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Production of cellulases by *Penicillium* sp. in a solid-state fermentation of oil palm empty fruit bunch

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To use oil palm empty fruit bunch (EFB) for cellulase production, a novel fungus was isolated from moistened EFB. This fungus was classified as *Penicillium* sp. by sequence analysis of the internal transcribing space and it was named *Penicillium* sp. GDX01. The *Penicillium* sp. strain secreted cellulases under solid-state fermentation of EFB. The fermentation conditions were optimized for maximal enzyme production. Of the different substrates tested, both EFB and rice straw gave the maximum production of filter paper activity (FPase). Five percent yeast extract and 40-50% initial moisture content were found to be optimal for enzyme production. In addition, the pretreatment of EFB with NaOH before fermentation inhibited the cellulase production of *Penicillium* sp. GDX01. Saccharification of pretreated EFB by cellulases from *Penicillium* sp. GDX01 resulted in a more than 80% release of glucose during a 72 h incubation, which is a better result than when using Celluclast 1.5L without the addition of β -glucosidase. Our results show that the cellulases produced by *Penicillium* sp. GDX01 are more efficient at the saccharification of EFB than Celluclast 1.5 L.

Key words: Cellulase, oil palm empty fruit bunch, solid-state fermentation, *Penicillium*.

INTRODUCTION

Lignocellulose is an important source for the production of renewable energy and chemicals. It is composed of cellulose, hemicellulose, and lignin. The former, cellulose and hemicellulose can be hydrolyzed into glucose or xylose which can in turn be used to produce valuable products. Thus far, cellulolysis has been performed through enzymatic saccharification by cellulases and hemicellulases. For economical saccharification, cellulase production is the most important step. Cellulase is a mixture of various hydrolyzing enzymes which synergistically convert cellulose into glucose and its major components are endo-D-glucanase, exo-D-glucanase and β -glucosidase.

Trichoderma reesei is a model fungus for studying cellulase production (Sukumaran et al., 2005; Kubicek et al., 2009) and is a major industrial source of cellulase. Besides *T. reesei*, other fungi such as *Humicola*, *Penicillium*, and *Aspergillus* are capable of producing high levels of extracellular cellulases (Sukumaran et al., 2005). *Aspergillus niger* (Ong et al., 2004), *Aspergillus nidulans* (Kwon et al., 1992), *Aspergillus terreus* (Kotaka et al., 2008), *Aspergillus japonicus* (Gao et al., 2008), and *Aspergillus oryzae* (Herculano et al., 2011) have been employed in cellulase production. *Humicola insolens* (Karlsson et al., 2002) and *Humicola grisea* (Takashima et al., 1996) were also found to be cellulase producers.

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Abbreviations: SSF, Solid-state fermentation; SMF, submerged fermentation; EFB, empty fruit bunch; CMC, carboxymethylcellulose; PDA, potato dextrose agar; MEA, malt extract agar; ITS, internal transcribed spacer; PCR, polymerase chain reaction; DNS, 3, 5-dinitrosalicylic acid; CBase, β -glucosidase; CMCCase, endo-glucanase; FPase, filter paper activity; BSA, bovine serum albumin; IMC, initial moisture content.

Many studies reported that several *Penicillium* species secrete a complete cellulase with high β -glucosidase activity (Singh et al., 2009; Dillon et al., 2011; Liu et al., 2011; Singhvi et al., 2011; Castro et al., 2010; Dutta et al., 2008). Cellulase from *Penicillium funiculosum* exhibited better-balanced β -glucosidase, endo- and exoglucanase activities than commercial preparations had done (Castro et al., 2010), and produced glucose faster during corn cob hydrolysis than commercialized enzymes. In addition, *Penicillium citrinum* produced alkali-tolerant and thermostable cellulases (Dutta et al., 2008). In order to improve the production rate and specific activity of cellulases, chemical and physical mutagenesis have been applied to several fungal strains (Dillon et al., 2011; Liu et al., 2011).

Specifically, Dillon et al. (2011) employed hydrogen peroxide to generate mutants, and isolated a new *Penicillium echinulatum* strain showing faster cellulase secretion than the wild type. Similarly, Liu et al. (2011) used ethylmethanesulfonate and UV irradiation to mutate *Penicillium decumbens* and created a mutant exhibiting higher cellulase activity.

Cellulases can be produced by solid-state fermentation (SSF) or submerged fermentation (SF), which is widely employed in the production of industrial enzymes, is a fermentation process wherein the solid material acts as both a physical support and nutrient source. It has several advantages over submerged fermentation, including high enzyme concentrations, high volumetric productivity, low operating expenses and low sterility requirements. The high volumetric productivity of SSF also leads to lower water requirements and reduces the volume of waste generated. In addition, the agro-industrial by-products and crop residues can be used as carbon sources at a lower cost.

Oil palm empty fruit bunch (EFB) is an agricultural by-product of the extraction of palm oil from fresh fruit bunches. EFB is a useful biomass for bioethanol production, and produced abundantly in Indonesia and Malaysia. EFB biomass is rich in cellulose and hemicelluloses, typically containing 35-40% cellulose and 20-25% hemicellulose. Palm oil mills in Malaysia generate 2.4 million tons of EFB annually, and while a small fraction of that is used as a fuel for the generation of steam or electricity, the most of them are dumped as waste.

