Food Engineering Progress
Vol. 16, No. 3. pp. 199~206 (2012.08)

산업식품공학
Food Engineering Progress

Pretreatment Effects on the Rice Bran Saccharification with Newly Identified Fungal Enzymatic Activities

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Abstract

In order to develop an efficient microbial source of cellulase enzyme system, cellulolytic fungal strain was newly isolated from traditional Korean *nuruk* samples in the present study. The crude enzyme extract of this fungal strain, avicelase, CMCase and β-glucosidase activities reached the maximal points of 6.73, 3.22, and 5.64 units/mL, respectively and was used for the subsequent enzymatic saccharification on pretreated deproteinised and lipid-extracted rice bran. This strain was identified as *Penicillium* sp. determined by cellular fatty acid composition analysis. Three different pretreatment conditions were evaluated on the deproteinised and lipid-extracted rice bran at 121°C/1.5 psi for 5 different residence times: one with 0.1 N sulfuric acid, another with 0.1 N sodium hydroxide, and the last with distilled water. The greatest enzymatic saccharification yield increased up to 75.2% from acid-catalyzed autoclaving pretreatment for 30 min. The acid-catalyzed autoclaving pretreatment enhanced the saccharifying ability of the newly isolated cellulolytic fungal strain on the deproteinised and lipid-extracted rice bran.

Key words: cellulolytic fungal strain, cellulase, enzymatic saccharification, pretreatment

Introduction

Bioethanol can be produced by pretreating lignocellulosic materials followed by enzymatic saccharification and fermentation (Öhgren et al., 2007). Unlike cereal endosperm where the major carbohydrate is starch, lignocellulosic material is composed of cellulose, hemicelluloses, and lignin. The complexity of lignocellulosic material makes it much more difficult than starch to be enzymatically decomposed to fermentable sugars (Banerjee et al., 2009). The pretreatment is a prerequisite step to alter structural characteristics of lignocellulosic substrate prior to enzymatic saccharification. These structural modiifications are highly dependent on the conditions of pretreatments employed (Kumar et al., 2009; Alvira et al., 2010). Deproteinised and lipid-extracted rice bran obtained from food processing industries has a potential to serve as a low cost feedstock for bioethanol production. Therefore, it is necessary to properly pretreat deproteinised and lipid-extracted rice bran before enzymatic saccharification step to enhance the subsequent fermentable sugar yield. For this purpose, the effect of different pretreatment strategies, such as 0.1 N acid and alkaline pretreatment on the saccharification of deproteinised and lipid-extracted rice bran were investigated in this study.

The widely accepted mechanism for enzymatic saccharification involves synergistically combined actions by endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Ferreira et al., 2009). There has, therefore, been extensive research for an efficient microbial source of cellulase enzyme system. *Trichoderma* sp. has been considered to be the most powerful source of cellulose-active enzymes among fungi, but its β-glucosidase activity is low for achieving good cellulose hydrolysis (Peciulyte, 2007). β-Glucosidase plays an important role in the complete saccharification of cellulose to glucose by catalyzing the hydrolysis of cellobiose (Ng et al., 2010). In this study, we report a newly isolated fungal strain from traditional Korean *nuruk* samples with higher β-glucosidase activity among wild-type strains. Traditional Korean *nuruk* is a kind of fermentation starter for rice wine. Various airborne microorganisms such as fungi, yeast, and bacteria are naturally inoculated during the traditional manufacturing process of *nuruk*. To our knowledge, the research regarding a newly isolated cellulolytic fungal strain from traditional Korean *nuruk* samples and its crude enzyme extract for the subsequent enzymatic saccharification on the pretreated deproteinised and lipid-extracted rice bran is being presented for the first time. The objective of this study was to investigate the pretreatment effects on saccharification

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Received July 11, 2012; revised July 27, 2012; accepted August 6, 2012

