Production of Cellulolytic Enzyme and Glucose from Paper Sludge by *Geobacillus kaustophilus* C1

V. Phakeenuya, K. Ratanakhanokchai, R. Waeonukul, P. Pason and C. Tachaapikoon

***Abstract*—** The objective of this study was to produce a cellulolytic enzyme and glucose from paper sludge (PS), which is a solid waste from pulp and paper manufacturing, by using *Geobacillus kaustophilus* strain C1. When PS was used as a sole carbon source, a cellulolytic enzyme, mainly β-glucosidase, was produced by the strain C1, while cellulose in PS was utilized at 90%. The cellulolytic enzyme from strain C1 was applied afterward to hydrolyze PS for glucose production. The glucose was produced as a major product. The degree of saccharification of cellulose in PS to glucose was improved when the crude enzyme of strain C1 was combined with commercial *Trichoderma reesei* cellulase (Celluclast 1.5 L). The results indicated that PS could be utilized as feedstock for cellulolytic enzyme and glucose production. Also, that glucose from PS can be used as raw material for the production of value-added products in many industries.

***Keywords*—**Cellulolytic enzyme, *Geobacillus kaustophilus*, glucose, paper sludge

I. Introduction

Paper sludge is a solid waste generated from pulp and paper manufacturing. Most of the PS is disposed by burning or landfills, which creates economic and environmental problems, air pollution, and ground water contamination [1], [2]. It has been estimated that by 2020 the production of PS worldwide will be 500 million tons per year [3]. Moreover, in 2012 the Taiwan Ministry of Economic Affairs (MOEA) reported that in Taiwan alone the average annual quantity of paper sludge was approximately 227 tons per year. Hence, what is needed is a positive PS disposal method, as this would have a positive environmental and economic effect.

PS is complex structural material that contains cellulose (major component), hemicelluloses, and some amounts of lignin and ash. Physical characteristics of PS exhibit small-short fiber and are well-dispersed [4]. PS cannot be reused as a material for paper production. However, it is suitable as a carbon source for cellulolytic enzyme production and as a substrate for glucose production, without pretreatment processes (most of the lignin and hemicellulose have already been removed during the pulping process). This is important because glucose produced from PS can be converted to many products, such as ethanol, organic acids and biogas [5]. Nevertheless, although the potential for using PS as a carbon source to produce cellulolytic enzyme by filamentous fungi have been reported, there are currently no information for this production from bacteria.

Cellulose in biomass was hydrolyzed to glucose by two processes, acid or enzymatic hydrolysis processes. However, enzymatic hydrolysis is an excellent process for biomass hydrolysis because of cellulolytic enzyme that specifically hydrolyzes cellulose to glucose. It is also a green technology and is cost effective [6]. Cellulose is hydrolyzed to glucose by cellulolytic enzyme involving synergistic action of endo-glucanase (EC 3.2.1.4), exo-glucanases (EC 3.2.1.91 and EC 3.2.1.176) and β-glucosidase (EC 3.2.1.21). Efficient saccharification of cellulose in PS is important for the disposal of cellulosic waste and the utilization of cellulose for glucose production. Current literature on this topic has reported that glucose production processes from PS is conducted using endo- and exo-glucanase, which needs to be combined with β-glucosidase to enhance saccharification and glucose yield [7], [8]. Therefore, β-glucosidase is the key enzyme in cellulose hydrolysis processes, completing the final step by converting cellobiose to glucose. Moreover, one of the major challenges in the conversion of biomass into glucose is finding good properties of β-glucosidase.

In this study, we isolated a thermophilic bacterium, *Geobacillus kaustophilus* strain C1 from a composting wood waste soil sample in Northern Taiwan. The strain C1 produced high β-glucosidase activity and exhibited good thermostability and glucose tolerance. The objective of this study was to use PS for β-glucosidase and glucose productions by strain C1.

