Mycobiology 37(2) : 133-140 (2009)

Improved Bioethanol Production Using Activated Carbon-treated Acid Hydrolysate from Corn Hull in *Pachysolen tannophilus*

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(Received March 9, 2009. Accepted April 8, 2009)

To optimally convert corn hull, a byproduct from corn processing, into bioethanol using *Pachysolen tannophlius*, we investigated the optimal conditions for hydrolysis and removal of toxic substances in the hydrolysate via activated carbon treatment as well as the effects of this detoxification process on the kinetic parameters of bioethanol production. Maximum monosaccharide concentrations were obtained in hydrolysates in which 20 g of corn hull was hydrolyzed in 4% (v/v) H_2SO_4. Activated carbon treatment removed 92.3% of phenolic compounds from the hydrolysate. When untreated hydrolysate was used, the monosaccharides were not completely consumed, even at 480 h of culture. When activated carbon–treated hydrolysate was used, the monosaccharides were mostly consumed at 192 h of culture. In particular, when activated carbon-treated hydrolysate was used, bioethanol productivity (P) and specific bioethanol production rate (Q_p) were 2.4 times and 3.4 times greater, respectively, compared to untreated hydrolysate. This was due to sustained bioethanol production during the period of xylose/arabinose utilization, which occurred only when activated carbon-treated hydrolysate was used.

KEYWORDS : Acid hydrolysis, Activated-carbon treatment, Bioethanol, Corn hull, *Pachysolen tannophilus*

Recent reports describe the technical and economical implications of bioethanol production (Cardona and Sánchez, 2007; Demirbas, 2007; Hamelinck *et al*., 2005; Sánchez and Cardona, 2008). In particular, there has been a substantial interest in the use of lignocellulosic material from agricultural byproducts as a resource for the production of bioethanol, especially because of the recent price increase in crop production (e.g. corn, rice, etc.). Among these, sugar cane bagasses (Aguilar *et al*., 2002; Gámez *et al*., 2006), sorghum straw (Herrera *et al*., 2003; Vázquez *et al*., 2007), wheat straw (Saha *et al*., 2005; Yang *et al*., 2008), rice straw (Karimi *et al*., 2006a, b), corn stover (Agbogbo and Wenger, 2007; Georgieva and Ahring, 2007; Lau *et al*., 2008; Ohgren *et al*., 2006a, b), corncob (Chen *et al*., 2007; Qu *et al*., 2006; Vázquez *et al*., 2006), and corn hull (i.e., corn fiber) (Gáspár *et al*., 2007; Hespell, 1998; O'Brien *et al*., 2004; Schell *et al*., 2004) are currently being investigated for use as resources of bioethanol production. To this end, their fermentable sugar content, optimal fermentation process, and strategies for their hydrolyses have been examined. Prior to bioethanol fermentation, hydrolyses of these byproducts has been typically carried out using dilute solutions of sulfuric acid (Aguilar *et al*., 2002; Karimi *et al*., 2006a), phosphoric acid (Gámez *et al*., 2006; Vázquez *et al*., 2007), or hydrochloric acid (Bustos *et al*., 2003; Herrera *et al*., 2003). In addition, utilization of enzymatic hydrolysis (Lau *et al*., 2008; Ohgren *et al*., 2006a) and combined hydrolysis protocols using enzymes and dilute acid has been reported (Chen *et al*., 2007; Saha *et al*., 2005).

Corn hull is a residue produced from the corn wet-milling process. Its sugar composition varies (glucose 10~50%, xylose 13~49%, arabinose 10~31%, and galactose 3~10%) depending on its origin, hydrolysis method, and corn processing method (Gáspár *et al*., 2007; Hespell, 1998; O'Brien *et al*., 2004; Schell *et al*., 2004). Nowadays, most corn hull is used as animal feedstuff, and its utilization for bioethanol production has only recently been examined (Dale *et al*., 1996; Grohmann and Bothast, 1997; Gulati *et al*., 1996; Saha *et al*., 1998; Schell *et al*., 2004). However, it has been reported that furan derivatives (furfural and 5-hydroxymethlyfurfural) and phenolic compounds within the acid hydrolysate are toxic and can inhibit bioethanol production (Carvalheiro *et al*., 2005; Chandel *et al*., 2007; Larsson *et al*., 1999; Palmqvist and Hahn-Hägerdal, 2000a, b), with levels of about 0.6~3.0 g/*l* found in the acid hydrolysate (Carvalheiro *et al*., 2005; Chandel *et al*., 2007). Detoxification of acid hydrolysates was reviewed in previous reports (Palmqvist and Hahn-Hägerdal, 2000a; Sánchez and Cardona, 2008). Activated carbon treatment led to a 92% reduction in furan derivatives and 68% reduction of 5-hydroxymethlyfurfural as well as a reduction in total phenolic compounds from about 1.25 to 0.5 g/*l* (Carvalheiro *et al*., 2005).