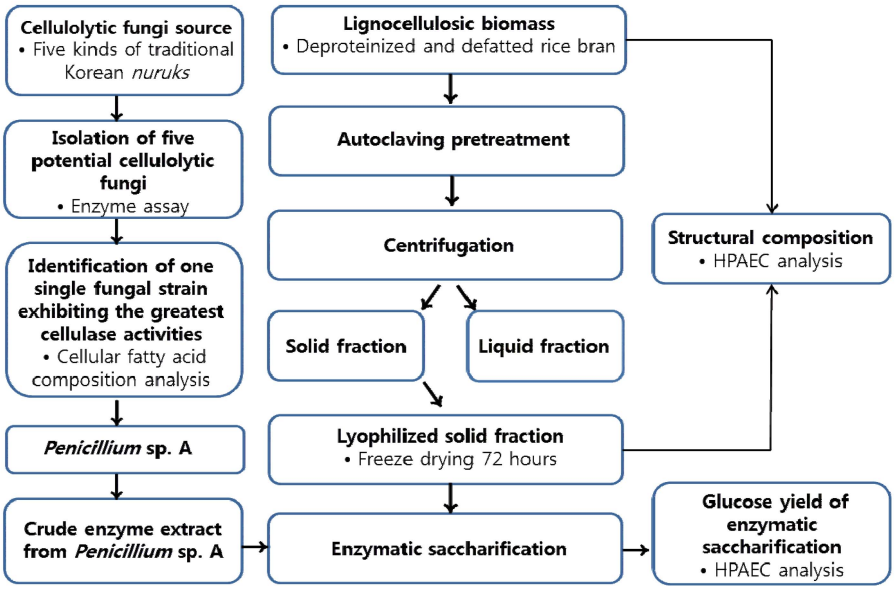

Fig. 1. Schematic experimental procedure for celluloytic fungal strain isolation and enzymatic saccharification of pretreated rice bran material.

of deproteinised and lipid-extracted rice bran by using crude enzyme extract from the newly isolated fungal strain. The whole experimental procedures employed in the present study are summarized in Fig. 1.

Materials and Method

Isolation of cellulolytic fungi

Five samples of traditional Korean *nuruk*s, which were collected from different areas in South Korea, were used for isolating strong cellulolytic fungi. Ten grams of each sample were suspended in 200 mL of NaCl solution (1%, w/v) for 3 h. The suspension was diluted from 10^{-1} to 10^{-5} and then 100 μL of the dilutions were seeded on the isolation plates. Czapek-Dox medium was used as an isolation plate with addition of 1% (w/v) carboxymethyl cellulose (CMC, Sigma-Aldrich Chemical Co., St. Louis, MO, USA), 1% (w/v) microcrystalline cellulose (Avicel PH 101, Fluka, Buchs, Switzerland), and 1.5% (w/v) agar (Dae-Jung Chemical Co., Seoul, Korea). The inoculated plates were incubated at 30°C for 7 days, and the growing fungal strains on the isolation plates were examined for their cellulase activities.

Enzyme preparation

Conical flasks (500 mL volume) containing 200 mL of the selection medium were inoculated with each colony of fungal strains growing on the isolation plates, respectively. The selection medium (pH 7.21) consisted of 3% sucrose, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.2% $NaNO_3$, 0.001% $FeSO_4 \cdot 7H_2O$, 1% CMC, 1% Avicel PH 101, and 0.02% sodium azide. The flasks were incubated at 30°C for 7 days. The aliquots (10 mL) of culture were taken on daily basis, which were centrifuged at 4°C and 11,000×*g* for 15 min (Eppendorf 5804R, Germany). The clear supernatants were used as crude enzyme extracts for the subsequent enzyme assays.