II. Material and Methods

A. Analysis of Paper Sludge Composition

The PS used in this study was primary sludge obtained from a paper manufacturing company in Taiwan. Composition of the PS was determined following the laboratory analytical procedures of the National Renewable Energy Laboratory [9]. Oven-dried paper sludge was hydrolyzed by 72% sulfuric acid at 30 °C for 1 h, then diluted to 4% sulfuric acid for autoclaving at 121 C for 1 h. The PS hydrolysate was neutralized to pH 6.0 with calcium carbonate and vacuum filtered through a filtering crucible. Mono and oligosaccharide components were detected by high performance liquid chromatography (HPLC), with a refractive index detector (Shimadzu RID-10A) on a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA), which operated at 80 °C with MilliQ-filtered water (EMD Millipore Corp., MA, USA) at a flow rate of 0.6 mL/min.

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V. Phakeenuya and K. Ratanakhanokchai (corresponding author) are with School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand

R. Waeonukul, P. Pason and C. Tachaapikoon are with Pilot Plant Development and Training Institute King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand

Acid insoluble lignin (Klason lignin) content was defined as the weight of the filter cake (oven-dried at 70°C to constant weight).

B. Medium Culture

The bacterial strain C1 was cultivated in a mineral salt (MS) medium that was used as the selective medium for *Bacillus* spp. [10]. This medium was supplemented with 0.5% PS as a carbon source and subsequently sterilized (20 min, 121°C).

C. Strain Isolation

More than 25 samples were collected from composting wood waste soil samples in Northern Taiwan. Approximately 1 g of each soil sample collected was inoculated directly into an MS medium that was supplemented with 0.5 % of PS as a sole carbon source at pH 7.0 and 60°C. After 3 days culture, samples showed distinct appearance of PS, which were selected for bacteria isolation. Thereafter, each culture was spread on MS agar plate containing 0.5% carboxymethyl cellulose (Sigma Chemical Co., St. Louis, MO, USA) and 1.5% of agar (Wako Pure Chemical Industries Ltd., Japan), at 60°C. Different colonies were collected and isolated by subculturing. The Congo red staining method was utilized to screen the cellulase-producing strains. Individual colonies that produced a clear zone were collected from the agar plate and incubated in an MS medium that was supplemented with paper sludge at 60°C and 200 rpm for 3 days. The crude enzyme of each pure isolated bacterium was used for hydrolysis paper sludge at 60°C, and then the hydrolysis products were analyzed by HPLC.

D. Identification of Bacterium

The isolated target strain was inoculated into an MS medium that was supplemented with 0.5% glucose at 60°C and 200 rpm for 1 day. An appropriate amount of cells were taken from the culture, and the genomic DNA of the cells was extracted by Genomic DNA Extraction Miniprep for the sequence analysis (Viogene, New Taipei City, Taiwan). Amplification of the 16S rDNA gene was conducted by a polymerase chain reaction (PCR) using F8 (5'-AGAGTTTGATCCTGGCTCAG-3') and R1541 (5'-AAGGAGGTGATCCAGCCGCA-5') primers. The PCR products were sequenced and the obtained sequence was analyzed and compared using BLAST software at NCBI to determine the identity of the isolated strain. The phylogenetic tree was also constructed using the MegAlign software 5.01. The 16S rRNA gene sequence of the *Geobacillus kaustophilus* strain C1 (1,430 bp) was deposited in GenBank under accession number KF879569.

E. Production of Cellulolytic Enzyme

To study the time courses of growth, productions of β-glucosidase and CMCase and PS remaining by strain C1 were achieved in an MS medium supplemented with 0.5% (w/v) PS at 60°C, pH 6.0 (The highest level of β-glucosidase production condition). After bacterium grown in a PS medium in the above conditions, culture samples at various times were centrifuged at 8,000g for 10 min at 4°C. Each of the culture supernatants was concentrated by ultrafiltration (10 kDa cut-off membrane). Next, crude enzyme samples were taken to assayed extacellular enzyme activities that contained β-glucosidase and CMCase activities. Pellet samples (cells and residual PS) were washed by a phosphate buffered saline (PBS), as described previously by Wang [11]. Cells were eluted from PS by adding a large amount of PBS, pH 6.0. This was followed by votexed for 5 min. The eluted samples were collected and centrifuged at 8,000 rpm for 10 min at 4 °C. After that, the supernatant was discarded and sediment of strain C1 cells was collected. Thereafter, the cells were resuspended in 500 µl of 100 mM phosphate buffer (pH 6.0). Strain C1 cell suspensions were stained and analyzed by electron microscopy for an aspect of the lysed cell study. The viable cell numbers of the C1 cell suspensions were determined by counting the colony-forming unit (CFU) on an MS agar plate. C1 cell suspensions (300 µl) were placed within an ultrasonic cell disintegrator that was operated at 200 W (10 s interval time, 30 s of ultrasonic time) for a total of 5 min, and were kept in an ice bath during the ultrasonic process to prevent heating. After centrifugation, the supernatant was assayed for the activity of intracellular β-glucosidase and the precipitate was determined for the remaining PS. Finally, the time that best showed the highest β-glucosidase and CMCase activities and PS consumption was chosen to recover enzymes for use in a glucose production process.

