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Production of Cellulolytic Enzyme from Paper Sludge by *Geobacillus kaustophilus* C1 and Its Application for Glucose Production from Rice Straw

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***Abstract*—The objective of this study was to produce cellulolytic enzyme from paper sludge (PS), a solid waste from pulp and paper manufacturer by *Geobacillus kaustophilus* strain C1. When PS was used as sole carbon source, cellulolytic enzyme, mainly β-glucosidase was produced by the strain C1 which exhibited good properties, high temperature and high glucose concentration tolerance. Cellulolytic enzyme from strain C1 was afterward applied to hydrolyze ammonia-treated rice straw (ARS) for glucose production. Glucose was produced as a major hydrolysis product. Degree of saccharification of cellulose in ARS to glucose was improved when crude enzyme of strain C1 was combined with commercial *Trichoderma reesei* cellulase (Celluclast 1.5 L) and revealed 78.1% glucan saccharification. Glucose from ARS can be subsequently used as raw material for production of value-added products in many industries.**

***Keywords*—*Geobacillus kaustophilus*, Glucose, β-glucosidase, Paper sludge, Rice straw**

I. Introduction

Lignocellulose, consisting of the three major polymers that are cellulose, hemicellulose and lignin, is expected to utilize as an abundant renewable resource. Cellulose is the main component of plant cell wall and is being cheap and easily available can replace food crops for production of glucose which can be subsequently used as raw material for value-added products production such as ethanol, organic acids and biogas. Cellulose in biomass was hydrolyzed to glucose by two processes, acid or enzymatic hydrolysis process. However, enzymatic hydrolysis is the excellent process for biomass hydrolysis because of cellulolytic enzyme specifically hydrolyzed cellulose to glucose, green technology and gives maintenance costs [1]. The complete enzymatic degradation of cellulosic substrates depends upon the production of a complex enzyme system consisting of endo-glucanase (EG) (EC 3.2.1.4), exo-glucanases (CBHs) (EC 3.2.1.91 and EC 3.2.1.176) and β-glucosidase (BGL) (EC 3.2.1.21) that are involved in the conversion of cellulose to glucose. EGs act randomly along the cellulose chains to produce cellulose fragments. CBHs act as exoglucanase to release cellobiose, and BGLs hydrolyze cellobiose to yield glucose [2]. BGL is the key enzyme in cellulose hydrolysis process, complete the final step by converting cellobiose to glucose. BGL not only produces glucose but also reduces the product inhibition exerted by cellobiose, allowing efficient functioning of the other cellulolytic enzymes. One of the major challenges in the conversion of biomass into glucose is finding good properties of BGL that are high BGL activity, high temperature and glucose concentration tolerance for efficient enzymatic saccharification of biomass [3, 4, 5].

Paper sludge is a solid waste generating from pulp and paper manufacturer and physical characteristics of PS exhibit small-short fiber and well-dispersed [6]. Consequently, PS cannot reuse as the material for paper production. PS is complex structure material that contains of cellulose (major component), hemicelluloses, some amount of lignin and ash. Thereby, it is suitable as carbon source for cellulolytic enzyme production without pretreatment process because of most of lignin and hemicelluloses have already removed during the pulping process. Moreover, potential for using PS as a carbon source to produce cellulolytic enzyme by filamentous fungi have been reported [7, 8].

Rice straw (RS) is a lignocellulosic waste derived from rice grain collection. RS are generated annually more than 37.8 million tons in Thailand. Farmers usually remove RS by fire before the next cultivation season because of its low value, including its low protein content, high mineral content, high bulk density, and slow degradation in soil, [9, 10]. The burning of RS generated air pollution and negatively impacts human health. To increase the value of RS and protect the environment, RS can be used as a feedstock for glucose production because straw is a non-food and abundant resource, especially in Thailand and other Asian countries. According to its chemical composition, up to 70% of the dry biomass consists of polysaccharides, which represent a rich source of glucose and other fermentable sugars [11].

In the present study, we isolated and identified a potent BGL-producing bacterium, *Geobacillus kaustophilus* strain C1 from composing wood waste soil sample in Northern Taiwan. When grown on PS as sole carbon source, the strain C1 produced high BGL activity and exhibited good thermostability and glucose tolerance. We also studied the enzymatic saccharification of lignocellulosic substrate, RS by crude enzyme from strain C1 for glucose production.

