Production of Endoglucanase, Beta-glucosidase and Xylanase by *Bacillus licheniformis* Grown on Minimal Nutrient Medium Containing Agriculture Residues

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ABSTRACT: *Bacillus licheniformis* was grown in minimal nutrient medium containing 1% (w/v) of distillers dried grain with soluble (DDGS), palm kernel meal (PKM), wheat bran (WB) or copra meal (CM), and the enzyme activity of endoglucanase, β-glucosidase, xylanase and reducing sugars was measured to investigate a possibility of using cost-effective agricultural residues in producing cellulolytic and hemicellulolytic enzymes. The CM gave the highest endoglucanase activity of 0.68 units/mL among added substrates at 48 h. CM yielded the highest titres of 0.58 units/ml of β-glucosidase, compared to 0.33, 0.23, and 0.16 units/mL by PKM, WB, and DDGS, respectively, at 72 h. Xylanase production was the highest (0.34 units/mL) when CM was added. The supernatant from fermentation of CM had the highest reducing sugars than other additional substrates at all intervals (0.10, 0.12, 0.10, and 0.11 mg/mL respectively). It is concluded that *Bacillus licheniformis* is capable of producing multiple cellulo- and hemicellololytic enzymes for bioethanol production using cost-effective agricultural residues, especially CM, as a sole nutrient source. (**Key Words:** Agricultural Residues, *Bacillus licheniformis*, β-Glucosidase, Endoglucanase, Xylanase)

INTRODUCTION

Bioethanol production using lignocellulosic materials replacing high starch grains has been of interest in many applied sciences because grains are also the main energy source in livestock feed and thus competition may increase the cost of substrates which can be a potential barrier for efficient bioethanol production (Hsu et al., 2011). Bioethanol production from cellulosic biomass is achieved by stepwise processes which consist of pretreatment of biomass, hydrolyzation of biomass to fermentable sugar, and ethanol production from reducing sugar (Kumar et al., 2008).

The acid or enzyme treatment is conventionally used for hydrolysis of cellulosic biomass (Sukumaran et al., 2009;

Hamzah et al., 2011). Although acid hydrolysis is more common process, it produces dangerous acid wastes and has technical difficulties in recovering sugars from the acid (Sukumaran et al., 2009). Compared to acid hydrolysis, enzymatic hydrolysis has several advantages including mild reaction condition and high production of pure sugar without any toxic wastes production (Hamzah et al., 2011). However, high cost and low yield efficiency of the enzyme production by microbial fermentation are significant barriers for industrial application of the enzyme method to produce fermentable sugars from lignocellulosic biomass (Maki et al., 2009). In addition, both cellulase and hemicellulase and their synergistic action are needed to hydrolyze lignocellulosic biomass efficiently since both cellulose and hemicellulose are abundant and cross-linked in the plant structure.

It is therefore beneficial in terms of reducing production cost if a microorganism releases cellulase and xylanase simultaneously and it can effectively use cheap medium nutrients such as agricultural residues for microbial enzyme production. Previously, a facultative bacterium, *Bacillus*

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licheniformis which can simultaneously release endoglucanase, β-glucosidase and xylanase, was isolated from Korea native goat's rumen (Seo et al., 2013). The objective of this study was thus to investigate production profiles of endoglucanase, β-glucosidase, xylanase, and reducing sugars grown on mineral minimum nutrient media containing different agricultural residues.

MATERIALS AND METHODS

Strain of microorganism

The cellulase and xylanase producing bacteria was isolated from Korea native goat's rumen and identified as *Bacillus licheniformis* based on its biochemical, morphological characteristics as well as 16s rDNA sequences (Seo et al., 2013).

Agricultural residues

Agricultural residues tested in this study as substrates for growing *Bacillus licheniformis* for enzyme production were distillers dried grain with soluble (DDGS), wheat bran (WB), palm kernel meal (PKM), and copra meal (CM). These residues were purchased from Cargill co. Ltd., dried at 65°C for 48 h in a dry oven, and grounded to pass through a 1 mm screen using stainless-steel grinder. Ground substrates were analyzed to determine dry matter, crude protein, ether extract (EE), fiber, and ash contents as described by AOAC (1990). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed using Ankom 200 fiber analyzer. The chemical compositions of four agricultural residues tested in this study were listed in Table 1.