F. Enzyme and Protein Assays

The endoglucanase activity assay was performed in 1% (v/w), with a final concentration of carboxymethyl cellulose at 60° C in a 50 mM acetate buffer (pH 5.0) for 10 min. Endoglucanase activity was measured by the amount of reducing sugars liberated from carboxymethyl cellulose. Reducing sugars were determined using the Somogyi-Nelson method [12]. 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 exoglucanase and β-glucosidase activities were performed at 60 °C in 50 mM phosphate buffer (pH 6.0) for 30 min, and were based on measurements of the release of *p*–nitrophenol from *p*-nitrophenyl-D-cellobioside and *p*-nitrophenyl-β-D-glucoside, respectively. One unit of the enzyme releases 1 µmole equivalent of *p*-nitrophenol per min. Protein concentration was determined by the Lowry method with bovine serum albumin as the standard.

G. Glucose Production from Paper Sludge

Enzymatic digestibility experiments were performed at 60° C in 1.5 mL Eppendrof tube, which contained 6% (w/v) PS in the presence of 50 mM acetate buffer (pH 6.0) with 1 mL total working volume for 18 h. For glucose production optimization, PS was hydrolyzed with 10 (U/g PS) of *T. reesei* cellulase (Celluclast 1.5 L) and was supplied with 10, 20, and 30 of β-glucosidase activity per g paper 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 a glucose assay kit (Human, Germany). Sugar profiles were detected by HPLC.

III. Results and Discussion

A. Composition of Paper Sludge

PS composition varies widely depending on the source [11]. The major component of dry paper sludge contained organic material consisting of cellulose and hemicellulose. The compositions of paper sludge was determined to be 45.96%

cellulose, 9.17% xylan, 8.20 % lignin, 32.62% ash and 4.05% other compositions.

B. Isolation and Identification of the Isolated Strain

Bacillus spp. are interesting bacteria because of their fast and high growth rate, their ability to grow at high temperatures, low toxicity, and the low cost of their medium and carbon sources. Moreover, *Bacillus* spp. can grow and produce cellulolytic enzymes from lignocellulosic materials at high temperatures. Therefore, we isolated thermophilic bacteria, *Bacillus* spp., to produce cellulolytic enzymes and glucose from paper sludge. Eighteen pure strains were isolated from composting wood waste soil samples. We required bacteria that have the ability to convert cellulose in PS to glucose, a simple compound for the conversion of many platform chemicals [13]. Among these strains, the 6 with high cellulase activity were chosen for cellulolytic enzyme production. After that, 2% (w/v) of paper sludge was hydrolyzed by the crude enzyme of each of the 6 bacterial strains (5 unit of CMCase activity/g substrate) at 60°C. Hydrolysis products of PS with the crude enzyme of the strain C1 showed glucose as a major product, when analyzed by HPLC. Among the other strains, only one exhibited glucose as major product. However, strain C1 showed the higher glucose concentration and the other 4 strains produced a series of sugars. It was quite difficult to find a bacterium like strain C1 that produced glucose as a major product. Thus, bacterium strain C1 was selected as target bacterium to produce cellulolytic enzyme and glucose from PS. The result of the 16S rDNA gene phylogenic tree indicated that strain C1 belonged to the genus *Geobacillus* and is very similar to *Geobacillus kaustophilus* (99.3% sequence similarity). Thus, the strain C1 was identified as *Geobacillus kaustophilus* strain C1.