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II. Material and Methods

A. Medium Culture

The bacterial strain C1 isolated from composting wood waste soil samples in Northern Taiwan. The strain C1 was cultivated in a mineral salt (MS) medium that was used as the selective medium for *Bacillus* spp. [12]. MS medium contained the following dissolved in 1 L of water: 2 g of $NaNO_3$, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.02 g of $MnSO_4.7H_2O$, 0.02 g of $FeSO_4.7H_2O$ and 0.02 g of $CaCl_2.H_2O$. This medium was supplemented with 0.5% PS as a carbon source and subsequently sterilized (20 min, 121°C).

B. Production of Cellulolytic Enzyme

Cellulolytic enzyme production of strain C1 was achieved in MS medium supplemented with 0.5% (w/v) PS at 60°C, pH 6.0 for 3 days. After bacterium grown in PS medium at above condition, the culture supernatant was centrifuged at 8,000g for 10 min at 4°C and then was concentrated by ultrafiltration (10 kDa cut-off membrane). The crude enzyme was taken to assayed enzyme activities.

C. Enzyme and Protein Assays

The EG activity assay was performed in 1% (v/w) final concentration of carboxymethyl cellulose at 60 °C in 50 mM acetate buffer (pH 5.0) for 10 min. EG activity was measured by the amount of reducing sugars liberated from carboxymethyl cellulose. Reducing sugars were determined using the Somogyi-Nelson method [13]. One unit of the enzyme activity was defined as the amount of enzyme to release 1 µmole of reducing sugar in 1 min under the above condition. The CBH and BGL activities were performed at 60 °C in 50 mM phosphate buffer (pH 6.0) for 30 min and were based on measurement of the release of *p*–nitrophenol from *p*-nitrophenyl-D-cellobioside (pNPC) and *p*-nitrophenyl-β-D-glucoside (pNPG), respectively [14]. One unit of enzyme releases 1 µmole equivalent of *p*-nitrophenol per min. Optimum and stability of temperature and pH were determined for the crude enzyme using pNPG as the substrate. Optimum temperature was determined at temperatures from 35 to 70 °C at pH 6.0. Optimum pH range profile was determined using different pH conditions (sodium acetate buffer (pH 4.0–6.0), sodium phosphate buffer (pH 7.0) or Tris–HCl buffer (pH 8.0)) at 60 °C. For thermostability assessment, crude enzyme was incubated in 0.1 M sodium acetate buffer (pH 6.0) at 60 °C for 12 h. Glucose inhibition of BGL activity was measured by adding glucose at different concentrations (0–1 M) to the standard reaction mixture with pNPG as the substrate. The concentration of glucose required to inhibit 50% of the initial BGL activity under the assay condition was determined. Glucose inhibition of BGL activity was measured by adding glucose at different concentrations (0–400 mM) to the standard reaction mixture with *p*-nitrophenyl-β-D-glucoside as the substrate under the assay condition. Protein concentration was determined by the Lowry method with bovine serum albumin as the standard.

D. Pretreatment of Rice Straw by Aqueous Ammonia Soaking

RS was obtained from rice fields in the Ayutthaya Province of Thailand. The RS was air-dried and cut into small pieces using scissors prior to pretreatment. The RS was pretreated with aqueous ammonia, as described previously by Phitsuwan [15]. The pretreatment was carried out by soaking RS in 27% (w/w) of ammonium hydroxide at a solid:liquid ratio of 1:12 at 25°C for 14 days. After the pretreatment, the sample was filtrated to remove the liquid phase and the retrieved solid was washed with water. The biomass suspension was neutralised with 1 N HCl/NaOH and washed with water. The pretreated solid was dried at 60 °C and milled a small particle size (0.5 cm). This material was then used as a substrate for glucose production.

E. Compositional Analysis

The chemical composition of ARS was analyzed following NREL Chemical Analysis and Testing Standard Procedure [16]. The sample was subjected to 72% sulfuric acid hydrolysis at 30 °C for 60 min, followed by 3% sulfuric acid hydrolysis at 121 °C for 60 min. The autoclaved hydrolysis solution was neutralized to pH 6.0 with calcium carbonate and vacuum filtered through a filtering crucible. This filtrate was then analysed using quantified by HPLC to determine the carbohydrate contents.