The use of EFB lowers the cost of cellulase production by SSF. Cellulases produced during the SSF of EFB would more efficiently hydrolyze EFB. However, only a few reports have showed that EFB can be used to produce cellulases under SSF. Alam et al. (2009) used a rotator drum bioreactor to produce cellulases from EFB with *Trichoderma hazianum*. Bahrin et al. (2011) investigated cellulase production under EFB solid-state fermentation using *Botryosphaeria* sp. isolated from EFB. Others used pretreated EFB as the carbon source for submerged fermentation (Ariffin et al., 2008; Umikalsan

et al., 1997). This study reports the isolation of *Penicillium* sp. GDX01 from moistened EFB and its ability to produce cellulases in SSF. The results showed that cellulases from *Penicillium* sp. GDX01 can be applied to the saccharification of EFB for bioethanol production.

MATERIALS AND METHODS

Strain isolation and identification

The filamentous fungus *Penicillium* sp. GDX01 was isolated from the surface of EFB. The cellulolytic strain was screened using carboxymethylcellulose (CMC, low viscosity) medium containing 2% agar (w/v), 1% yeast extract, 2% peptone, and 1% CMC. A potato dextrose agar (PDA) medium and malt extract agar (MEA) medium were used for strain maintenance. Also, *Penicillium diversum* (KCTC16052) and *Penicillium oxalicum* (KCTC6440 and KCTC16912) were purchased from the Korean Collection for Type Culture (KCTC). The strains were grown on PDA or MEA media for up to 7 days at 28°C until conidia formed, and then stored at 4°C until needed. The internal transcribed spacer (ITS) sequence of *Penicillium* sp. GDX01 was amplified by polymerase chain reaction (PCR) with the primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2 (5'-GCTGCGTTCATCGATGC-3'). The amplification was performed using a PCR cyclor with the following cycling parameters: 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 52°C, and 1 min at 72°C, and a final extension for 5 min at 72°C. The resulting PCR product of ITS rDNA was cloned and sequenced. Basic Alignment Search Tool (BLAST) was used to search for fungi with the most homologous ITS sequences.

Enzyme production by solid-state fermentation (SSF)

The solid-state fermentation was carried out in a 400 ml glass bottle (81 x 132mm). *Penicillium* sp. GDX01 was grown in 400 ml glass bottle containing 40 g of substrate (EFB and rice straw) and yeast extract as a nitrogen source. The moisture content was adjusted to 40-80% (w/w) by adding deionized water and all bottles were sterilized at 121°C for 15 min. Next, four agar blocks (15 x 15 mm) of fungi grown for one to two weeks (1×10^6 - 10^8 conidia) were inoculated into each of the bottles, and then incubated statically at 30°C for a further one to two weeks.

Enzyme extraction

The enzymes were extracted from the fermented substrate with 6 ml of 0.05 M sodium citrate buffer (pH 4.8) per gram of biomass by shaking at 250 rpm and 30°C for 1 h. Then, the eluted enzyme mixture was separated from the fungal biomass by centrifugation (10,000 x g for 10 min) and the clarified supernatant used as the crude enzyme source.

Enzyme assay and protein determination

Next, the total cellulase, endo-glucanase, and β -glucosidase activities were determined. The total cellulase activity (filter paper activity, FPase) was assayed by incubating 500 μ l of crude enzyme with 1 ml of 0.05 M sodium citrate buffer containing Whatman No. 1 filter paper (50 mg, 1 x 6 cm) at 50°C for 1 h. The amount of reducing sugars released was determined by the 3, 5-dinitrosalicylic acid (DNS) method. Next, the endo-glucanase activity (CMCase)

was assayed in a total reaction mixture of 200 μ l which contained 50 μ l of crude enzymes, 100 μ l of 0.1 M potassium phosphate buffer (pH 6.0) and 50 μ l of 1% (w/v) CMC solution. The mixture was incubated at 50°C for 30 min, and the amount of reducing sugars released was again determined by the DNS method. Lastly, the activity of β -glucosidase (CBase) was determined by measuring hydrolyzed glucose from cellobiose. A total reaction mixture of 1 ml containing 500 μ l of crude enzyme and 500 μ l of 15 mM cellobiose solution in sodium citrate buffer was incubated at 50°C for 30 min. After incubation, 10 μ l of the reaction mixture was mixed with 1 ml of glucose kit reagent (Young-Dong Diagnostics, Yong-in, Korea) and incubated at 37°C for 10 min. Then, the amount of glucose in the final reaction solution was measured with a UV-VIS spectrophotometer at 600 nm with glucose as the standard. In addition, the total protein in the crude enzyme extract was determined by the Bradford method with bovine serum albumin (BSA) as the standard.

Enzyme activity

One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 μ mole of products (reducing sugar or glucose) from the appropriate substrates per minute under the assay conditions. In this study, the enzyme activities are expressed as units per gram of EFB (U/g).

Enzyme concentration and saccharification of pretreated EFB

After enzyme extraction, fungal cells were eliminated from the crude enzyme by membrane filtration (0.22 μ m). This solution was then concentrated approximately 10-fold with an ultra filtration kit (Sartorius, Germany).