There has been only a limited report which observed

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the effect of detoxification of the acid hydrolysate of corn hull on kinetic parameters of bioethanol production. In this work, the kinetic analysis of physiological parameters was carried out as a tool to assess how this detoxification process affects the kinetic parameters of bioethanol production using *Pachysolen tannophlius*. Furthermore, we explored an optimized corn hull hydrolysis protocol and the removal of toxic substances from the hydrolysate via activated carbon treatment.

Materials and Methods

Hydrolysis of corn hull. For this study, the dried corn hull was kindly supplied by Corn Products Korea, Inc. (Icheon, Republic of Korea). Prior to hydrolysis, 150 g of dried corn hull was ground for 5 min using a blender and then filtered using a sieve (mesh size 25). Hydrolysis was carried out by mixing the ground corn hull and 100 *ml* of sulfuric acid (H_2SO_4) solution, which was then autoclaved at 121°C for 45 min. The hydrolysate was obtained from the supernatant by centrifugation at 4,000 rpm. To prepare the hydrolysate for flask culture experiments, the hydrolysis was carried out in 2 *l* of H_2SO_4 solution.

Activated carbon treatment of hydrolysate. After the pH of the hydrolysate was adjusted to 5.5 with NaOH, 100 *ml* of hydrolysate and 10 g of activated carbon (Daejung Chemicals & Metals Co, Republic of Korea) were mixed, and then the activated carbon was removed by the vacuum filtration (Carvalheiro *et al.*, 2005).

Yeast strain and flask culture. Yeast *Pachysolen tannophilus* (*P. tannophilus*) ATCC 32691 was used in this study. YPX (1% yeast extract, 2% peptone, and 2% xylose) medium was used for seed culture, and YPC (1% yeast extract and 2% peptone in 100 *ml* corn hull hydrolysate) medium was used for the flask culture during bioethanol production. One hundred milliliters of flask culture were grown in a 250 *ml* flask at 30°C and shaken at 150 rpm. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (Spectronic, Thermo Scientific, USA). The residual reducing sugar in the culture medium was analyzed using the dinitrosalicylic acid (DNS) method (Chaplin and Kennedy, 1986).

Phenolic compounds in hydrolysate. Total phenolic compounds were measured using Folin-Ciocalteu reagent (Hayashi Pure Chemical Co., Japan) (McDonald *et al.*, 2001) with gallic acid (Sigma) as the standard. After the color reaction was complete, 200 μl of colored solution was transferred to a 96-well microtiter plate, and its absorbance was measured using a plate reader (Bio-Rad) at 655 nm.

Thin-layer chromatography. Monosaccharide content of the hydrolysate was analyzed via thin-layer chromatography (TLC) using a 20 × 10 cm Partisil® K5F (Whatman) as a TLC plate, acetonitrile solution (acetonitrile : water = 85 : 15, v/v) for the mobile phase, and a sample loading volume of 1.0 μl. For the visualization of bands, TLC plates were soaked in 0.5% α-naphtol and 5% H_2SO_4 in ethanol and then dried in an oven at 80°C for 15 min. Specifically, the amounts of reducing monosaccharides (glucose, xylose, and arabinose) in the hydrolysate and the yeast culture broth were quantitatively determined via TLC. Glucose (0 to 7%), xylose (0 to 7%), and arabinose (0 to 2%) standards were simultaneously developed on the same TLC plate. The monosaccharide bands in the scanned images were converted to peaks using the AlphaEase FC software (Alpha Innotech, U.S.A.), and their quantities were calculated from the standard curve for each monosaccharide.