F. Glucose Production from Rice Straw Pretreated

Enzymatic digestibility experiments were performed at 60° C in 1.5 mL Eppendrof tube containing 5% (w/v) ARS in the presence of 50 mM acetate buffer (pH 6.0) with 1 mL total working volume for 72 h. For glucose production optimization, ARS was hydrolyzed with 20 (U/g RST) of *T. reesei* cellulase (Celluclast 1.5 L) and was supplied with 0, 20, 40 and 60 of BGL activity per g RST of strain C1 (ratio 1:1, 1:2, 1:3). At the appropriate time, the hydrolysis products were taken and glucose contents were analyzed by glucose assay kit (Human, Germany). Sugar profiles were detected by Thin layer chromatography (TLC), as described previously by Sornyotha [17]. The hydrolysis products were applied to aluminum sheet silica gel 60, F254 (Merck, Darmstadt, Germany) and were applied with the mixture of *n*-butanol:acetic acid:distilled water (2:1:1). After spraying the plates with 1 g α-diphenylamime dissolved in solution of aniline:phosphoric acid:acetone (1:7.5:50) and heating them over 100 °C, the spots were appeared. Cellooligosaccharides (G1-G6) and xylooligosaccharides (X1-X6) were used as standards (Megazyme).

III. Results and Discussion

A. Enzyme Production and Activity Assay

Cellulolytic enzyme of strain C1 was harvested at 60 h because of high cellulase activities in the culture medium and high reduction of cellulose content in PS (90% decreasing of cellulose contents) was detected. This is a

good advantage of the strain C1 that could reduce high amount of PS that was generally disposed by burning or landfills, which created economic and environmental problems. Cellulolytic enzyme activities were determined and exhibited dominate BGL activity (3.0 U/mg protein) and CMCase activity (0.6 U/mg protein). In addition, crude enzyme of strain C1 also exhibited CBH activity (0.22 U/mg protein). The result indicated that strain C1 has potential for cellulolytic enzyme production by using PS as a sole carbon source. There have been studied on cellulolytic enzymes produced from PS as sole substrate by filamentus fungi. Wang et al. (2010) reported that *T. reesei* Rut C-30, produced xylanolytic and cellulolytic enzymes, mainly endoxylanase while BGL showed low activity (0.5 U/mg protein) when grown on PS [8]. While, *Acremonium cellulolyticus* could use PS as sole carbon source for cellulolytic enzyme production but it could not produce BGL [18]. Thus, strain C1 is unique bacterium that produced high BGL activity.

B. *Effects of Temperature and pH on BGL Activity*

This is the first report of BGL produced by *G. kaustophilus*. Thus, the optimum conditions of enzyme properties are interesting point to study for getting high saccharification and glucose concentration from ARS. The effects of pH on the activity and stability of BGL from strain C1 are shown in Fig. 1a. Results exhibited their optimal activity at pH 6.0 and the stability of BGL was maintained more than 80% between pH 4.0 to 6.0. The optimal temperature of BGL was 60°C and the thermostability of BGL was maintained more than 60% between 35-60°C for 12 h (Fig. 1b). A number of thermostable of BGL have been reported. Table 1 showed a comparison of thermostability of strain C1 BGL with other sources. Results indicated that BGL of strain C1 could maintain 40% activity at optimum temperature for long time incubation (12 h) as well as among the BGLs reported from thermophiles. Therefore, BGL of strain C1 can be used in industrial process.

C. *RS Chemical Composition*

The major components of plant cell walls consisted cellulose, hemicelluloses (mostly xylan), and lignin. The major polysaccharide content of RS suggests that it is the good lignocellulosic biomass for glucose production. However, the RS is difficult to hydrolyze because lignin is associated with polysaccharides that limit the accessibility of cellulose to cellulases [23]. Therefore, aqueous ammonia, a delignifying agent was applied to RS for lignin removal. The lignin content of RS significantly decreased from 23.3 to 13.4% when 27% (w/w) aqueous ammonia at a solid:liquid ratio of 1:12 was applied to RS. Phitsuwan et al. (2016) reported that per gram of ARS, the cellulose and xylan contents significantly increased by 32.6% and 12.5%, respectively [15].