One gram of pretreated EFB was digested with 5 FPU of concentrated enzymes (GDX-10) or Celluclast 1.5L (Novozymes, Denmark) in 100 ml of 0.05 M sodium citrate buffer as a control. This mixture was incubated at 50°C for 3 days with gentle shaking. Finally, the glucose yield (%) was analyzed using high performance liquid chromatography.

RESULTS AND DISCUSSION

Screening of fungi producing cellulase using EFB as substrate

To isolate fungi producing cellulases using EFB as the substrate we screened for fungi that could grow on EFB as their sole carbon source. A cultivation bottle containing wet EFB was exposed to the air to seed fungal spores. Then the bottle was incubated at 30°C for several days until fungal growth was observed. The fungi were then re-grown on PDA plates containing antibiotics to suppress bacterial growth. We observed the growth of two kinds of fungi on the plate and repeatedly cultivated each fungus on new PDA plates to isolate pure strains. Then each fungus was grown on EFB once again to check for cellulase production and both were found to produce cellulases using EFB as their sole carbon source. To identify these fungal strains, the ITS sequence was analyzed. One of them was classified as *Penicillium* sp. showing a 96% identity with *P. oxalicum* and the other

showed a 97% identity with *Aspergillus fumigatus* (data not shown). Because the latter is pathogenic to humans, we characterized only the former, naming it *Penicillium* sp. GDX01.

Optimization of conditions for cellulase production

Effect of biomass

To compare the effects of biomass on the cellulase production of *Penicillium* sp. GDX01, we performed a SSF of the four different biomasses, EFB, rice straw, reed, and *Miscanthus*. Enzymatic assays showed no cellulase activity in the SSF of reed and *Miscanthus*. However, we observed similar levels of cellulase activities with both rice straw and EFB (Figure 1). In both cases, FPase activity increased up until the 10th day of SSF, the limit of our study. This showed that *Penicillium* sp. GDX01 prefers rice straw and EFB as carbon sources for cellulase production. The maximum FPase activity obtained was 606 IU/g EFB, which is much higher than the several *Penicillium* species' cellulase activities previously reported; more specifically our observed cellulase production rate under SSF was much higher than, for example, a mutant strain of *Penicillium janthinellum* which produced 67 IU of FPase activity/g cellulose (Singhvi et al., 2011); Chahal (1985) showed the Cellulase yields of 250 to 430 IU/g of cellulose were recorded in a new approach to solid-state fermentation of wheat straw with *Trichoderma reesei* QMY-1. (Chahal, 1985), which was more than two times lower than our result. Whereas Arpan et al. (2013) showed FPase of 826.2 IU/g was obtained by *A. fumigatus* ABK9 in wheat bran-rice straw mixed substrate which was higher than our result

Effect of nitrogen source

Nitrogen is supplemented usually in organic or inorganic form and sometimes provided with the substrate itself. The use of different nitrogen sources have been shown to influence the production of cellulase. Among those tested, NH_4NO_3 gave the highest production of cellulase by *T. reesei* under SSF (Singhania et al., 2006). However, several studies have reported that organic nitrogen sources result in a higher production of cellulases than inorganic (Deswal et al., 2011; Jeya et al., 2010).

This study analyzed three different organic nitrogen sources (peptone, yeast extract, and wheat bran, rape seed meal) to test their effects on the cellulase production. Peptone produced limited activity whereas yeast extract showed higher cellulase production than did wheat bran (data not shown). Rape seed meal which has economical price showed cellulase activity as yeast extract (data not shown) therefore it is suitable for

