermentation experiment was carried out in sterile 250-mL Erlenmeyer flasks with cap and needle to remove CO_2 in a shaking incubator at 30 °C and 170 rpm for 48 h. Each flask was filled with 50 mL fermentation medium inoculated with 10% (v/v) growth culture. The fermentation medium was supplied with glucose 35 g/L, xylose 15 g/L, KH_2PO_4 5 g/L, $MgSO_4$ 0.4 g/L and trace element. The trace element solution contains (in 1 L 0.08 M HCl): $FeCl_2{\cdot}4H_2O$ 1.5 g, $ZnCl_2$ 70 mg, $MnCl_2{\cdot}4H_2O$ 100 mg, H_3BO_3 6 mg, $CoCl_2{\cdot}6H_2O$ 190 mg, $CuCl_2{\cdot}2H_2O$ 2 mg, $NiCl_2{\cdot}6H_2O$ 240 mg, $Na_2MoO_4{\cdot}2H_2O$ 36 mg. Diluted HNO_3 solution was added as a nitrogen source and pH was adjusted to 5.4. The samples were withdrawn at designated times for analysis.

2.5. Analytical methods

The composition of rice straw was determined following National Renewable Energy Laboratory (NREL) protocols (Sluiter et al., 2008). At first, a sample was treated with 72% H_2SO_4 at 30 °C in a shaking water bath for 2 h. The reaction mixture was then diluted to 4% H_2SO_4 and autoclaved at 121 °C for 1 h. The hydrolysis solution was filtered and analyzed for sugar contents by HPLC. The remaining solid part was dried at 105 °C overnight and further placed in the muffle furnace at 575 °C for lignin analysis.

The amount of sugars and inhibitors in hydrolysate and ethanol concentration were quantified using HPLC system (Agilent 1200 series, USA) with an Aminex HPX-87P column (Bio-Rad Inc., USA) and a reflective index detector. The mobile phase was 4 mM H_2SO_4 at a flow rate of 0.6 mL/min.

2.6. Experimental design

Response surface methodology (RSM) was utilized to optimize the condition of pretreatment with HNO_3 and to evaluate the effect of variables including HNO_3 concentration (X_1), pretreatment temperature (X_2), and reaction time (X_3) on xylose yield (Y). The pretreatment conditions were HNO_3 concentrations of 0.2–1.0% (w/w), temperatures of 140–180 °C, and reaction times of 1–20 min. The effect of three variables on the response was studied according to Box–Behnken Design (Box & Behnken, 1960) using Design-Expert software (version 8.0, Stat-Ease, USA).

3. Results and discussions

3.1. Compositional changes of pretreated rice straw

Table 1 summarizes the compositions of liquid phase (hydrolysate) and solid residue produced by the HNO_3 pretreatment under different conditions. Furfural and HMF were regarded as the main inhibitors in this process (Chen, Ye, & Sheen, 2012). The mass recovery of solid residue ranged from 49.5% to 76.6% depending on the pretreatment condition. Also, xylan content decreased at more severe conditions. This happened because at high temperature a strong acid such as HNO_3 does hydrolyze and dissolve hemicellulose fraction and does so too much especially when the treatment is prolonged. Because of the removal of hemicellulose, on the other hand, relative amount of glucan and lignin increased in the solid residue.

Since the mechanism of acid pretreatment is primarily to dissolve out hemicellulose and it certainly improves the enzymatic hydrolysis, the amount of xylose in the liquid fraction can be used as an indicator of the pretreatment efficiency. Yield from the conversion of hemicellulose to xylose varied from 24.8% to 89.9% depending on the pretreatment condition. The maximum xylose yield of 89.9% was obtained at a condition of 0.6% HNO_3 at180 °C for 1 min. The amount of released xylose was measured to be 16.44 g for 100 g of raw dry biomass. When either acid concentration or reaction time increased, the xylose recovery decreased. This occurred probably because of the partial decomposition of the released xylose to furfural. It is typical that such degradation products are accumulated in the hydrolysate under harsh reaction conditions. When the xylose yield was greater than 80%, the maximum concentrations of acetic acid, furfural and HMF were 1.68 g/L, 1.18 g/L and 0.15 g/L, respectively. These amounts, lower than values reported to be inhibitory to *P. stipitis* fermentation (Bellido et al., 2011), were anticipated not to interfere with the subsequent fermentation process. Most of existing pretreatment technologies generate the byproducts higher than critical levels and thus suffer the degraded ethanol productivity; thus an additional detoxification step is required. Adjustment of pH is attempted to reduce the detrimental effect of acetic acid on xylose fermentation and various detoxification methods, such as over-liming, vacuum evaporation, and activated charcoal, are also proposed for furfural (Hsu et al., 2010). Therefore, the unnecessity of this extra step is an added advantage of the proposed nitric acid-based pretreatment.