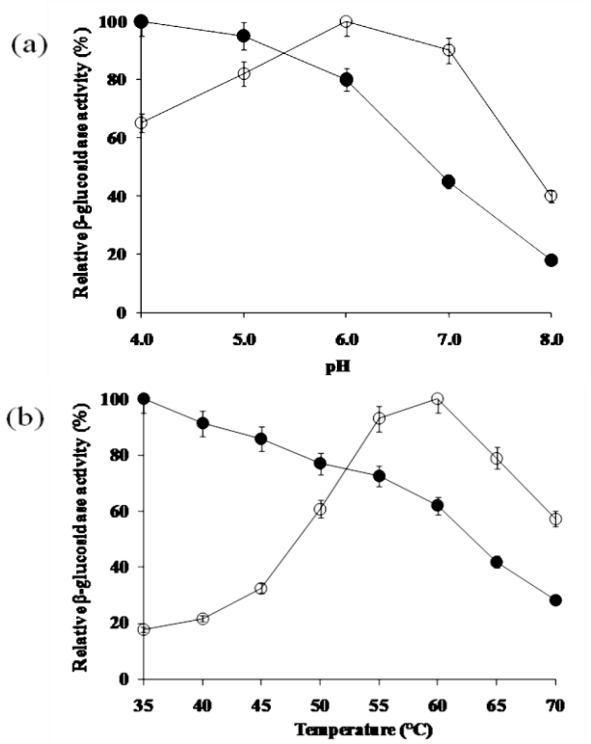

Figure 1. Effects of pH (a) and temperature (b) on BGL produced by *G. kaustophilus* strain C1. *Open circle* represent optimum of BGL and *close circle* represent stability of BGL

TABLE I. OPTIMUM AND STABILITY TEMPERATURES OF β -GLUCOSIDASE FROM VARIOUS SOURCES

Enzymes	Optimum Temperature (°C)	Thermostability (%)	Ref.
Crude enzymes			
G. kaustophilus strain C1	60	40 (18 h)	This study
T. thermohydrosulfuricus YM3	60-70	50 (18 h)	[19]
T. thermosaccharolyticum NOI	60-70	40 (18 h)	[19]
Novozyme-188	50-60	25 (18 h)	[19]
Recombinant enzymes			
rBglB (*C. thermocellum*)	50-60	43 (18 h)	[19]
BglB (*C. thermocellum*)	60	50 (20 min)	[20]
BGL (*S. thermophilum*)	60	50 (40 min)	[21]
BGL (*C. thermophilum*)	65	50 (2 h)	[22]

D. *Glucose Production from Rice Straw Pretreated*

Cellulolytic enzyme of strain C1 (20 U of BGL/g ARS) was applied to hydrolyze ARS for glucose production. Although glucose was produced as a major product, low glucose concentration (5.2 g/L) was found (data not shown). The crude enzyme of strain C1 is a unique enzyme that could hydrolyze ARS to glucose as a major product, which diverge with other enzymes. Although *Trichoderma* spp. and *C. thermocellum*, have been reported to be the most

potent cellulose degrading microbial known to produce large variety of cellulolytic enzyme, they produce less amount of BGL. EG cleaves the internal bindings of the cellulose fiber to cellodextrins, CBH released cellobiose from at external region of the cellulose and BGL hydrolyses cellobiose to glucose [24]. Cellulolytic enzyme of strain C1 revealed high level of BGL, which caused to excellently hydrolyzed cellulose to glucose as a major product that stead to handily for value-added products production in the next steps. However, low concentration of glucose probably due to low EG and CBH activities. Therefore, to increase the concentration of glucose, combination of BGL of C1 with another cellulolytic enzyme that rich in EG and CBH activities are required.