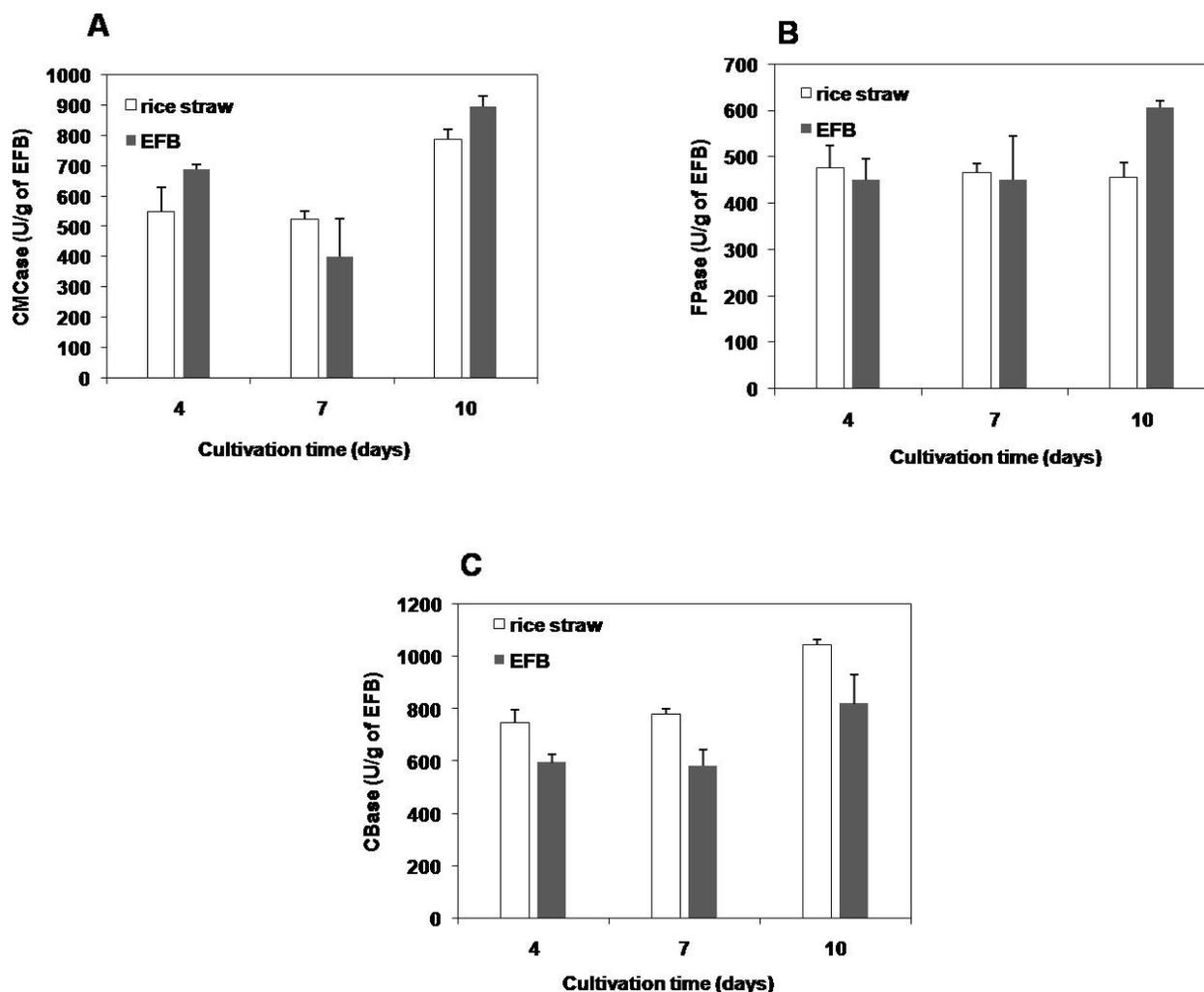


Figure 1. Comparison of FPase activity by the SSF using EFB and rice straw as substrates. SSF Conditions: pH 7; temperature, 30°C; moisture content, 50%; nitrogen source, 5% yeast extract; particle size, 2 mm; no pretreatment.

alternative.

We used different concentrations of yeast extract to determine the optimum yeast extract concentration for cellulase production (Figure 2). The results showed that 5% yeast extract produced the maximum FPase activity (Figure 2B). Without yeast extract, *Penicillium* sp. GDX01 could not produce cellulase, indicating that nitrogen is essential for cellulase production. With 10% yeast extract however, cellulase production decreased due to the growth inhibition of *Penicillium* sp. GDX01. These results clearly show the need to determine optimal nitrogen source concentration and, in this case, the maximum CMCase and β -glucosidase activities were observed with 5% yeast extract.

Effect of initial pH

pH is also regarded as an important factor for SSF. Spray

et al. has previously reported that the cellulase expression of *T. reesei* is pH-dependent (Sprey and Lambert, 1983). The final pH of each the enzyme extract was pH 4.8. As cultivation time increased, the cellulase activity of *Penicillium* sp. GDX01 increased at all pH levels and the highest activity was observed on the 10th day of cultivation. Both the CMCase and CBase activities of *Penicillium* sp. GDX01 were only slightly higher at pH 4 than at pH 6 or pH 9 on the 10th day (Figures 3A and C). In contrast, the, FPase activity at pH 4 was in fact slightly lower than at pH 6 or 9 by the 10th day (Figure 3B). These results for *Penicillium* sp. GDX01 differed from previous ones for *T. reesei*. Singhania et al. (2006) reported that *T. reesei* NRRL 11460 produced higher yields of cellulase at pH 7 and Xiong et al. (2004) revealed that considerable levels of xylanase were produced by *T. reesei* Rut C-30 when the pH of the medium was 4-4.5. In other words, while the maximum enzyme production of *T. reesei* was found to be strongly