Enzyme assays

Avicelase (crystalline cellulose hydrolase) activity was assayed by incubating 100 μL of the same enzyme extracts with 400 μL of 0.2% (w/v) Avicel PH 101 in 50 mM sodium acetate buffer (pH 4.8) at 45°C for 60 min (Yamanobe et al., 1987). CMCase (Carboxymethyl cellulase) activity was determined by incubating 100 μL of appropriately diluted enzyme extracts with 400 μL of 0.2% (w/v) CMC in 50 mM sodium acetate buffer (pH 4.8) at 45°C for 30 min (Yamanobe et al., 1987). β-Glucosidase activity was determined under the same conditions at 45°C for 30 min as described above, except for 10 mM salicin solution (D-(-)-salicin, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) as a substrate (Yamanobe et al., 1987). After the incubation, the amount of released reducing sugars were determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959; Tabka et al., 2006). One unit of each enzyme activity was defined as 1 μmol of

reducing sugar as glucose equivalent released per mL per min.

Identification of isolated cellulolytic fungus

A fungal strain exhibiting the highest cellulase activities among the isolated strains was selected and identified by cellular fatty acid composition analysis. Forty milligrams of cells were transferred from the surface of the isolation plate to a screw-capped glass tube sealed with a teflon-lined cap. Cellular lipids were saponified by heating for 30 min after vigorously vortexing in 1 mL of 15% (w/v) NaOH in 50% (v/v) methanol. After cooling down the mixture, fatty acids were methylated by adding of 2 mL of 3.25 M HCl in 45.8% (v/v) methanol, homogenizing, and heating at 80°C for 10 min. The resulting fatty acid methyl esters were gently mixed with 1.25 mL of hexane/methyl-tert-butyl ether (1:1, v/v) on a shaker for 10 min. After two phases were allowed to separate, the lower aqueous layer was removed, and 3 mL of diluted NaOH (10.8 g NaOH in 900 mL distilled water) was added. After separating the phases again, an aliquot of the supernatant was then transferred to a screw-capped sample vial (Agilent Technologies 5182-0714). The fatty acid methyl esters extracts were analyzed using an gas chromatograph with a flame ionization detector (Agilent Technologies 6890, Palto Alto, CA, USA) and an HP-1 column (30 m × 0.25 mm i.d., Agilent Technologies 19091S-633LTM). Identification of the fatty acid methyl ester profiles was handled by the Sherlock MIS Software (Miller, 1982; Yang et al., 1993; Huys et al., 1997).

Autoclaving pretreatment

Deproteinised and lipid-extracted rice bran was kindly provided by CJ CheilJedang Corp., Seoul, Korea. The deproteinised and lipid-extracted rice bran was stored in sealed plastic bags at room temperature until the pretreatment experiments. Dilute acid solution containing 0.1 N sulfuric acid, dilute alkali solution containing 0.1 N sodium hydroxide, and distilled water as control (uncatalyzed) were used to pretreat 3 g milled deproteinised and lipid-extracted rice bran at a solid loading of 10% (w/v). Autoclaving pretreatments were carried out at a desired reaction time (5, 15, 30, 45, and 60 min) using an autoclave at 121°C with 15 psi pressure. These ranges of pretreatment parameters were similar with those previously reported on starch-free triticale bran (Garcìa-Aparicio et al., 2011) and cotton stalks (Silverstein et al., 2007). After the pretreating reaction, the slurry was separated into the solid and liquid fractions by centrifugation at 10,000×*g* and 4°C for 15 min, and then the separated solid fraction was washed with distilled water until the pH of the wash liquid became approximately 7.0. Wet solid fraction was then lyophilized using a freeze-dryer (FDA 8512, Ilshin Lab Co., Ltd., Seoul, Korea). The lyophilized solid fraction was analyzed for structural composition and used as substrate for the enzymatic saccharification experiment.