Table 1
The composition of solid and liquid phase (hydrolysate) after nitric acid pretreatment of RS.

	HNO_3 (%)	Temp. (°C)	Time (min)	Solid recovery (%)	Solid composition (%)			Liquid composition (g/L)					Xylose yield (%)
					Glucan	Xylan	Lignin	Glucose	Xylose	Acetic acid	Furfural	HMF	
1	0.2	140	10.5	76.57	43.49	21.05	18.03	3.26	4.53	1.31	0	0	24.78
2	0.2	160	1.0	70.91	45.11	17.44	18.57	2.76	6.12	1.37	0	0	33.48
3	0.2	160	20.0	61.62	56.43	9.97	21.33	3.92	9.47	1.54	0.24	0	51.81
4	0.2	180	10.5	55.96	56.03	6.45	22.27	5.99	11.43	1.58	0.56	0	62.53
5	0.6	140	1.0	65.45	50.41	12.56	19.90	4.08	9.11	1.60	0	0	49.84
6	0.6	140	20.0	55.15	55.77	5.51	24.57	7.99	12.93	1.62	0.61	0.06	70.73
7	0.6	160	10.5	53.94	58.38	4.33	24.10	7.85	15.78	1.65	1.03	0.12	86.32
8	0.6	160	10.5	53.33	61.19	2.44	25.13	9.04	16.33	1.66	1.06	0.13	89.33
9	0.6	160	10.5	54.34	60.32	2.88	23.40	8.01	16.01	1.65	1.13	0.15	87.58
10	0.6	180	1.0	52.37	61.88	2.10	24.00	9.07	16.44	1.68	1.18	0.15	89.93
11	0.6	180	20.0	48.28	53.63	0	31.17	11.09	9.08	1.72	1.74	0.25	49.67
12	1.0	140	10.5	57.17	56.38	4.19	22.20	7.78	15.49	1.68	0.88	0.10	79.27
13	1.0	160	1.0	52.93	56.70	4.60	23.57	9.56	14.64	1.84	0.83	0.08	80.09
14	1.0	160	20.0	50.10	56.17	2.24	25.27	10.95	12.67	2.18	1.31	0.18	63.84
15	1.0	180	10.5	49.49	56.26	0	27.93	10.41	10.82	2.32	1.73	0.27	59.19

3.2. Optimization of nitric acid pretreatment condition

The optimization of the pretreatment condition was done using Box–Behnken analysis and the quadratic equation for xylose yield in terms of actual parameters was obtained:

$$Y = -1101.43344 + 493.74063X_1 + 11.58386X_2 + 16.55193X_3 - 1.80719X_1X_2 - 2.27500X_1X_3 - 0.080461X_2X_3 - 121.99479X_1^2 - 0.029454X_2^2 - 0.12099X_3^2$$

where X_1, X_2, X_3, and Y represent the HNO_3 concentration, temperature, time, and xylose yield respectively. In order to study the significance and adequacy of this model, analysis of variance (ANOVA) was carried out and summarized in Table 2. The model F-value of 48.07 and a low p-value ($p < 0.001$) implies that this model was statistically significant. In addition, the p-value for each model term that $X_1, X_2, X_1X_2, X_1X_3, X_2X_3, {X_1}^2, {X_2}^2$, and ${X_3}^2$ had significant effect on the xylose yield. Although X_3 was insignificant ($p > 0.05$), this factor could not be eliminated to support hierarchy of the model, because the value of adjusted determination coefficient (Adj. $R^2 = 0.9673$) indicated a high significance of the model. The optimized condition for maximum xylose yield considering efficiency were $X_1 = 0.65\%$, $X_2 = 158.8\,^{\circ}C$ and $X_3 = 5.86$ min with a predicted xylose yield of 87.3%. To verify the validity of the predicted value, experiment was performed at the optimized condition, and the xylose yield was 86.5%.