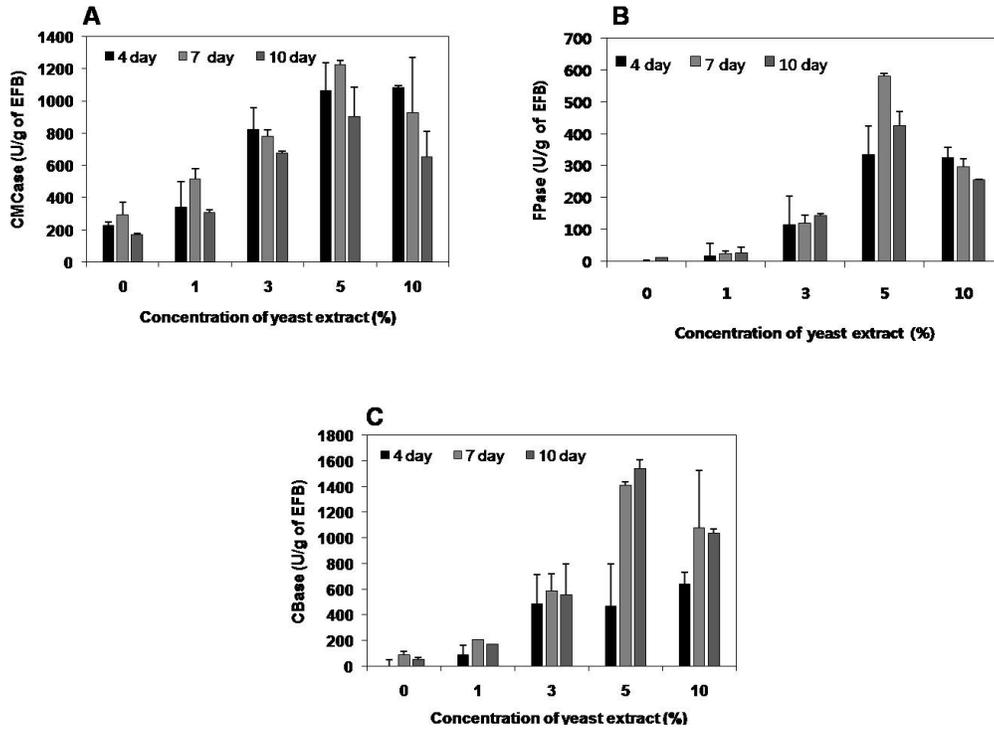


Figure 2. Effects of different concentrations of yeast extract on cellulase production. Varying concentrations (w/w) of yeast extract were added at the beginning of SSF (before the spore inoculation). (A) CMCase, (B) FPase, (C) CBase. SSF Conditions: pH 7; temperature, 30°C; moisture content, 50%; nitrogen source, 0~10% yeast extract; particle size, 2 mm; no pretreatment.

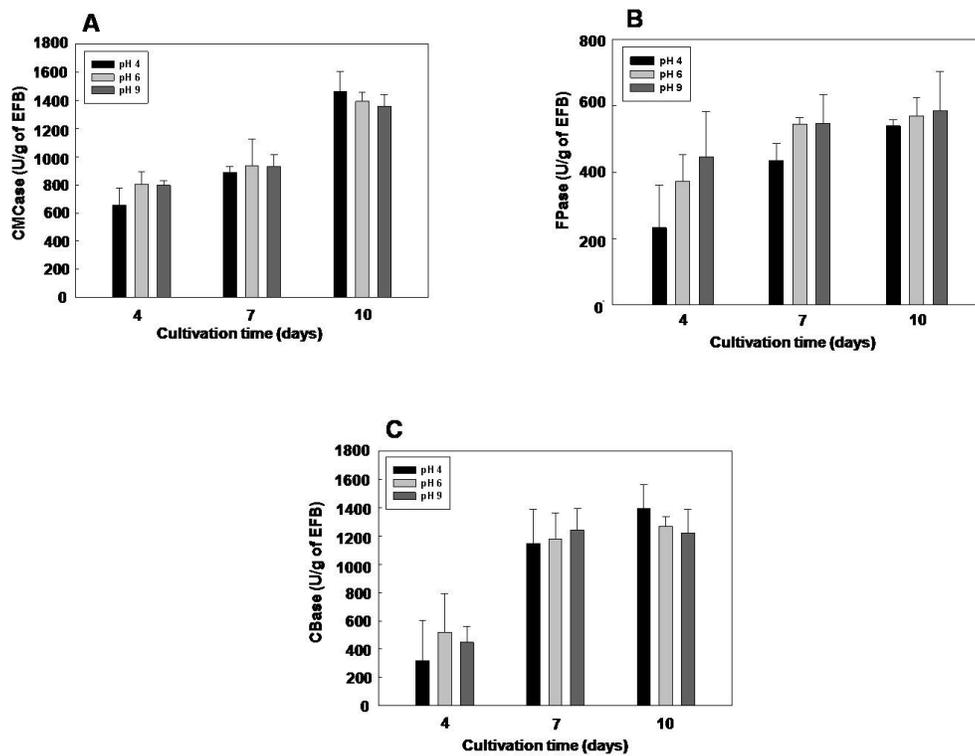


Figure 3. Effect of initial pH on cellulase production. (A) CMCase, (B) FPase, (C) CBase. SSF conditions: pH 4~9; temperature, 30°C; moisture content, 50%; nitrogen source, 5% yeast extract; particle size, 2 mm; no pretreatment.