Gas chromatography. The concentration of bioethanol was also analyzed by gas chromatography (HP 6890, Agilent technologies, USA) using a flame ionization detector (FID). An HP INNOWax column (Agilent 19091N-113; film thickness, 0.25 μm; length, 30 m; inner diameter, 0.32 mm) was used. The initial temperature, maximum temperature, and temperature rate in the oven were 50°C, 170°C, and 10°C/min, respectively. Both the injector and FID temperatures were controlled at 250°C. Nitrogen was used as the carrier gas with a flow rate of 40 *ml*/min. For quantitative analysis, *n*-butanol was used as an internal standard.

Productivity and specific production rate of bioethanol. Productivity and specific production rate were calculated as follows:

$$\text{Bioethanol productivity (P)} = \frac{\Delta E}{\Delta t} = \frac{(E_2 - E_1)}{(t_2 - t_1)} (g/l/h)$$

$$\text{Specific bioethanol production rate } (Q_p) = \frac{P}{(OD_{avg})} = \frac{\Delta E}{\Delta t \cdot (OD_{avg})} = \frac{(E_2 - E_1)}{(t_2 - t_1) \cdot (OD_{avg})} (g/l/h/OD)$$

where E_2 and E_1 are the bioethanol concentrations in the culture broth at time t_1 and t_2, respectively, and OD_{avg} is the average cell growth (OD_{600}) between time t_1 and t_2.

Results

Acid hydrolysis of corn hull. Corn hull was hydrolyzed in various concentrations of H_2SO_4 (0.5 to 2.5%, v/v) (Fig. 1). When the hydrolysate was prepared using 10 g of corn hull and a 100 *ml* H_2SO_4 solution, the hydrolysate was mostly composed of glucose, xylose, and arabinose. The

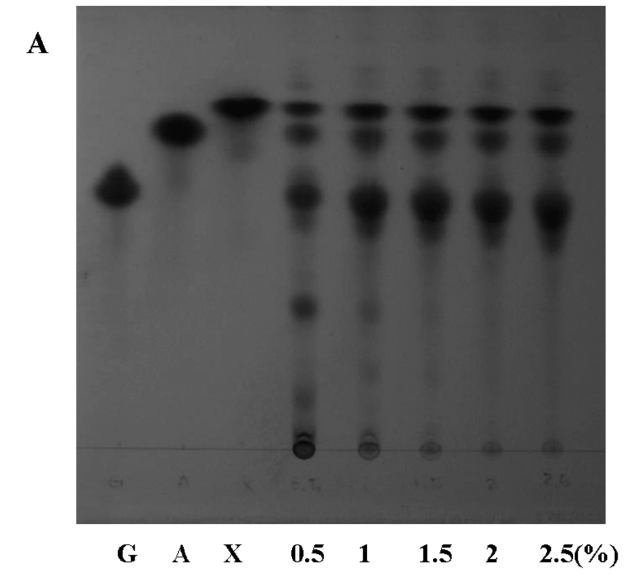

Fig. 1. Acid hydrolysis of corn hull. A, TLC of corn hull hydrolysate. G, A, and X indicates the glucose, arabinose, and xylose standards, respectively. H_2SO_4 concentrations are 0.5, 1, 1.5, 2, and 2.5% (v/v). B, Glucose, xylose, and arabinose concentrations in the hydrolysate relative to the changes in H_2SO_4 concentrations. These data were obtained from quantitative TLC.

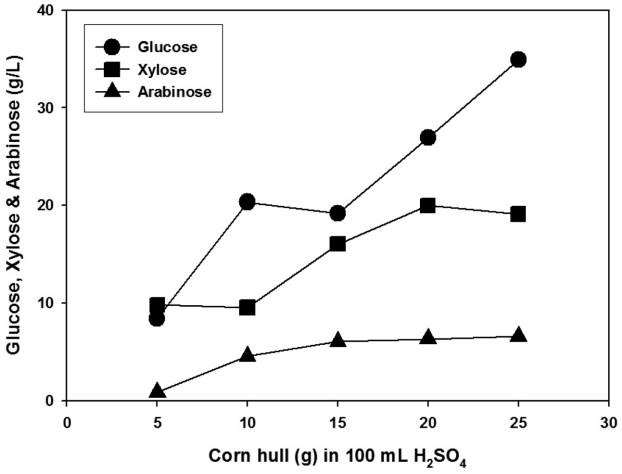

Fig. 2. Glucose, xylose, and arabinose concentrations in hydrolysates relative to the increase in corn hull concentrations. Two percent (v/v) H_2SO_4 was used for the hydrolysis. These data were obtained from quantitative TLC.