C. Enzyme Production and Activity Assay

In previous studies, a few literature reviews have reported using PS as feedstock for cellulolytic enzyme production, especially from *Bacillus*. However, literature reviews of bacterial cellulolytic enzyme production from PS is rare and do not give enough information to understand cellulolytic enzyme patterns or estimate their enzymatic properties. In our study, after strain C1 was cultivated, culture supernatant (extracellular enzyme) and pellet (cells and residual PS) were separated by centrifugation. Culture supernatant was concentrated by ultrafiltration (10 kDa cut-off membrane) and then used for enzyme activity assays (CMCase and β-glucosidase). Whereas the pellet was determined by the viable cell numbers, the remaining PS and intracellular β-glucosidase activity. β–Glucosidase is an enzyme that catalyses cellobiose and short chain substrates from hydrolyzated products of cellulose with endo- and exo-cellulases. After that, bacterial can introduce cellobiose into cells, then β–glucosidase hydrolyze cellobiose to 2 glucoses [14]. However, β–glucosidase can also be detected in culture supernatant. Thus, β–glucosidase activity needs to determine in both in the cell and culture supernatant.

Fig. 1 shows that during the growth of strain C1 on PS, very low activity of intracellular β-glucosidase and CMCase activities were detected, particularly during the lag phase (0-24 h) because cells had to adapt on a new culture medium. Thus, the C1 cells did not produce enzymes to hydrolyze substrate. After 24 h (starting log phase), CMCase activity could assay in the culture medium and the CMCase was being produced during the growth phase of the strain C1. This is in agreement with the previous study, which showed that CMCase is a growth-associated enzyme of bacteria [15], [16]. Intracellular β-glucosidase activity could assay in the C1 cell after 30 h and dramatically increased until 42 h (late log phase). At the late stationary growth phase (48-54 h), cells began lysis (data not shown), which caused them to secrete β-glucosidase into culture supernatant. Thus, at this period, β-glucosidase activity could assay in culture supernatant and reach nearly constant activity, between 60-96 h (death phase). At this time, the C1 cells dramatically lysed and secreted high amounts of β-glucosidase, which appeared in culture supernatant. In contrast, cellulose contents of PS dramatically decreased after 48 h because of the action of cellulolytic enzyme in culture supernatant. The cellulolytic enzyme of strain C1 was harvested at 60 h, and high β-glucosidase and CMCase activities in the culture medium and high reduction of cellulose content in PS (90% decreasing of cellulose contents) were detected. The latter is a good advantage of the strain C1 that can reduce high amount of PS. Furthermore, this method is a better alternative than sending PS to a landfill, which creates environmental problems. Although the strain C1 exhibited high saccharification of PS, glucose was not detected (data not shown) because the strain C1 utilized glucose for cell growth. Thereby, cellulolytic enzyme of strain C1 was used to produce glucose from PS.

At 60 h, cellulolytic enzyme activities were determined and exhibited dominate β-glucosidase activity (3.0 U/mg protein) and CMCase activity (0.6 U/mg protein). In addition, the crude enzyme of strain C1 also exhibited exo-glucanase activity (0.22 U/mg protein). The result indicated that strain C1 has the potential for cellulolytic enzyme production by using PS as a sole carbon source. As numerous studies have shown, cellulolytic enzymes are produced from PS as sole substrate by filamentus fungi. For instance, Wang et al. (2010) reported that *T. reesei* Rut C-30, produced xylanolytic and cellulolytic enzymes, mainly endoxylanase, while β-glucosidase showed low activity (0.5 U/mg protein) when grown on PS [11]. Also, studies have shown that *Acremonium cellulolyticus* can use PS as sole carbon source for cellulolytic enzyme production but it cannot produce β-glucosidase [5]. Thus, strain C1 is a unique bacterium that produces high β-glucosidase activity.