In order to determine the interactive effects of independent variables on the response, three-dimensional surface plots were described in Fig. 1. The response surface plot of xylose yield at a fixed time of 10.5 min with different HNO_3 concentration and temperature is shown in Fig. 1a. Increases in acid concentration and temperature provided indeed better solubilization of hemicellulose, leading to an increase in the amount of released xylose. However, a further increase in HNO_3 concentration and temperature resulted in a decrease in xylose yield due to secondary decomposition of the xylose to other derivatives such as furfural. Fig. 1b shows the interaction between HNO_3 concentration and pretreatment time at the temperature of 160 °C. It was found that the effect of HNO_3 concentration was more significant than that of the pretreatment time, which was also evident from the ANOVA test. Fig. 1c suggested that the effect of the temperature and pretreatment time on the xylose yield was significant. It was observed that temperature had a strong positive interaction with reaction time as indicated by the high F-value and temperature was more influential compared to reaction time.

3.3. Enzymatic hydrolysis

In order to evaluate the effectiveness of the pretreatment, enzymatic hydrolysis was also carried out using rice straw pretreated under the optimal condition, i.e., 0.65% HNO_3 at 158.8 °C for 5.86 min. Sulfuric acid (H_2SO_4) was used for comparison. Enzymatic hydrolysis yield was monitored by quantifying glucose released from the pretreated solid fraction over time (Fig. 2). The HNO_3-treated rice straw exhibited much higher enzymatic digestibility than that of the untreated control, indicating that HNO_3, as an effective catalytic agent, indeed improved the enzyme accessibility to cellulose. The highest glucose yield was achieved after 72 h hydrolysis (47.7 g/L), and it was higher than that with H_2SO_4 (43.8 g/L). In addition, most of xylan (>99%) was converted into xylose monomer

Table 2
Analysis of variance (ANOVA) for the quadratic model.

Source	Sum of squares	*df*	Mean square	*F* value	*p* value Prob > F	
Model	5863.11	9	651.46	46.98	0.0003	Significant
X_1-HNO_3	1506.73	1	1506.73	108.67	0.0001	
X_2-Temp.	168.36	1	168.36	12.14	0.0176	
X_3-Time	37.37	1	37.37	2.70	0.1616	
$X_1\,X_2$	836.08	1	836.08	60.30	0.0006	
$X_1\,X_3$	298.94	1	298.94	21.56	0.0056	
$X_2\,X_3$	934.83	1	934.83	67.42	0.0004	
${X_1}^2$	1406.76	1	1406.76	101.46	0.0002	
${X_2}^2$	512.52	1	512.52	36.96	0.0017	
${X_3}^2$	440.23	1	440.23	31.75	0.0024	
Residual	69.33	5	13.87			
Lack of fit	64.76	3	21.59	9.45	0.0972	Not significant
Pure error	4.57	2	2.29			