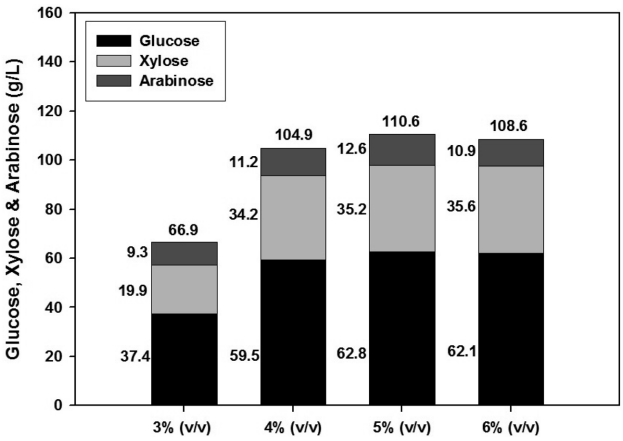

Fig. 3. Glucose, xylose, and arabinose concentrations in the hydrolysates relative to the increase in concentrations of H_2SO_4. Twenty grams of corn hull were used for the hydrolysis. The values on the left of vertical bars indicate glucose, xylose, and arabinose concentrations in the hydrolysate, and those on the top indicate the sum of three monosaccharides (i.e. total monosaccharides in the hydrolysate). These data (n = 5) were obtained from quantitative TLC.

maximum concentration of each of these monosaccharides was observed using a 2.0% (v/v) H_2SO_4 (Fig. 1A & B). Furthermore, the amount of each monosaccharide in the hydrolysate increased relative to the increasing amount of corn hull in 100 *ml* of 2.0% (v/v) H_2SO_4 (Fig. 2). However, when more than 25 g of corn hull was used in 100 *ml* of 2.0% (v/v) H_2SO_4, most of the H_2SO_4 solution was adsorbed into corn hull and only a minimal amount of hydrolysate could be obtained. Therefore, we decided to use 20 g of corn hull for hydrolysis, and the optimum concentration of H_2SO_4 was examined once again. As shown in Fig. 3, when 20 g of corn hull was hydrolyzed in 100 *ml* of 3.0 to 6.0% (v/v) H_2SO_4, the total concentration of monosaccharides in the hydrolysate ranged from 66.9 to 110.6 g/*l*. At 4.0 to 6.0% (v/v) H_2SO_4, the maximum concentration of total momosaccharides reached 104.9~110.6 g/*l*. The average concentrations of glucose, xylose, and arabinose in the hydrolysate were 61.4, 35.0, and 11.6 g/*l*, respectively.

Removal of phenolic compounds by activated carbon treatment. In the bioethanol production process, the toxic furan derivatives (furfural and 5-hydroxymethylfur-

fural) and phenolic compounds are produced during the acid hydrolysis and are known to inhibit both cell growth and ethanol production (Palmqvist and Hahn-Hägerdal, 200b; Larsson *et al.*, 1999). We removed these compounds by activated carbon treatment following hydrolysate preparation using 20 g of corn hull and 4% (v/v) H_2SO_4. As shown in Fig. 4, this process resulted in total phenolic compounds values decreasing from 2015.2 to 153.3 mg/*l*, which is equivalent to a 92.3% reduction in total phenolic compounds. This activated carbon-treated hydrolysate was then used for bioethanol production and compared with untreated hydrolysate.

Bioethanol production in flask culture. To explore how the activated carbon treatment of acid-hydrolysized corn hull affects bioethanol production, we carried out two flask cultures: one used untreated hydrolysate and the other used activated carbon-treated hydrolysate as a carbon source for yeast culture. As shown in Fig. 5, the cell growth profiles of *P. tannophilus* were similar between the two media, although the OD_{600} was more severely affected by the medium color when the hydrolysate not

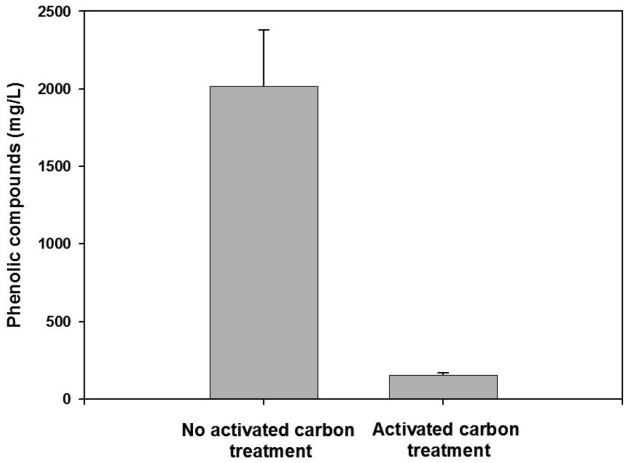

Fig. 4. Removal of phenolic compounds by activated carbon treatment (n = 3).