D. Glucose Production from Paper Sludge

1. Cellulolytic Enzyme from Strain C1

The cellulolytic enzyme of strain C1 (10 U of β-glucosidase activity/g PS) was applied to hydrolyze PS for glucose production. Although glucose was produced as a major product, low glucose concentration (3 g/L) was found (data not shown). The conversion of cellulose into glucose requires synergism of cellulolytic enzymes. Endo-glucanase cleaves the internal bindings of the cellulose fiber to cellodextrins, exo-glucanases released cellobiose from at external region of the cellulose and β-glucosidase hydrolyses cellobiose to glucose [14]. The crude enzyme of strain C1 is a unique enzyme that can hydrolyse PS to glucose as a major product, which diverges with other enzymes. However, although *Trichoderma* spp. and *Clostridium thermocellum* have been reported as the most potent cellulose

degrading microbial known to produce a large variety of cellulolytic, they produce less amounts of β-glucosidase.

Accordingly, the end product, cellobiose, inhibits endo-glucanase and exo-glucanase, which collects oligosaccharide products [17], [18]. Cellulolytic enzymes of strain C1 revealed high levels of β-glucosidase, which excellently hydrolyze cellulose to glucose as a major product. However, low concentrations of glucose are probably due to the low endo-glucanase and exo-glucanase activities. Therefore, to increase the concentration of glucose, a combination of β-glucosidase of C1 with another cellulolytic enzyme that is rich in endo- and exo-glucanase activities are required.

2. Commercial Cellulases

The cellulolytic enzyme of strain C1 exhibited dominate β-glucosides. In order to improve hydrolysis of PS and get more glucose concentration, β-glucosidase of strain C1 was combined with *T. reesei* (Celluclast 1.5 L), which works in the same range of conditions ranges as β-glucosidase of strain C1 and lacks β-glucosidase activity. Commercial *T. reesei* cellulase activities were 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, PS was hydrolyzed with 10 U of CMCase/g PS of *T. reesei* and supplemented with various concentrations of β-glucosidase, strain C1. However, glucose concentration was increased by its combination with β-glucosidase of strain C1. Optimum efficient degradation was achieved at a ratio of 1:2 (Fig. 2). A combination of *T. reesei* cellulase with β-glucosidase of strain C1 was required to increase glucose yield.

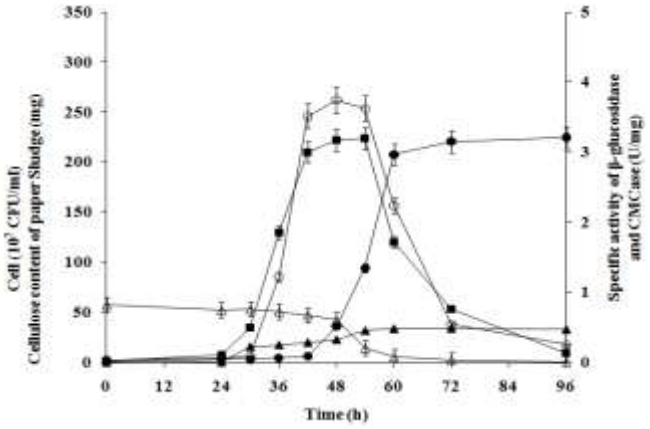

Fig. 1. Cell growth (*close square*), production of intracellular β-glucosidase (*open circle*), β-glucosidase in culture supernatant (*close circle*), CMCase (*close triangle*), and cellulose content of PS remaining (*open triangle*) by *G. kaustophilus* C1. All experiments were performed in triplicate.

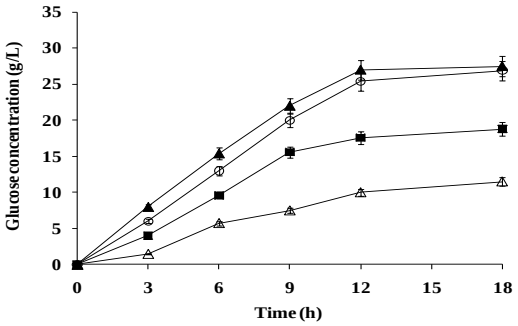

Fig. 2. Glucose production by hydrolysis of PS with commercial *T. reesei* cellulase alone (*open triangle*) and various ratios of combination *T. reesei* cellulase and β-glucosidase loading. *Close square* represent ratio 1:1, *open circle* represent ratio 1:2 and *close triangle* represent ratio 1:3.