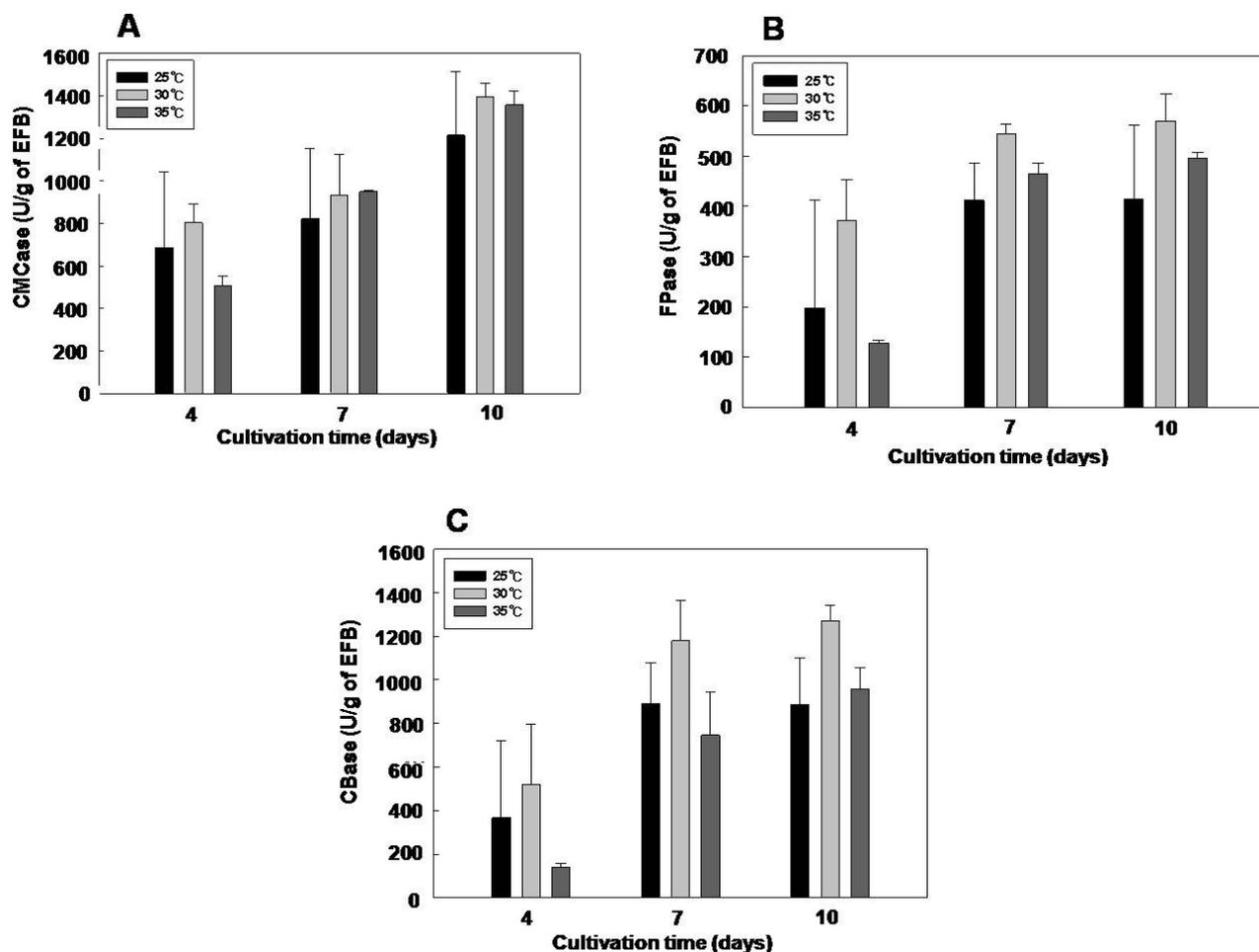


Figure 4. Effect of growth temperature on cellulase production (A) CMCase, (B) FPase, (C) CBase. SSF conditions: pH 7; temperature, 25-30°C; moisture content, 50%; nitrogen source, 5% yeast extract; particle size, 2 mm; no pretreatment.

pH-dependent, this was not the case for the cellulase production of the *Penicillium* sp.

Effect of temperature

The temperature normally employed in SSF is in the range of 25-35°C so the effect of temperature on cellulase production in SSF was studied by incubating the bottle at 20, 25 and 30°C. The results show that FPase and CBase production were the highest at 30°C and reduced at 35°C as shown in Figure 4.

However CMCase production at the two temperatures was comparable. Overall, the temperature most suitable for cellulase production by *Penicillium* sp. GDX01 was 30°C.

This observation is similar to a previous study carried out by Atif et al. (2004). Deswal et al. (2011) also reported increased cellulase production by *Fomitopsis* sp. RCK2012 up to 30°C, with further temperature increase strongly inhibiting enzyme production.

Effect of initial moisture content

The initial moisture content of growth media is an important variable for SSF. Kalogeris et al. (2003) reported that the optimal initial moisture content (IMC) depends on the microorganism, biomass, and the type of end product. *Penicillium* sp. GDX01 showed maximal production of cellulases at 40 or 50% IMC (Figure 5). More specifically, CMCase and FPase activities were highest at 50% IMC (Figures 5A and B) whereas that of CBase was at 40%.