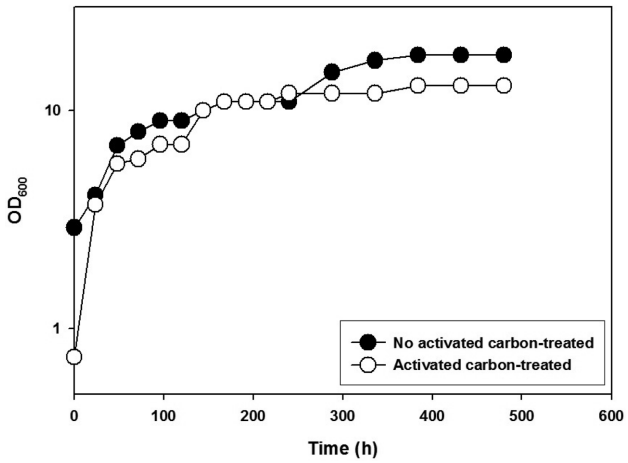

Fig. 5. The growth profiles of *P. tannophilus* in the flask culture.

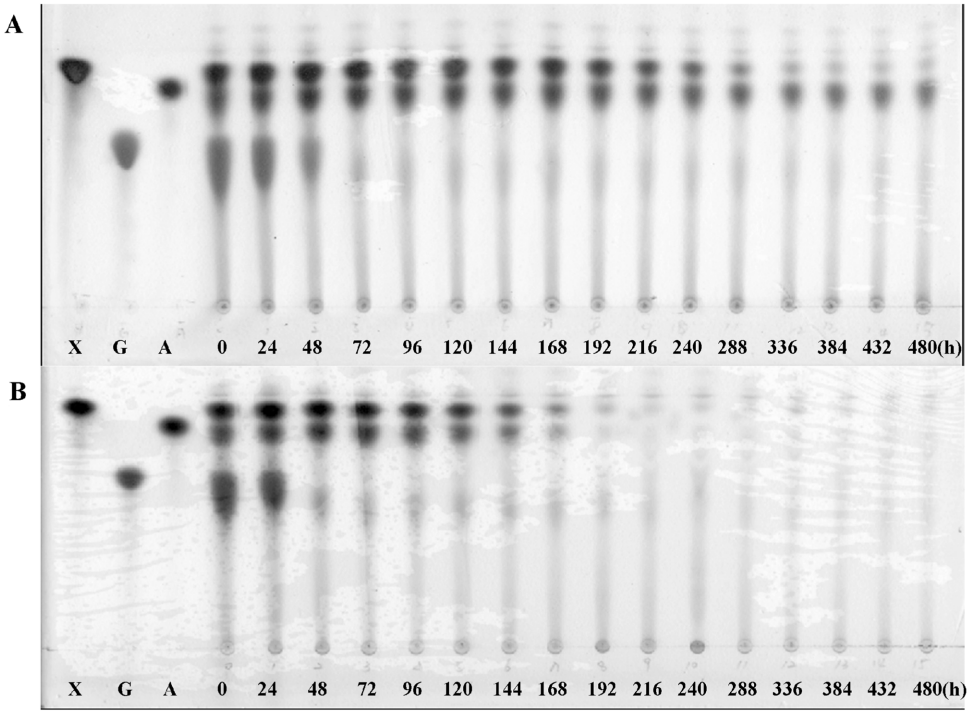

Fig. 6. TLC analysis of the flask culture broths of *P. tannophilus*. G, A, and X indicate the glucose, arabinose, and xylose standards, respectively.