3. Combination of β-glucosidase of Strain C1 with Commercial Cellulase

Fig. 3 shows sugar profiles of hydrolysis product of PS by cellulolytic enzyme at optimum conditions of glucose production (6% of PS, pH 6.0, ratio of *T. reesei*: strain C1 = 1:2 at 60°C for 18 h). When PS was hydrolyzed by β-glucosidase of strain C1 (20 U/g PS) alone, glucose was detected as a major product with low concentration (7 g/L) (Fig. 3a). In contrast, commercial *T. reesei* cellulase alone exhibited cellobiose, glucose and xylose as major products (Fig. 3b). Xylose was detected as a hydrolysis product from PS, which indicated that xylanolytic enzymes also contained commercial *T. reesei* cellulase. The general cellulolytic enzymes of fungi, especially *T. reesei*, exhibited low β-glucosidase activity. Thus, cellobiose cannot completely convert to glucose and retain its high cellobiose contents because endocellulase and exocellulase inhibition that exhibited low saccharification [19]. In contrast to *T. reesei* cellulase, when the enzyme is combined with β-glucosidase of strain C1, cellobiose was lost and glucose concentration dramatically increased (Fig. 3C). β-Glucosidase not only prevented the inhibition by cellobiose but also enhanced the activities of endo- and exo-glucanase in cellulosic bioconversion [20]. By adding β-glucosidase of strain C1 to *T. reesei* cellulase, glucose production from PS was increased from 11.5 g/L to 26.11 g/L (2.27 times) and exhibited 95.4% saccharification. This is a significant improvement over recent publications using PS as a cellulose source. For instance, Kang et al. (2010) studied PS hydrolysis by combining commercial cellulase and Novozyme 188 β-glucosidase. The results exhibited a 94.6% of glucan saccharification, which is close to the glucan saccharification of this study [8]. Thus, the advantage of β-glucosidase of strain C1 is good synergy between cellulases and β-glucosidase.

This report illustrates one of the methods for using PS to produce glucose. In particular, it illustrtes a method that uses green tecnology for PS eliminatation. Figure 4 provides flowchart diagram estimation for cellulolytic enzyme and glucose production from PS by strain C1. When 5 g of PS is utilized as sole carbon source for cellulolytic enzyme production, around 2.35 g is used by *G. kastophilus* C1. In this step retrained 2.85 g of PS residual and obtained cellulolytic

enzyme (dominate β-glucosidase). β-Glucosidase was combined with commercial *T. reesei* cellulase for glucose production from another 60 g of PS. The glucose concentration was determined and exhibited 26.11 g/L. In this complete process, 65 g of PS was utilized for cellulolytic enzyme and glucose production. After the process 34.9 g of PS residual remained. This demonstrates that the residual of PS can be used for composing for improving soil quality and that the residual xylan in PS can be used as a substrate for xylose conversion. Moreover, it may also be of use for biofuels and biochemicals production [21].

IV. Conclusion

Paper sludge, generated from paper making industrial was eliminated by using as carbon source for the strain C1 to produce a cellulolytic enzyme. The strain C1 produced a cellulolytic enzyme that dominated β-glucosidase activity and could decrease 90% of cellulose content in PS. Moreover, strain C1 could convert paper sludge to glucose as major product. Thus, the strain C1 may be a beneficial alternative for PS waste disposal and glucose production, which can subsequently be used as a raw material in the production of many products.