Cultivation of the bacterial strain for enzyme production

Bacillus licheniformis was grown in 500 mL Erlenmeyer flasks containing 100 mL mineral salts media (g/L, 2.5 KH_2PO_4, 2.5 K_2HPO_4, 0.1 NaCl, 0.2 $MgSO_4 \cdot 7H_2O$, 0.01 $FeSO_4 \cdot 7H_2O$, 0.007 $MnSO_4 \cdot 7H_2O$, 0.05 $CaCl_2 \cdot 2H_2O$, 1 $[NH_4]_2SO_4$) and 1% (w/v) of each agricultural residue. The culture flasks were incubated at

37°C for 4 days shake at 125 rpm. Crude enzyme extract was obtained by centrifugation of a sample at 13,000 g×10 min at 4°C, and clear supernatants were used for enzyme assay. All the incubations were performed in triplicate.

Enzyme assays

The amount of reducing sugars and the enzyme activities of endoglucanase, β-glucosidase and xylanase were measured by spectrometric determination of reducing sugars by 3, 5-dinitrosalicylic acid (DNS) method as described by Ghose (1987). Endoglucanase and β-glucosidase activities were determined using carboxy-methyl cellulose and salicin (2-[hydroxymethyl]-phenyl-β-D-glucopyranoside) as substrates, respectively. Xylanase activity was determined by measuring the release of xylose from birch wood xylan. The amount of released reducing sugar was determined based on a standard curve which was constructed using the standard solutions of glucose and xylose. One unit of enzymatic activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute. All the assays were performed in triplicate.

Statistical analysis

Enzyme and reducing sugar production data from the study were analyzed with PROC general linear model of SAS (SAS Institute Inc., Carey, NC, USA) with a two factor analysis of variance test. The linear model for this is as follows:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

y_{ijk} is the kth observation of the incubation of ith feed sample with j^{th} incubation time, μ is the overall mean, α_i is the fixed effect of different feed samples, β_j is the fixed effect of different time of incubation, $(\alpha\beta)_{ij}$ is the interaction between feed sample and different incubation time, and ε_{ijk} is unexplained random effect. Pair-wise comparisons of the least square means were conducted within feed samples or incubation time using the least significance difference test with Tukey-Kramer adjustment. Significance was declared at p<0.05.

Table 1. Chemical composition of the agricultural residues tested in this study

Component (%, dry matter basis)	DDGS	PKM	WB	CM
Dry matter (%, as fed)	89.54	93.03	87.17	89.22
Crude protein	30.19	17.64	17.59	24.59
Ether extract	11.26	10.43	4.91	2.53
Crude ash	4.36	4.71	5.52	7.3
Neutral detergent fiber	30.1	61.78	39.45	57.64
Acid detergent fiber	8.77	35.87	12.62	29.52
Hemicellulose[1]	21.63	25.91	26.83	28.12

DDGS, distillers dried grain with soluble; PKM, palm kernel meal; WB, wheat bran; CM, copra meal.

[1] Hemicellulose = neutral detergent fiber – acid detergent fiber.

RESULTS AND DISCUSSION

The use of cheap agricultural cellulosic residues as substrates for cultivating microorganism would decrease the cost for enzyme production. Various kinds of lingocellulosic materials such as sugarcane bagasse (Song and Wei, 2010), corn cob residues (Hsu et al., 2011), rice straw (Sukumaran et al., 2009) and WB (Chandra et al., 2007) have been tested for their use in cellulolytic enzyme production. In this study, DDGS, PKM, and WB as well as CM were tested for the possibility of their use as substrates for microbial growth as well as enzyme production.