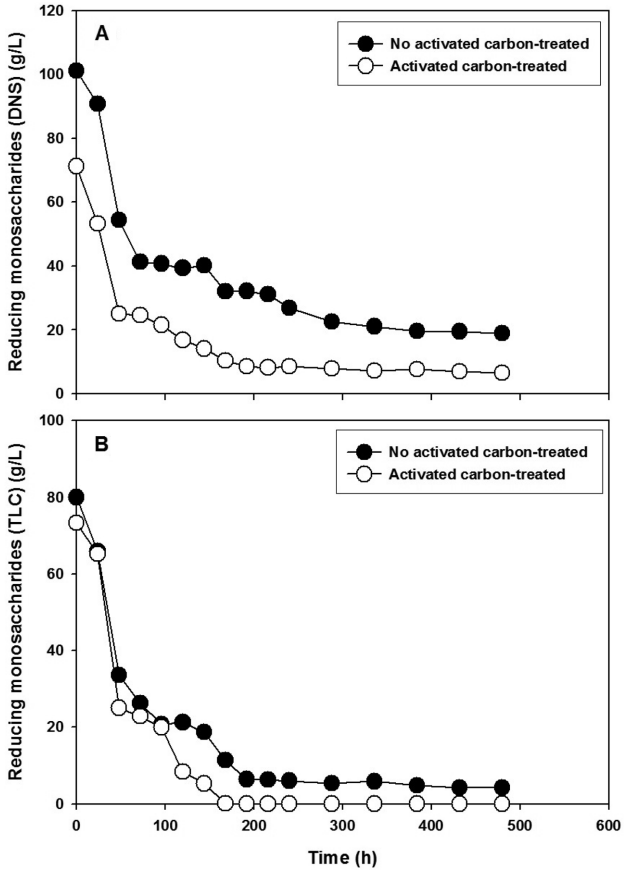

Fig. 7. Consumption profiles of the reducing monosaccharide (glucose, xylose, and arabinose) in the *P. tannophilus* flask culture. A, DNS method; B, quantitative TLC method.

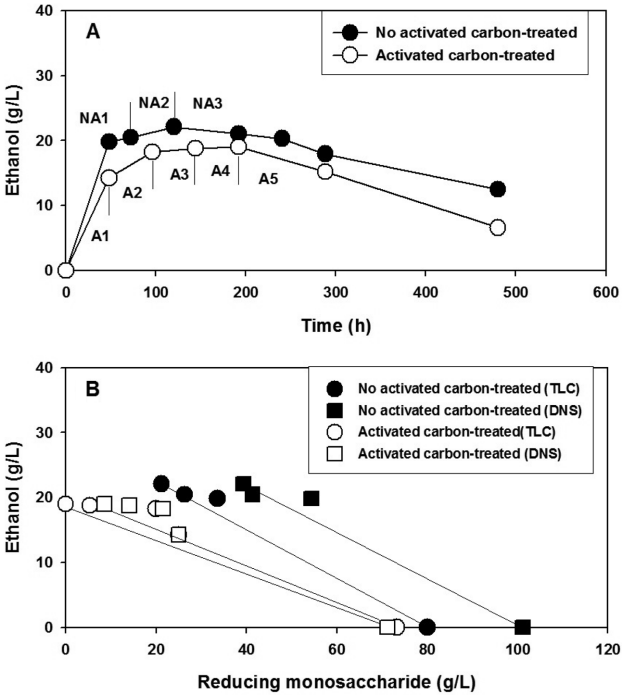

Fig. 8. Profiles of bioethanol production. A, Profiles of bioethanol production in the *P. tannophilus* flask culture. The meanings of NA1-NA3 and A1-A5 are described in the text. B, The plot of bioethanol produced vs. reducing monosaccharide consumed. Data used in this plot are from time zero until the bioethanol concentrations were at their maximum. The slopes of straight lines indicate the overall bioethanol yields.

treated. However, the consumption profiles of glucose, xylose, and arabinose were remarkably different (Fig. 6). While monosaccharides were almost completely consumed at 192 h of culture when the activated carbon-treated hydrolysate was used (Fig. 6B), they were not completely consumed, even at 480 h of culture, when the untreated hydrolysate was used (Fig. 6A). For example, glucose consumption was retarded when the untreated hydrolysate was used; specifically, glucose was completely consumed at 72 h when the untreated hydrolysate was used, while glucose was completely consumed at 24 h when activated carbon-treated hydrolysate was used.