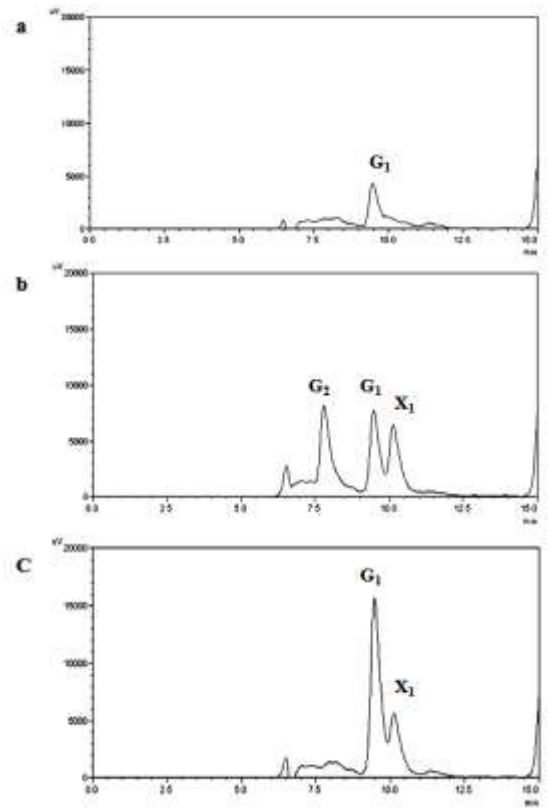

Fig. 3. HPLC analysis of PS hydrolysed with individual enzyme from (a) *G. kaustophilus* strain C1 or (b) *T. reesei* or (c) combination of *T. reesei* cellulase (10 U/g substrate) and β-glucosidase from *G. kaustophilus* strain C1 (20 U/g substrate).

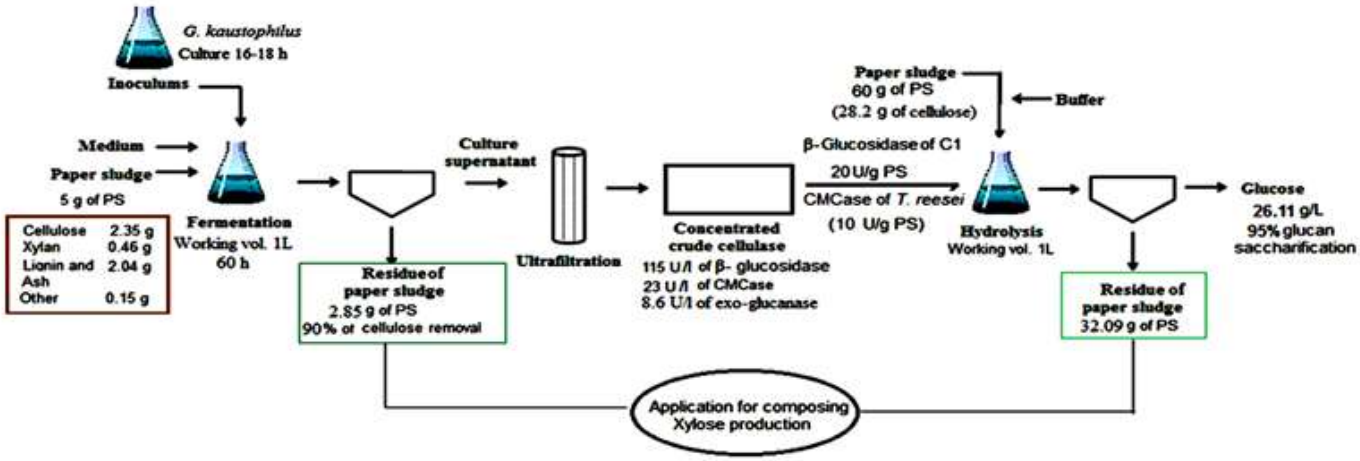

Fig. 4. Flowchart diagram for cellulolytic enzyme and glucose production from PS by *G. kaustophilus* strain C1

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V. Phakeenuya is from Thailand and was born in Ayutthaya Province. I was born in June 2nd, 1987. I started college for a B.S. degree in Biotechnology at Faculty of Applied Science, King Mongkut's University of Technology North Bangkok (KMUTNB). Currently, I am a Ph.D. candidate in Biochemistry program at Faculty of School of Bioresources and Technology, King Mongkut's University of Technology Thonburi (KMUTT), Thailand.

For my thesis, I have been working on production, purification, characterization and application of lignocellulosic enzymes from novel bacterium under supervision of Assoc. Prof. Khanok Ratanakhanokchai. He has expertise in enzyme technology and biomass conversion technology.