Enzymatic saccharification

Enzymatic saccharification of the pretreated solid fraction was carried out at 10% solids concentration in 50 mM sodium acetate buffer (pH 4.8) with 1 mL of the crude enzyme extract (avicelase, 3.22 units/mL; CMCase, 6.73 units/mL; β-glucosidase 5.64 units/mL) of the isolated cellulolytic fungus. The reaction mixture was incubated at 45°C for 24 h with constant agitation at 150 rpm. The enzymatic saccharification was terminated by boiling the reaction mixture for 15 min, which was then centrifuged at 11,000×*g* for 15 min. The supernatant was analyzed for glucose content by high-performance anion exchange chromatography (HPAEC; Dionex, Sunnyvale, CA, USA) as described below. The glucose yield of enzymatic saccharification was expressed as the percentage of the released amount of glucose in supernatant after enzymatic saccharification from the amount of glucose left in the pretreated solids as 100% (kumar & Wyman, 2009).

Analytical assays

The contents of glucose, xylose, arabinose and lignin in the untreated material and the pretreated solid fraction were determined according to the National Renewable Energy Laboratory (NREL) procedure (Sluiter et al., 2008). The samples (160 mg) were treated with 72% (w/w) H_2SO_4 1.5 mL at 30°C for 1 h. The solutions were adjusted to 4% (w/w) H_2SO_4 with 42 mL of water and autoclaved at 121°C for 1 h. The hydrolysate was separated into the acid insoluble residue fraction and the filtrate fraction. The latter fraction was neutralized with barium hydroxide ($Ba(OH)_2 \cdot 8H_2O$), and then analyzed for sugar contents of the pretreated solid fraction with HPAEC . The acid insoluble residue fraction was dried overnight at 105°C and weighed. The sample was thereafter incinerated in the muffle furnace at 550°C for 24 h to determine the ash content. The acid insoluble lignin content was determined as the weight of the acid insoluble residue subtracted the ash content. The HPAEC system was equipped with CarboPac PA-1 anion exchange column (4 × 250 mm, Dionex 016158), an automated sampler, a gradient pump, and an electrochemical detector (ED 50, Dionex, Sunnyvale, CA, USA). The column temperature was fixed at 30°C. The mobile phase was 18 mM NaOH at a flow rate of 1 mL/min. All

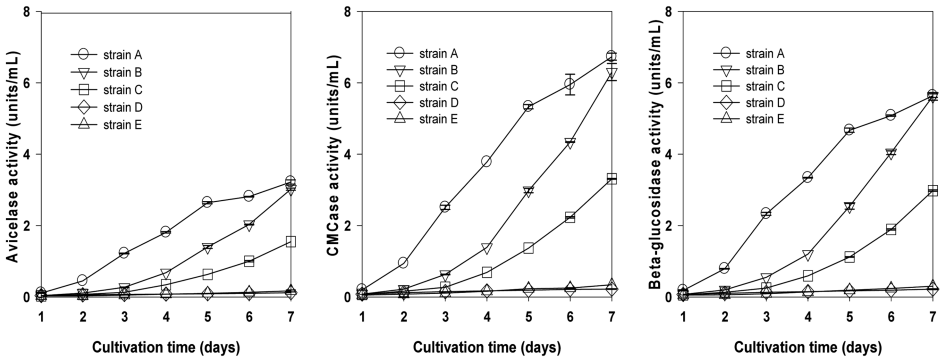

Fig. 2. Cellulase activities of isolated cellulolytic strains during the cultivation time. These five isolated strains were named strains A, B, C, D, and E after their sources. The data presented are means of two individual experiments.

samples were filtered through a 0.45-μm filter prior to HPAEC injection. All analytical determinations were performed in duplicate.