During the culture period, the total residual reducing monosaccharides were measured by two methods, DNS (Fig. 7A) and quantitative TLC methods (Fig. 7B). Although the profiles of DNS and TLC might show a similar pattern, they were in fact different. Specifically, the untreated hydrolysate was measured to have 10~15 g/*l* higher monosaccharides via DNS as compared to TLC. The activated carbon-treated hydrolysate demonstrated the same initial profiles via DNS or TLC; however, after 192 h DNS measured 4~5 g/*l* of monosaccharides and TLC indicated undetectable levels in the culture broth.

Bioethanol production reached the maximum value of 22.1 g/*l* at 120 h when untreated hydrolysate was used and reached 19.01 g/*l* at 192 h when activated carbon-treated hydrolysate was used (Fig. 8A). Moreover, with activated carbon-treated hydrolysate the bioethanol production was sustained for 48 to 192 h, from 14.2 to 19.01 g/*l*, while production was discontinued at 120 h with untreated hydrolysate. Meanwhile, the bioethanol produced in the culture began being consumed at 120 h when untreated hydrolysate was used, while this occurred at 192 h when activated carbon-treated hydrolysate was used. As shown in Fig. 8B, the overall bioethanol yield was calculated only in the range when ethanol concentration was increasing. The overall bioethanol yield was about 0.36 when untreated hydrolysate was used, while with activated carbon-treated hydrolysate the yield was about 0.3. The slopes of the straight lines presented in Fig. 8B represent the overall bioethanol yields.

Effect of activated carbon treatment on the kinetic parameters for bioethanol production. To evaluate how activated carbon treatment of the hydrolysate affects the kinetic parameters of bioethanol production, the bioet-

Table 1. Bioethanol productivity (P) and specific bioethanol production rate (Q_p)

Pretreatment	Culture period	P (g/l/h)	Q_p (g/l/h/OD)	Monosaccharides consumed
No activated carbon	0~72 h (NA1)	2.84E-1	5.18E-2	Glucose
	72~120 h (NA2)	3.39E-2	3.91E-3	Xylose, Arabinose
	120~192 h (NA3)	-3.37E-2	-3.37E-3	Xylose, Arabinose, Ethanol
Activated carbon	0~48 h (A1)	2.96E-1	8.75E-2	Glucose
	48~96 h (A2)	8.33E-2	1.33E-2	Xylose, Arabinose
	96~144 h (A3)	1.08E-2	1.35E-3	Xylose, Arabinose
	144~192 h (A4)	5.20E-3	4.80E-4	Xylose, Arabinose
	192~288 h (A5)	-3.90E-2	-3.45E-3	Ethanol

hanol productivity (P) and specific bioethanol production rate (Q_p) were calculated as shown in Table 1. When activated carbon-treated hydrolysate was used, during the period NA1 and A1 (i.e., glucose consumption period) P was slightly higher and Q_p was 1.6 times greater than when untreated hydrolysate was used. Additionally, when activated carbon-treated hydrolysate was used, during the period NA2 and A2 (i.e., xylose and arabinose consumption period) P and Q_p were 2.4 times and 3.4 times greater, respectively, compared to untreated hydrolysate. Interestingly, xylose and arabinose continued to be consumed during the production of bioethanol (periods A2, A3, and A4) when activated carbon-treated hydrolysate was used, while xylose and arabinose only started being consumed at period NA2 when untreated hydrolysate was used; thereafter, bioethanol started to be consumed simultaneously with xylose and arabinose (period NA3). However, when activated carbon-treated hydrolysate was used, by period A5 xylose and arabinose had been consumed completely and only then the bioethanol started to be consumed.