At higher than 60% IMC, the activities of CMCase, FPase and β -glucosidase were all drastically reduced. Interestingly, FPase activity was more sensitive overall to changes in IMC (Figure 5B). However, Bahrin et al. (2012) reported highest cellulase production when the IMC was at 24-32% and Devendra et al. (2012) reported that 70% moisture content was suitable for cellulase production. Therefore, our report taken with these other two indicates that although optimal temperatures may differ depending on the exact system, cellulase production

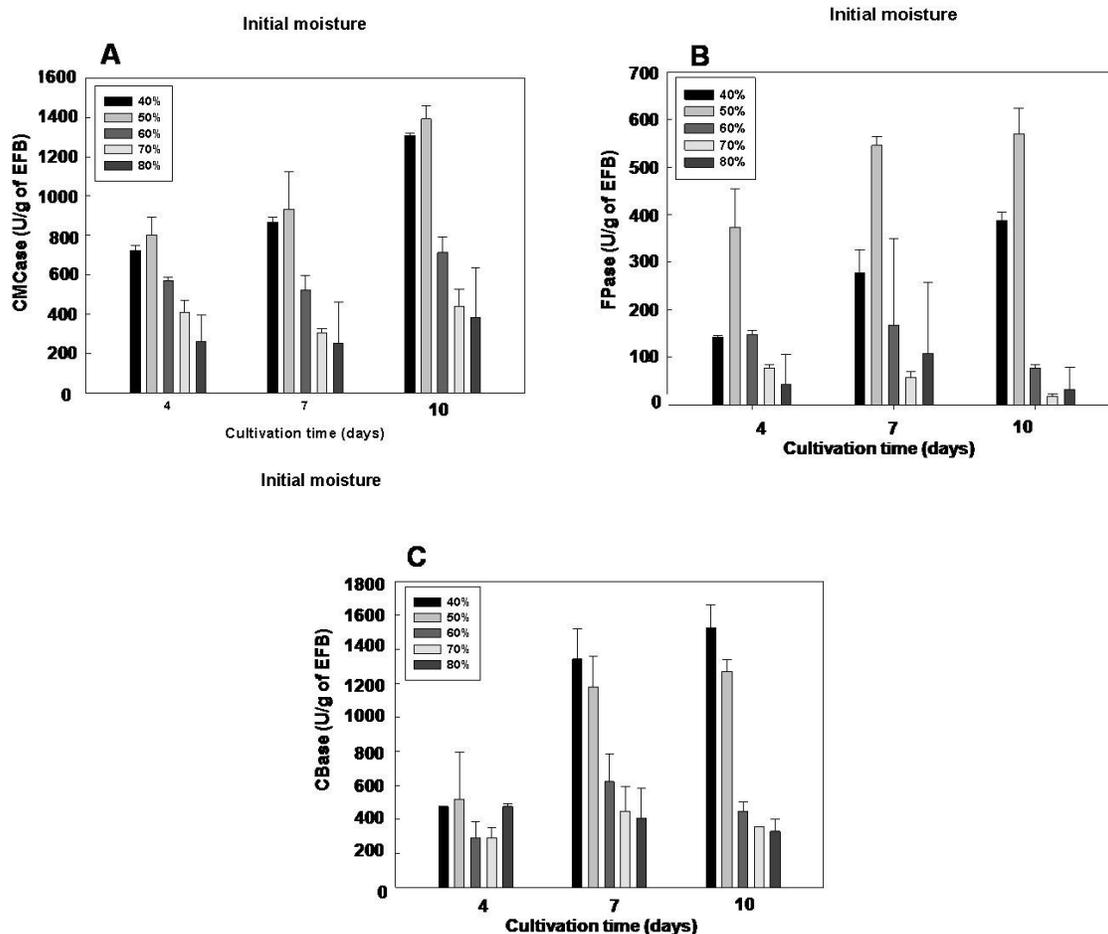


Figure 5. Cellulase activity using EFB with different initial moisture contents. Different volumes of deionized water were added before sterilizing the biomass. Data are presented as the mean percentage of initial water content of the total weight of the SSF mixture. (A) CMCase, (B) FPase, (C) CBase. SSF Conditions: pH7; temperature, 30°C; moisture content, 40-80 %; nitrogen source, 5% yeast extract; particle size, 2 mm; no pretreatment.

in SSF is highly sensitive to IMC.

Effect of biomass particle size

Biomass particle size could also affect enzyme production in SSF. While smaller-sized particles have a surface/volume ratio, leading to better nutrient absorption, a too finely-powdered biomass may inhibit gas exchange and heat transfer. Therefore, an optimal range of biomass particle size must be determined for maximal enzyme production.

Two different EFB particle sizes (2 and 5 mm) were tried to elucidate the relationship between biomass size and cellulase production in SSF. As shown in Figure 6, all cellulase activity was higher with the smaller-sized EFB particles. This was consistent with the report of Bahrin et al. (2011) where higher FPase and CMCase activities were observed with smaller-sized EFB particles. In our

study, β -glucosidase showed the same relationship, albeit less pronounced.

Effect of pretreatment

Pretreatment of the lignocellulosic biomass improves the enzymatic hydrolysis rates by changing its chemical and physical structure. Pretreatment could also help fungi have easier access to biomass and to test this, a sample of EFB was treated with sodium hydroxide before SSF. However, the results showed that more cellulases were obtained from untreated than treated EFB, suggesting that some unknown components involved in the induction of cellulase expression may be removed during pretreatment or that chemicals generated during pretreatment may inhibit cellulase induction (Figure 7).