The DDGS is the soluble and solid residues by-produced in ethanol fermentation process using grain sources (Liu, 2011), and has been used as an animal feed ingredient (Wu et al., 2011). There has been an interest in the use of DDGS for industrial enzyme production since it is inexpensive relative to high protein and carbohydrate contents. Ximenes et al. (2007) suggested that DDGS could become a suitable source for cellulolytic and hemicellulolytic enzymes production due to its carbohydrate profile including starch, cellulose and xylan. The PKM is a by-product produced after extracting oil from palm kernels. The PKM has been mainly used for a feed ingredient in a ruminant diet, but its purpose is limited due to high fiber content, low nutritive value and poor amino acid composition (Singhania et al., 2008). The WB is a major by-product of the wheat processing industry and commonly available. The WB has attracted attention as a substrate for cellulase and xylanase production (Chandra et al., 2007) because it contains various nutrients and provides large surfaces to cellulolytic microorganisms thereby producing enzymes quickly and effectively (Archana and Satyanarayana, 1997). The CM is a co-product generated by expeller extraction or solvent extraction of dried coconut kernels which is widely distributed around the tropical area including Philippines, India, Indonesia and South America (Singhania et al., 2008). The CM contains a relatively large amount of protein and degradable fiber (Creswell and Brooks, 1971), which indicates its possible use as a nutritious substrate for microbial growth to produce valuable enzymes.

Cultivation of *Bacillus licheniformis* in mineral minimal nutrient media containing 1% (w/v) of four different agricultural residues produced active endoglucanase (Figure 1[A]), β-glucosidase (Figure 1[B]), xylanase (Figure 1[C])
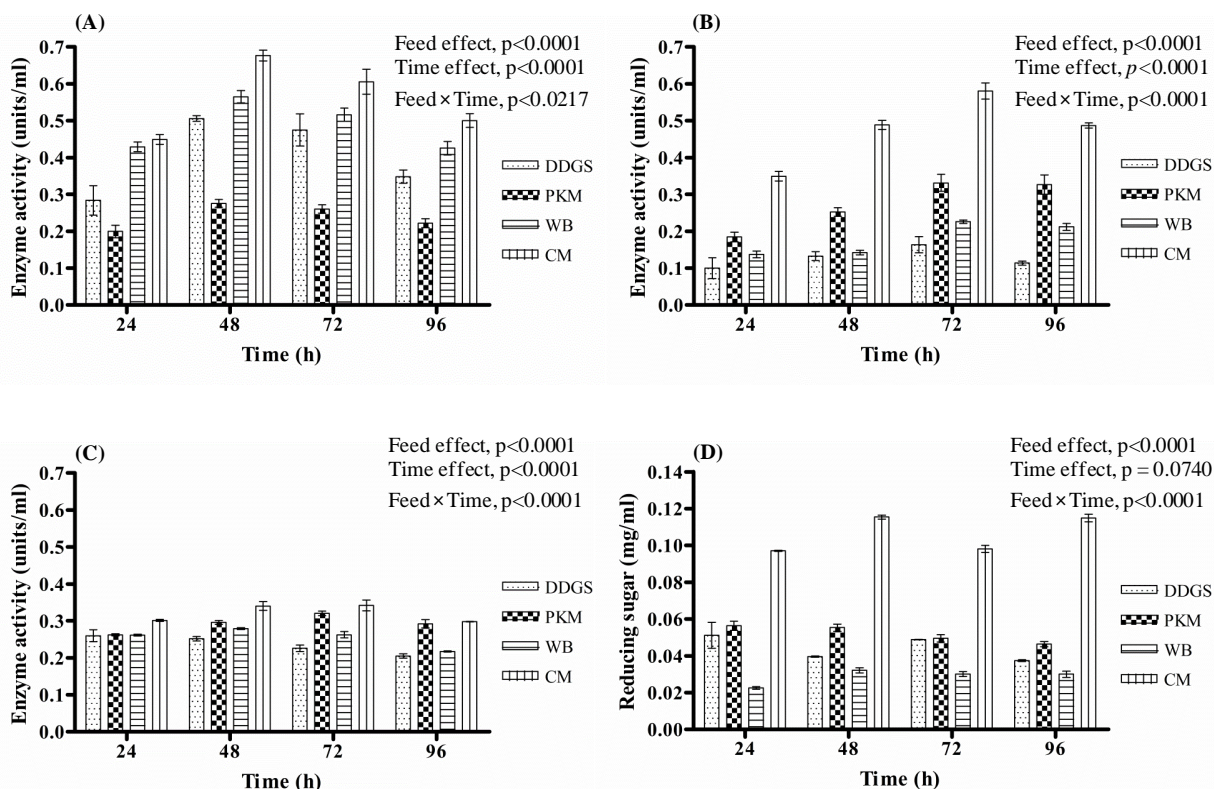
Figure 1. Production of endoglucanase (a), β-glucosidase (b), xylanase (c) (units/mL) and reducing sugars (d) (mg/mL) of *Bacillus licheniformis* grown in selected agricultural residues (DDGS, distillers dried grain with soluble; PKM, palm kernel meal; WB, wheat bran; CM, copra meal) and mineral minimum nutrient medium. The cultures were centrifuged at 13,000 g×10 min at 4℃ and the clear supernatants containing extracellular proteins were used for enzyme assay. Enzyme activity was determined at different time intervals. All assays were performed in triplicate. The data point and error bar indicate the average values and standard error.

and reducing sugar (Figure 1[D]) on the course of four-day incubation.