Discussion

As shown in the TLC data in Fig. 1A, corn hull was hydrolyzed completely by the H_2SO_4 solution. Although the TLC data was not shown for all the conditions tested, the optimized conditions for complete hydrolyzation consisted of 20 g of corn hull in 100 *ml* of 4% (v/v) H_2SO_4 (Fig. 3). Previous papers have reported on the various acid concentrations used for the hydrolysis of lignocellulosic byproducts, suggesting that 2~6% phosphoric acid (Gámez *et al.*, 2006; Vázquez *et al.*, 2007), 2~6% hydrochloric acid (Herrera *et al.*, 2003), or 1~6% sulfuric acid (Aguilar *et al.*, 2002; Karimi *et al.*, 2006a; O'Brien *et al.*, 2004; Vázquez *et al.*, 2006) are optimal. Additionally, total monosaccharides in acid hydrolysate reach 21~26 g/*l* for sugar cane bagasse (Aguilar *et al.*, 2002; Gámez *et al.*, 2006), 8~20 g/*l* for sorghum straw (Herrera *et al.*, 2003; Vázquez *et al.*, 2007), and 80~100 g/*l* for corn fiber (O'Brien *et al.*, 2004). In this work, more than 100 g/*l* of total monosaccharides were obtained when the hydrolysis was carried out in 4, 5, or 6% (v/v) H_2SO_4 (Fig. 3). This acid hydrolysis is a popular process and is well reviewed in many reports (Mosier *et al.*, 2005; Sánchez and Cardona, 2008). However, furan derivatives begin to severely inhibit bioethanol production when they reach the level of about 500 mg/*l* (Larsson *et al.*, 1999). These negatively affect the aerobic and anaerobic metabolism and the membrane permeability of yeast (Palmqvist and Hahn-Hägerdal, 2000b). To remove these toxic compounds from the corn hull hydrolysate, we attempted to treat the hydrolysate with activated carbon. This method has been intensively reviewed together with several other methods (Mosier *et al.*, 2005). In this work, although we did not investigate which specific toxic compounds were removed by our activated carbon treatment, total phenolic compounds were removed remarkably well, an approximate 92% reduction (Fig. 4). A previous report demonstrated a 60% reduction in phenolic compounds following activated carbon treatment (Carvalheiro *et al.*, 2005). Additionally, we observed that our activated carbon treatment led to the removal of the dark brown color present in the hydrolysate.

In the flask culture, the OD_{600} at time zero when untreated hydrolysate was used was significantly greater than when activated carbon-treated hydrolysate was used (Fig. 5). This was a result of the color difference H_2SO_4 and amount of debris in the hydrolysates. Moreover, the higher OD_{600} observed at latter periods of culture, when untreated hydrolysate was used, was due to the attached debris on the wall of flask separating into the media. From the profiles of total monosaccharide consumption generated using the DNS (Fig. 7A) and TLC methods (Fig. 7B), we deduced that DNS measured, to some degree, unknown reducing substances affecting the assay result. In other words, TLC represented the most reasonable approach toward the quantification of monosaccharide levels in these cultures. Furthermore, it was shown that total monosaccharides in the sample at time zero (Fig. 7B) were about 20% less than those presented in Fig. 3. This might be because the hydrolysate used in the flask culture was prepared at a scale of 2 *l*, while the hydrolysis in Fig. 1, 2, and 3 were carried out at the 100-*ml* scale. In

Fig. 8B, the overall bioethanol yield decreased from 0.36 to 0.3 when activated carbon-treated hydrolysate was used compared to when untreated hydrolysate was used. However, the reason for this was because the decrease in overall bioethanol yields when activated carbon-treated hydrolysate was used represented the utilization of both xylose and arabinose after glucose was completely consumed.

As shown in Fig. 6, 7, 8A, and Table 1, when activated carbon-treated hydrolysate was used, the glucose consumption rate increased (period A1) and xylose and arabinose continued to be converted to bioethanol (period A2, A3, and A4) when compared to untreated hydrolysate. In addition, the bioethanol productivity and specific bioethanol productivity rate was increased by the use of activated carbon-treated hydrolysate.

To utilize xylose in *P. tannophilus*, xylose are known to enter the pentose phosphate pathway through xylulose, in which the reducing power was supplied from tricarboxylic acid cycle (Palmqvist and Hahn-Hägerdal, 2000a, b). Therefore, the fine control of aeration was crucial for the ethanol production using *P. tannophilus* because microaeration is needed for ethanol production from xylose (Larsson *et al*., 1999). In this work, because the aeration was not finely controlled and the yeast strains as well as other process were quite different, the parameters (overall yield, P, and Q_p) might be relatively lower than other researcher's results (Chandel *et al*., 2007). In conclusion, this work determined that P and Q_p were improved and the sustainability of bioethanol production during the period of xylose/arabinose utilization was superior when activated carbon-treated hydrolysate was used for bioethanol production by *P. tannophilus*.

Acknowledgement

This study was carried out with the support of Research Cooperating Program for Agricultural Science & Technology Development (Project No.200802A01036002), Rural Development Administration, Republic of Korea, and the support of a grant from the Academic Research Program of Chungju National University in 2008.

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