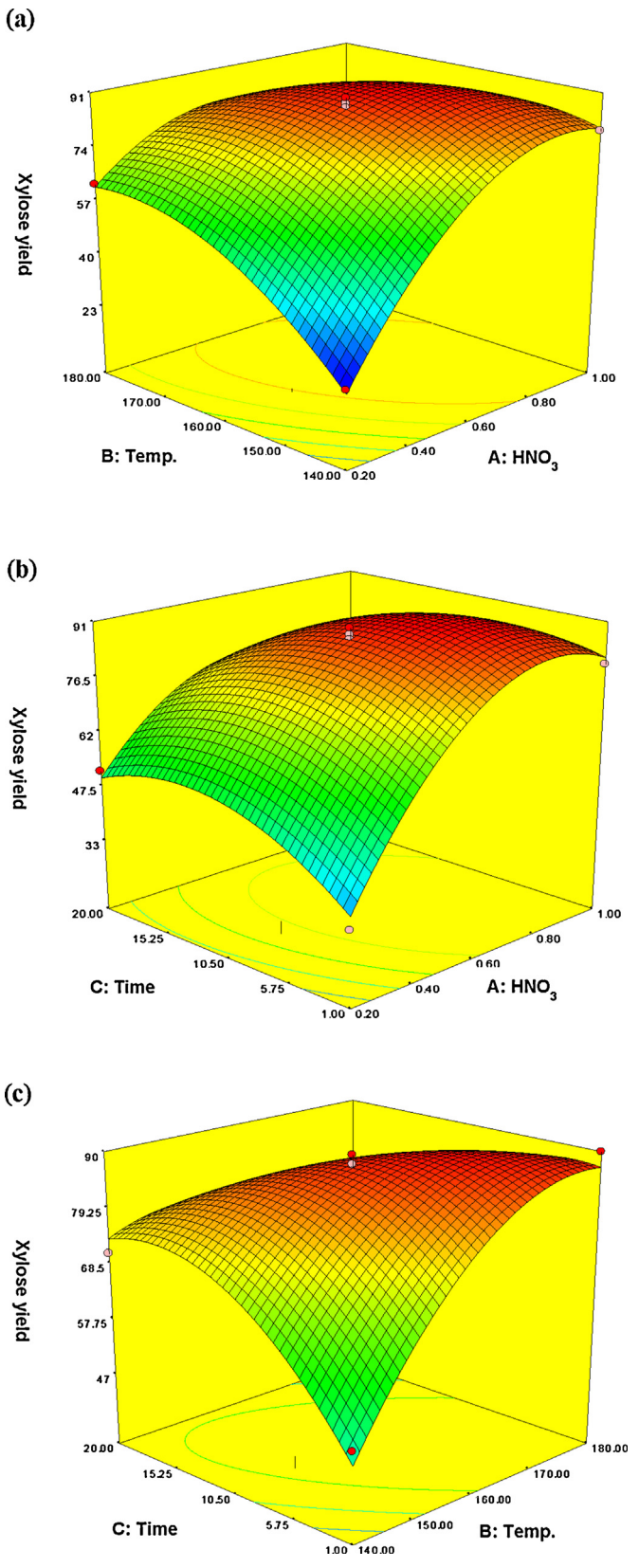

Fig. 1. The surface response plots of the effects of HNO_3 concentration, temperature and time on the xylose yield: (a) fixed time at 10.5 min; (b) fixed temperature at 160 °C; (c) fixed HNO_3 concentration at 0.6%.

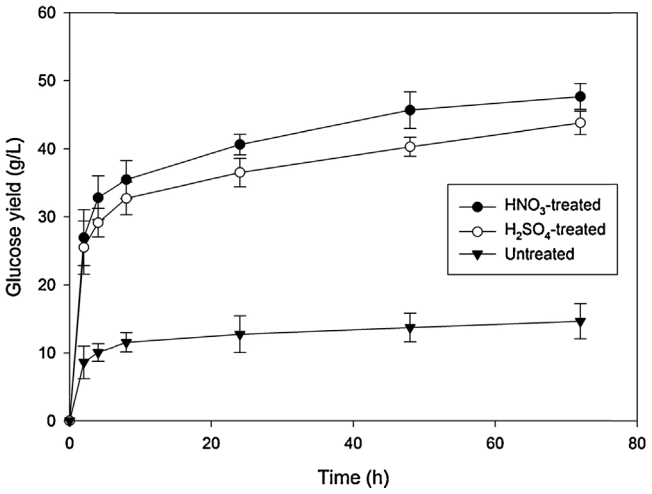

Fig. 2. Enzymatic hydrolysis yield of pretreated rice straw (HNO_3 or H_2SO_4 0.65%, 158.8 °C, and 5.86 min). Enzymatic hydrolysis was conducted at 50 °C and 170 rpm in 50 mM citrate buffer (pH 4.8) using 30 FPU enzyme blend Cellic C-Tec per gram of cellulose.

by HNO_3, while some, albeit a small part of xylan (6.3%), still remained after H_2SO_4 pretreatment (data not shown). These results support that HNO_3 is indeed a potent acid-catalyst, which is effective for xylose release from rice straw and enhances the recovery of hemicellulose and the enzymatic hydrolysis of cellulose.