The highest endoglucanase activity was observed at 48 h of incubation in all substrates. The enzyme extract from submerged fermentation on CM gave the highest endoglucanase activity of 0.68 units/mL, while WB, DDGS, PKM showed 0.56, 0.51, 0.28 units/mL at 48 h, respectively (Figure 1[A]). Compared to the other substrates, CM showed the highest endoglucanase activity at all of the time points (0.45, 0.68, 0.61, 0.5 units/mL respectively) and PKM gave the lowest activity (0.20, 0.28, 0.26, 0.22 units/mL respectively). The results thus suggested that CM, followed by WB, is the most suitable substrate among the tested agricultural residues for the endoglucanase production by *Bacillus licheniformis*.

The maximum β-glucosidase activity was observed at 72 h of incubation in all substrates (Figure 1[B]). The CM yielded the highest titres of 0.58 units/mL of β-glucosidase in submerged fermentation compared to 0.33, 0.23, 0.16 units/mL in PKM, WB, and DDGS at 72 h, respectively. In this study, production of endoglucanase was the highest at 48 h. The role of endoglucanase is to cleave the cellulolytic polymers randomly to produce new chain ends including cellobiose (Kumar et al., 2008). As cellobiose is a substrate for β-glucosidase (Kumar et al., 2008), an increase in the concentration of cellobiose seemed to simulate *Bacillus licheniformis* to produce β-glucosidase, and this may explain the reason for the observed maximum β-glucosidase activity at 72 h of incubation. Similar to endoglucanase, CM gave the highest β-glucosidase activity at all incubation time points (0.35, 0.49, 0.58, 0.49 units/mL, respectively), and DDGS gave the lowest activity (0.1, 0.13, 0.16, 0.11 units/mL respectively). β-Glucosidase activity declined at 96 h in all enzyme extracts.

The CM yielded the highest titres of xylanase activity throughout the enzymatic hydrolysis (Figure 1[C]). The PKM and CM showed the highest enzyme activity at 72 h (0.32 and 0.34 units/mL respectively) while the highest activities in DDGS (0.26 units/mL) and WB (0.28 units/mL) were observed at 24 h and 48 h, respectively. The differences in the xylanase activity among the enzyme extracts were relatively small, which might be due to similar amount of hemicellulose, calculated by NDF-ADF, content in the agricultural residues tested in this study (Table 1).

The supernatant from fermentation of CM had the highest reducing sugars than other additional substrates at all intervals (0.10, 0.12, 0.10, and 0.11 mg/mL, respectively) (Figure 1[D]), which was expected since production of endoglucanase, β-glucosidase and xylanase were the highest in CM. Hamzah et al. (2011) reported that combination of cellulase and β-glucosidase (Novozyme 188, Sigma-Aldrich, St-Luis, MO, USA) increased the production of soluble glucose in the reaction. Hence, the action of β-glucosidase is needed to avoid enzyme inhibition since accumulation of cellobiose during fermentation can inhibit the enzymatic action of endoglucanase (Hamzah et al., 2011).

Up to date, fungal species have been regarded as the most important microorganism for producing cellulase and hemicellulase due to its ability such as extra cellular secretion and sufficient productivity (Maki et al., 2009). However, bacteria have also been attracted in the last few decades because of their several advantages such as higher growth rate than fungi, multi enzyme production, and strong resistance of bacterial enzymes to harsh environments (Maki et al., 2009). All glycosyl hydrolases from *Bacillus licheniformis* were found to be stable in acidic condition and showed optimal activities at high temperature in previous study (Seo et al., 2013). The present study showed *Bacillus licheniformis* was able to secrete diverse enzymes simultaneously even though they were grown on minimal nutrient medium containing cheap agricultural residues.