Results and Discussion

Identification of the isolated cellulolytic fungus

A total of five potential cellulolytic fungi of traditional Korean *nuruk* samples were isolated in the isolation plates and were named strain A, B, C, D, and E, respectively. These five isolated strains as cellulase producing fungi were re-evaluated in the conical flasks containing 200 mL of the selection medium at 30°C for 7 days. During the cultivation, cellulase activities of each isolated strain were measured by analyzing the released amount of sugar reducing end groups, and the results were shown in Fig. 2. Cellulase activities of the strain A were significantly highest among all the five isolated strains during the whole cultivation period. After 7-day cultivation, avicelase, CMCase, and β-glucosidase activities from the strain A reached the maximal points of 3.22, 6.73, and 5.64 units/mL, respectively, at pH 4.8 and 45°C. Interestingly, the strain B also showed high cellulase activities along with the strain A on day 7, even though the cellulase activity levels of the strain B were significantly lower than the strain A at the beginning of cultivation. Thus, strain A was selected for its strong cellulolytic enzyme activities for further enzymatic saccharification process. The newly isolated fungal strain A produced relatively higher β-glucosidase activity (5.64 U/mL) than those produced by other wild-type strains. *Penicillium purpurogenum* was isolated from a soil sample by Steiner et al. (1994) and 0.46 U/mL of β-glucosidase activity was obtained by using Mandel's medium with 1% (w/v) cellulose as carbon source. The *Penicillium* YS40-5 was isolated from a rice straw compost by Ng et al. (2010) and 2.44 U/mL of β-glucosidase activity was obtained when using cellobiose as carbon source. The composition of the cellular fatty acids of the strain A showed the identity (74%) with *Penicillium* sp. (unpublished data).

Effect of pretreatment conditions on deproteinised and lipid-extracted rice bran

Deproteinised and lipid-extracted rice bran employed in this study contained 44.3% (w/w) glucose, 7.2% (w/w) xylose, 4.3% (w/w) arabinose, and 8.2% (w/w) acid-insoluble lignin. The high contents of sugars, together with its low lignin content, make this feedstock a promising candidate for subsequent ethanol production (Garcìa-Aparicio et al., 2011). The structural composition of the different solid fractions obtained after pretreatment at different conditions are given in Table 1.

The contents of glucose in the solid fractions after acid-catalyzed autoclaving pretreatment ranged from 29.6 to 33.5%, as compared to 38.3 to 47.4% in those after alkali-catalyzed autoclaving pretreatment. The glucose contents in the solid fractions after uncatalyzed (distilled water) autoclaving pretreatment were obtained ranging from 28.6 to 45.7%. Acid-catalysis condition (0.1 N sulfuric acid), when combined with autoclaving pretreatments (121°C, 15 psi), showed a significant reduction in the glucose contentsof the pretreated solid fractions. For example, acid-catalyzed pretreatment for 15 min provided the lowest glucose (28.7%) content after pretreatment, indicating that the acid-catalyzed autoclaving pretreatment was more effective in the disruption of cellulose than other two pretreating conditions employed in this study. Grohmann et al. (1986) reported that acid pretreatment temperatures between 140°C and 160°C produced highly digestible cellulose with aspen and straw

biomasses.

Unlike alkali-catalyzed autoclaving pretreatment, the acid-catalyzed autoclaving pretreatment showed a significant increase in the lignin contents of the pretreated solid fractions. The lignin contents in the solid fractions after pretreatment is affected by solubilization of glucose leading to a concentration of lignin in the pretreated solid fraction (Petersen et al., 2007) and insoluble degradation products from hemicellulose would be measured as klason lignin if these were not dissolved by the acidic hydrolysis (Thomsen et al, 2008). The increase in klason lignin content may be linked to other lignin-related reactions, turning into insoluble substances at high temperature as suggested by Kim & Lee (2006). The lignin contents were independent of the pretreating time though.

The lowest hemicellulose sugars and lignin content was found in the solid fraction from alkali-catalyzed autoclaving pretreatment, and the glucose content of the solid fraction after alkali-catalyzed autoclaving pretreatment increased as a result of reduction of hemicellulose sugars and lignin (Öhgren et al., 2007). Another potential reason for the higher glucose content from alkali-catalyzed autoclaving pretreatment could be contributed to the gelatinization of substrate during sodium hydroxide pretreatment that might impede releasing solubilized glucose from the solid fraction during rinsing off step (Park et al., 2010).