3.4. Effect of nitrate on ethanol production

One of distinct benefits to use nitric acid for the pretreatment lies in the downstream step; it is possible that the neutralized catalyst serves as a nitrogen source for yeast fermentation. Since a typical medium for ethanol fermentation contains 3 g/L yeast extract and 5 g/L of peptone, corresponding to a nitrogen amount of 1.13 g/L (Laopaiboon et al., 2009), the optimal amount of 0.65% HNO_3, corresponding to a nitrogen content of 1.44 g/L, might fall into a right range required for active ethanol production. To explore this possibility, fermentation experiments were conducted with *P. stipitis* with and without HNO_3 (Fig. 3). The total nitrogen concentration in the fermentation medium with 0.65% HNO_3 was found to be 1.71 g/L, which was slightly higher than that of pretreatment hydrolysate (1.63 g/L). As expected, the control medium containing yeast extract and peptone supported the best fermentation; even after 24 h, glucose was almost completely exhausted and after that xylose was gradually consumed, which is a typical pattern with *P. stipitis*. Ethanol concentration reached the highest value of 17.68 g/L after 120 h. With nitrate as a sole nitrogen source, the rates of sugar consumption and ethanol production yield were relatively lower than those obtained in the control. This result suggested that the type of nitrogen sources did affect the fermentation step: complex organic nitrogen sources are better than the inorganic source. Wang et al. (2012) also found the similar phenomenon; urea was better than ammonium in terms of ethanol production. Even so, the presence of nitrate in medium obviously enhanced the fermentation, e.g., final ethanol concentration from 10.92 g/L to 14.50 g/L as shown in Fig. 3b. This finding clearly supports a possibility that nitrate, derived from the nitric acid pretreatment, could be directly used as a nitrogen source in the subsequent fermentation process. This possibility expects not only to substantially simplify after-pretreatment-steps, since the acid catalyst needs not to be removed unlike the other acid catalysts, but also to reduce the overall process cost by providing an additional nitrogen source.

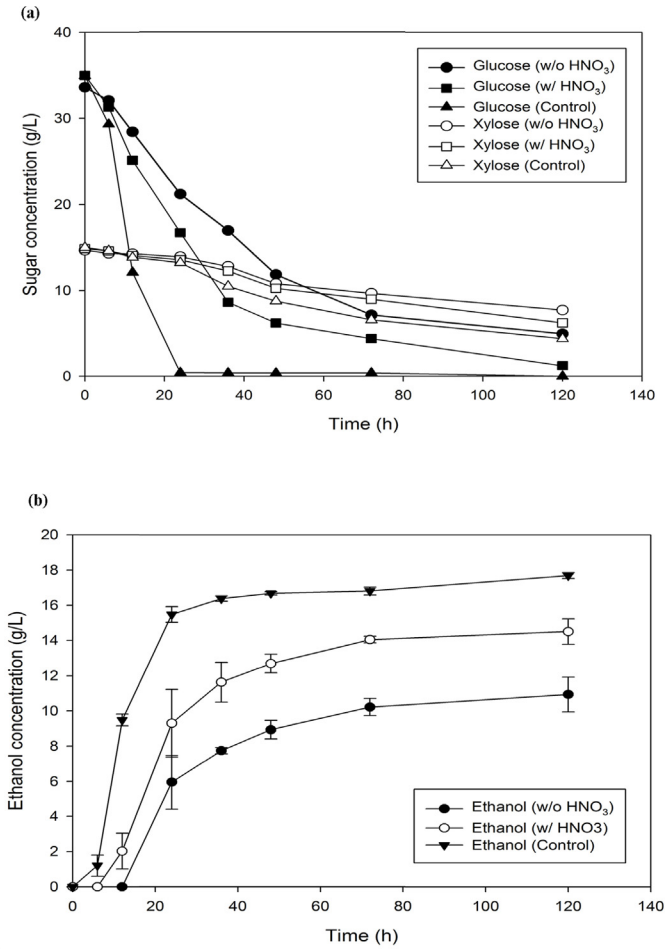

Fig. 3. (a) Sugar consumption and (b) ethanol production during fermentation of *P. stipitis*.

4. Conclusions

The present study demonstrated that nitric acid is a potent acid catalyst able to effectively release xylose from rice straw and allow for efficient enzymatic hydrolysis of cellulose. The maximum xylose yield of 86.5% in the pretreatment and enzymatic hydrolysis yield of 83.0% were obtained with 0.65% HNO_3 at 158.8 °C for the rather short reaction time (5.86 min). Furthermore, HNO_3 at the optimal amount improved ethanol yield by *P. stipitis* (14.50 g/L compared with 10.92 g/L of the control). Nitrate, the neutralized form of nitric acid, was found to be used as an acceptable nitrogen source for the bioethanol production. This powerful acid-catalyst is expected to reduce overall cost of bioethanol production.

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