CONCLUSIONS

During submerged fermentation, *Bacillus licheniformis* simultaneously produced endoglucanase, β-glucosidase and xylanase in the minimal nutrient medium containing selected agricultural residues. When CM was used only nutrient substrate, the activity of all three enzymes and the amount of reducing sugar generated were maximized suggesting that CM is an ideal substrate for cellulolytic and hemicellulolytic enzyme production from culturing *Bacillus licheniformis*, which may be helpful in decreasing the production cost for fermentable sugar formation from lignocellulosic materials. Further study on solid state fermentation of *Bacillus licheniformis* using agricultural residues is warranted to apply the technology into large-scale enzyme production.

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REFERENCES

AOAC. 1990. Official Methods of Analysis. 15th edn. Association of Official Analytical Chemists, Arlington, VA, USA.

Archana, A. and T. Satyanarayana. 1997. Xylanase production by thermophilic *Bacillus licheniformis* A99 in solid-state fermentation. Enzyme Microb. Technol. 21:12-17.

Chandra, M. S., B. Viswanath, and B. R. Reddy. 2007. Cellulolytic enzymes on lignocellulosic substrates in solid state fermentation by *Aspergillus niger*. Indian J. Microbiol. 47: 323-328.

Creswell, D. C. and C. C. Brooks. 1971. Composition, apparent digestibility and energy evaluation of coconut oil and coconut meal. J. Anim. Sci. 33:366-369.

Ghose, T. K. 1987. Measurement of cellulase activities. Pure Appl. Chem. 59:257-268.

Hamzah, F., A. Idris, and T. K. Shuan. 2011. Preliminary study on enzymatic hydrolysis of treated oil palm (*Elaeis*) empty fruit bunches fibre (EFB) by using combination of cellulase and β 1-4 glucosidase. Biomass Bioenergy 35:1055-1059.

Hsu, C. L., K. S. Chang, M. Z. Lai, T. C. Chang, Y. H. Chang, and H. D. Jang. 2011. Pretreatment and hydrolysis of cellulosic agricultural wastes with a cellulase-producing *Streptomyces* for bioethanol production. Biomass Bioenergy 35:1878-1884.

Kumar, R., S. Singh, and O. V. Singh. 2008. Bioconversion of lignocellulosic biomass: Biochemical and molecular perspectives. J. Ind. Microbiol. Biotechnol. 35:377-391.

Liu, K. 2011. Chemical composition of distillers grains, A review. J. Agric. Food Chem. 59:1508-1526.

Maki, M., K. T. Leung, and W. Qin. 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. Int. J. Biol. Sci. 5:500-516.

Seo, J. K., T. S. Park, I. H. Kwon, M. Y. Piao, C. H. Lee, and J. K. Ha. 2013. Characterization of cellulolytic and xylanolytic enzymes of *Bacillus licheniformis* JK7 isolated from the rumen of a native Korean goat. Asian Australas. J. Anim. Sci. 26:50-58.

Singhania, R. R., C. R. Soccol, and A. Pandey. 2008. Application of tropical agro-industrial residues as substrate for solid-state fermentation processes. In: Current Developments in Solid-state Fermentation (Ed. A. Pandey, C. R. Soccol, and C. Larroche). Springer, New York, USA. pp. 412-442.

Song, J. M. and D. Z. Wei. 2010. Production and characterization of cellulases and xylanases of *Cellulosimicrobium cellulans* grown in pretreated and extracted bagasse and minimal nutrient medium M9. Biomass Bioenergy 34:1930-1934.

Sukumaran, R. K., R. R. Singhania, G. M. Mathew, and A. Pandey. 2009. Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. Renew. Energy 34:421-424.

Wu, R. B., K. Munns, J. Q. Li, S. J. John, K. Wierenga, R. Sharma, and T. A. McAllister. 2011. Effect of triticale dried distillers grains with solubles on ruminal bacterial populations as revealed by real time polymerase chain reaction. Asian Australas. J. Anim. Sci. 24:1552-1559.

Ximenes, E. A., B. S. Dien, M. R. Ladisch, N. Mosier, M. A. Cotta, and X. L. Li. 2007. Enzyme production by industrially relevant fungi cultured on coproduct from corn dry grind ethanol plants. Appl. Biochem. Biotechnol. 136-140:171-183.