The contents of xylose and arabinose as major sugars derived from the hemicellulose decreased in the solid fractions by exposing rice bran along with pretreating time of all the conditions, except for xylose in the acid-catalysis condition. The contents of xylose and arabinose were greatest up to 11.8 and 9.2% after acid-catalyzed autoclaving pretreatment, whereas alkali-catalyzed autoclaving pretreatment significantly reduced the contents of xylose and arabinose to 6.4 and 6.2%, respectively. The treated solid fraction after alkali-catalyzed autoclaving pretreatment contained the low level of lignin contents ranging from 8.3-11.9%. This observation indicates that the hemicellulose and lignin fractions were solubilized most effectively with alkali-catalyzed autoclaving pretreatment condition.

The control, uncatalyzed autoclaving pretreatment resulted in lower lignin and hemicellulose sugars contents than expected. This was probably possible as a result of autohydrolysis at high pretreatment temperature (Öhgren et al., 2007). However, results from the present study suggest that the pretreatment condition for maximizing individual sugar yield would not often be the same as that for achieving the greatest yield of total

Table 1. Effect of different pretreatment conditions on composition and enzymatic saccarification of solid fractions after pretreatment (The data presented are means of two individual experiments).

Autoclaving pretreatment conditions		Compositional analysis of pretreated solid fraction (%, w/w)[1)]				Saccharification by the crude fungal extract
Reaction media	Reaction time (min)	Glucose	Xylose	Arabinose	Acid-insoluble lignin	Enzymatic saccharification (%, w/w)[2)]
Raw material	0	44.3±0.02	7.2±0.00	4.3±0.01	8.2±0.79	14.9±0.00
Acid-catalysis (0.1 N Sulfuric acid solution)	5	32.4±0.34	10.4±0.03	9.2±0.10	18.5±1.41	34.3±0.14
	15	28.7±0.00	10.9±0.00	8.3±0.00	18.6±0.06	67.6±1.08
	30	29.2±0.03	11.6±0.05	8.2±0.06	18.4±0.37	75.2±0.00
	45	30.6±0.42	11.5±0.06	7.6±0.04	18.3±1.03	58.1±0.45
	60	30.8±0.17	11.8±0.03	6.3±0.02	19.7±1.25	63.9±0.15
Alkali-catalysis (0.1 N Sodium hydroxide solution)	5	37.1±0.23	6.4±0.02	6.2±0.08	11.9±0.25	9.6±0.32
	15	39.7±0.74	5.2±0.00	5.0±0.07	8.3±0.12	14.6±0.15
	30	42.9±0.44	5.8±0.01	5.2±0.01	9.8±1.66	27.0±0.22
	45	45.9±0.44	6.1±0.04	5.5±0.07	9.9±0.16	15.8±1.38
	60	45.3±0.17	5.0±0.04	4.5±0.00	8.6±0.16	16.6±0.33
Control (Distilled water)	5	27.7±0.38	7.4±0.01	7.2±0.05	12.6±0.41	46.0±0.45
	15	35.4±0.07	7.4±0.07	6.4±0.05	10.4±1.75	43.0±1.90
	30	39.1±0.68	6.9±0.12	6.0±0.04	10.4±0.19	41.7±0.13
	45	44.2±0.05	5.2±0.06	4.8±0.03	8.1±0.50	44.1±0.30
	60	42.0±0.61	5.3±0.25	5.4±0.12	8.2±0.72	47.6±0.54

[1)] Contents of sugar and acid-insoluble lignin of raw material and pretreated solid fraction were expressed as the percentage (mg/100 mg of dry substrate).

[2)] These percentages (%) are based on the amount of glucose left in the solids after pretreatment (mg of glucose released in the supernatants after enzymatic saccharification/100 mg of glucose left in the solids after pretreatment).

sugar (Öhgren et al., 2005; Lloyd & Wyman, 2005).