In order to improve hydrolysis of ARS and get more glucose concentration, BGL of strain C1 was combined with cellulolytic enzyme from *T. reesei* (Celluclast 1.5 L), which work at the same condition ranges with BGL of strain C1 and had low BGL activity. Commercial *T. reesei* cellulase activities was determined and exhibited CMCase activity (15.5 U/mg protein), β-glucosidase activity (2.4 U/mg protein) and xylanase activity (5.3 U/mg protein). To enhance glucose yield, ARS was hydrolyzed with 20 U of CMCase/g RST of *T. reesei* and supplementary with various concentration of strain C1 BGL. Combination of *T. reesei* cellulase with BGL of strain C1 are required to increase glucose yield. Figure 2 exhibited sugars profile of hydrolysis products of ARS by cellulolytic enzymes at optimum condition of glucose production (5% of ARS, pH 6.0, ratio of *T. reesei*: strain C1 = 1:2 at 60°C for 72 h). When ARS was hydrolyzed by strain C1 BGL (40 U/g RS) alone, glucose was detected as a major product with low concentration (13 g/L). Whereas, commercial *T. reesei* cellulase alone exhibited cellobiose and glucoseas as major products. Xylose was detected as hydrolysis product from ARS indicated that xylanolytic enzyme also contained in commercial *T. reesei* cellulase. The general cellulolytic enzymes of fungi, especially *T. reesei* exhibited low BGL activity. Thus, cellobiose can not completely convert to glucose and remained high cellobiose contents cause inhibited EG and CBH activities [25]. Accordingly, the end product cellobiose, inhibits EG and CBH that cause to collected oligosaccharide products [26, 27]. Thus, conversion of cellulose into glucose requires synergism of cellulolytic enzyme. In contrast to *T. reesei* cellulase alone, when the enzyme combined with strain C1 BGL, cellobiose was lost while glucose concentration dramatically increased (Fig. 2). With the adding of BGL of strain C1 into *T. reesei* cellulase, glucose production from ARS was increased from 13 g/L to 22 g/L and exhibited 78.1% saccharification.

Glucose tolerance of BGL is one of an important property for efficient cellulose hydrolysis. High glucose concentration can either block active site of BGL [26]. Therefore, BGL of strain C1 was studied glucose tolerance ability and compared with commercial BGL, Novozyme188. Figure 3 illustrated, glucose concentrations were 300 mM and 200 mM required to inhibit 50% of BGL activity of strain C1 and Novozyme-188, respectively. This result indicated that glucose tolerance of strain C1 BGL was higher than Novozyme-188. Accordingly, BGL of strain C1 exhibited good properties and it may be as optional BGL for synergy which other cellulolytic enzymes, EG and CBH to effectively hydrolysis of cellulose.

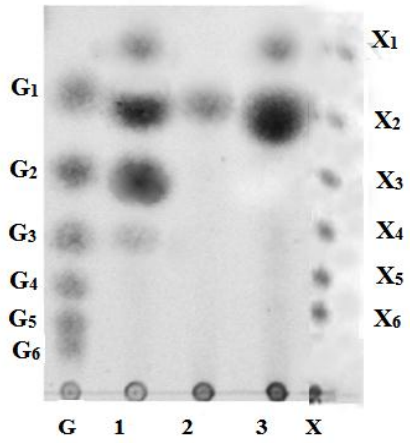

Figure 2. TLC of ARS hydrolysis with individual enzyme or combination between commercial cellulase from *T. reesei* (20 U of CMCase/ g substrate) and BGL from *G. kaustophilus* strain C1 (40 U/ g substrate). G = cellooligosaccharides standard, X = xylooligosaccharides standard, Lane 1 = *T. reesei*, Lane 2 = *G. kaustophilus* strain C1, Lane 3 = *T. reesei* and *G. kaustophilus* strain C1

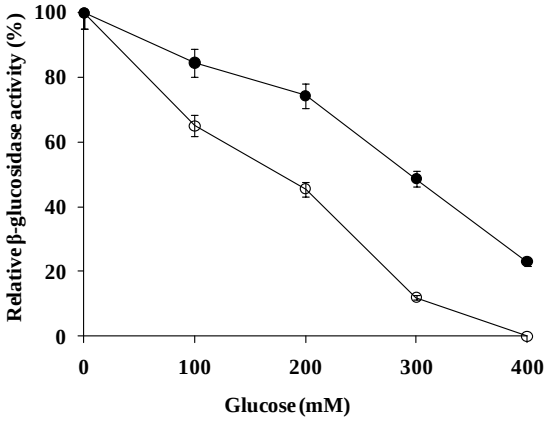

Figure 3. Effect of glucose concentration on BGL activity of *G. kaustophilus* strain C1 (*close circle)* and Novozyme-188 (*open circle)*, respectively.

IV. Conclusion

The strain C1 produced cellulolytic enzyme that dominate in BGL activity when cultivate on PS as carbon source. The BGL of strain C1 exhibited high thermostability and glucose tolerance. These properties are benefit of BGL for using in biorefinery industrial process. Thus, strain C1 is a good choice to utilize PS waste for disposal and cellulolytic enzyme production. BGL of strain C1 combination with *T. reesei* cellulase could efficient conversion of cellulose in ARS to glucose as a major product that can be subsequently used as a raw material in many industries.

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