Effect of pretreatment conditions on enzymatic saccharification

Enzymatic saccharification experiment was carried out on the solid fraction after pretreatment to investigate the effect of the different pretreatment conditions on the enzymatic saccharifying capability of newly isolated cellulolytic fungus. The effect of pretreatment conditions was evaluated on the basis of enzymatic saccharification yield in terms of the cellulose susceptibility of the pretreated solid fraction to enzymatic saccharification. The enzymatic saccharification of the different solid fractions obtained after pretreatment at different conditions was conducted by the crude enzyme extract of the newly identified *Penicillium* sp. strain. Glucose was mainly released during the enzymatic saccharification. Results of glucose yield from enzymatic saccharification are given in Table 1. The solid fractions obtained from acid-catalyzed autoclaving pretreatment resulted in the greatest glucose yield ranging from 34.3 to 75.2%, followed by uncatalyzed autoclaving pretreatment ranging from 41.7 to 47.6% and then alkali-catalyzed autoclaving pretreatment ranging from 9.6 to 27.0%. Based on the experiment data, it seemed that acid-catalyzed autoclaving pretreatment is more effective for enzymatic saccharification than other pretreatment conditions on rice bran employed in this study. Although producing high glucose yields, uncatalyzed autoclaving pretreatment as mild pretreatment condition, never equaled the yields after acid-catalyzed autoclaving pretreatment. The greatest glucose yield of the enzymatic saccharification increased up to 75.2% by acid-catalyzed autoclaving pretreatment for 30 min, while only 14.9% of the glucose yield was obtained from unpretreated deproteinised and lipid-extracted rice bran. Saha et al. (2005) showed that 60% highest yield of enzymatic saccharification from rice hulls on the basis of total carbohydrate content using dilute acid pretreatment (121^{o}C, 1 h). In addition, our results showed that the pretreated rice bran samples for longer more than 30 min resulted in lower glucose yield from the enzymatic saccharifica-tion, except for uncatalyzed autoclaving pretreatment .

For the alkali-catalyzed samples for 5 and 15 min, less amount of glucose was detected after the enzymatic saccharification than unpretreated sample. Results from the present study suggest that pretreatment of deproteinised and lipid-extracted rice bran by sodium hydroxide solution at 0.1 N was ineffective in enhancing enzymatic saccharification. During alkali catalyzed pretreatment process, absorption of some of the alkali into the biomass reduces the effect of alkali pretreatment (Balat et al., 2008; Silverstein et al., 2007) and causes hindering enzyme access to cellulose (Öhgren et al., 2007).

Therefore, the results confirm that acid-catalyzed autoclaving pretreatment made the main treatment effect on cellulose disruption in the pretreated solids, which can contribute to the higher susceptibility of the cellulosic substrate to cellulase enzyme, whereas alkali-catalyzed autoclaving pretreatment has a higher glucose content in pretreated solids after pretreatment. The solid fraction obtained from acid-catalyzed autoclaving pretreatment was rich in lignin (18.3-19.7%), but high lignin content did not seem to inhibit the cellulase activity (Thomsen et al., 2008). Increased cellulosic structure disintegration as a result of acid-catalyzed autoclaving pretreatment therefore improved the saccharifying ability of cellulase enzyme secreted by the newly isolated strain rather than hemicellulose and lignin removal effects as a result of alkali-catalyzed autoclaving pretreatment.

Conclusions

The present study is the first report on the newly isolated fungal strain from traditional Korean *nuruk* samples. The newly isolated cellulolytic fungus was identified as *Penicillium* sp. determined by cellular fatty acid composition analysis. Results from the present study showed that acid-catalyzed autoclaving pretreatment was effective in the disruption of cellulose of deproteinised and lipid-extracted rice bran. The optimum saccharification yield (75.2%) of the deproteinised and lipid-extracted rice bran by the newly isolated fungal strain was also obtained from acid-catalyzed autoclaving pretreatment. We clearly show increased cellulose disruption effect which improved the saccharifying ability of the newly isolated fungal strain than the other two pretreating conditions employed in this study.

Acknowledgements

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government [NRF-2009-351-F00025]. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (K21002001986-11B1200-10110)

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