Alkali-pretreated sugarcane bagasse has also been reported to yield less cellulase under liquid fermentation

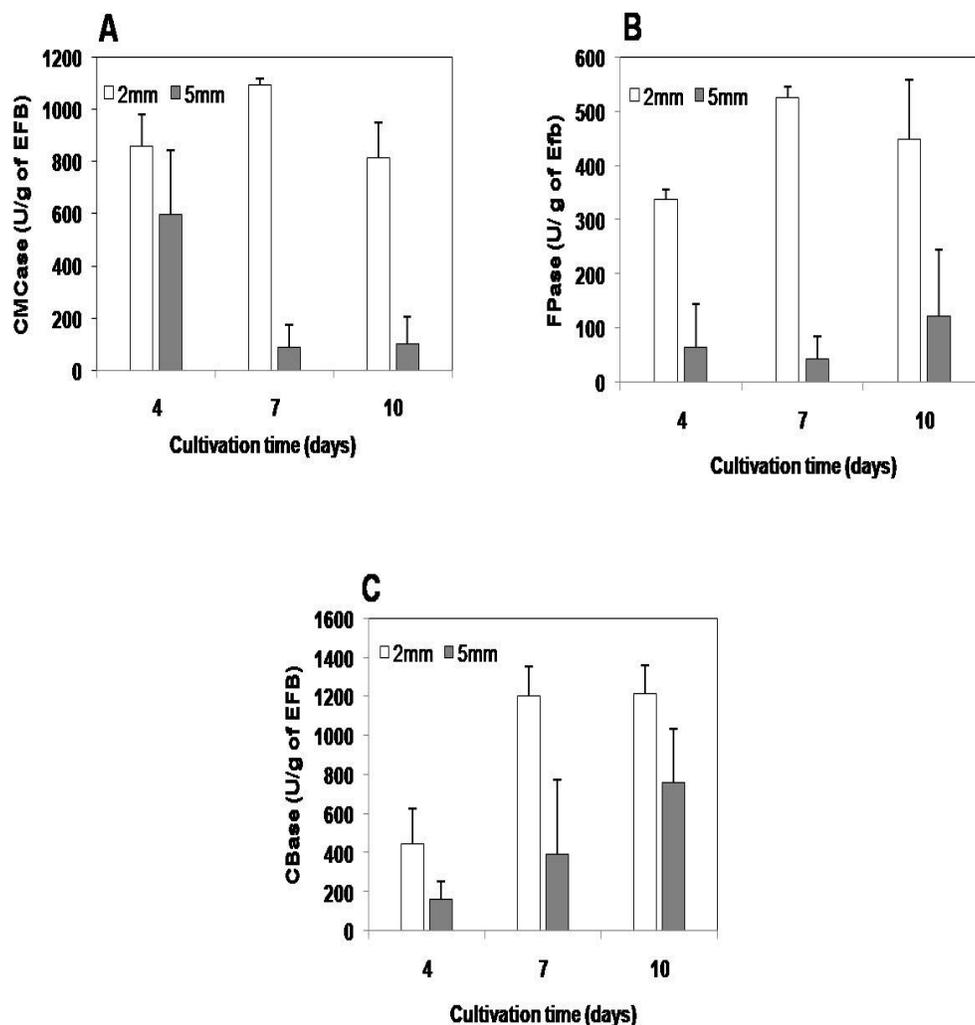


Figure 6. Cellulase activity using EFB with different particle sizes. The EFB was crushed through a cutter mill and sorted by particle size. (A) CMCCase, (B) FPase, (C) CBase. SSF conditions: pH 7; temperature, 30°C; moisture content, 50%; nitrogen source, 5% yeast extract; particle size, 2 mm and 5 mm; no pretreatment.

with *T. reesei* (Aiello et al., 1996). Brijwani and Vadlani, (2011) argued that changes in lignin and hemicelluloses and modification of cellulose structure could counter the positive effects of alkali-pretreatment. They also showed that acid pretreatment also lowered cellulase production. However, steam pretreatment resulted in a significant increase of approximately 4-fold in cellulase production in *T. reesei* fermentation of soybean hulls. In our experiment, the EFB was sterilized at 121°C for 15 min before seeding the fungi and this may have contributed to the high production of cellulase by *Penicillium* sp. GDX01.

Comparison of *Penicillium* species

Since *Penicillium* sp. GDX01 has a high cellulase activity

in the SSF of EFB, other *Penicillium* species may also have comparable cellulase activities. To investigate this, the cellulase activities during SSF of two different strains of *P. oxalicum* and 1 strain of *P. diversum* ordered from the KCTC were measured. At day 4, *Penicillium* sp. GDX01 showed a higher FPase activity than the other *Penicillium* species (Figure 8A). Although by day 10 a strain of *P. oxalicum* (KCTC6440) showed FPase activity, it was less than half of that of *Penicillium* sp. GDX01. *P. diversum* did not show any FPase activity, but exhibited a CMCCase activity that is weak compared to that of *Penicillium* sp. GDX01. The two KCTC *Penicillium* species have similar β -glucosidase activity to *Penicillium* sp. GDX01 by day 10 of SSF (Figure 8C). Thus while the ITS sequence of *Penicillium* sp. GDX01 is highly similar to that of *P. oxalicum*, the organism differs in being able to produce high amounts of cellulase during the SSF of EFB.

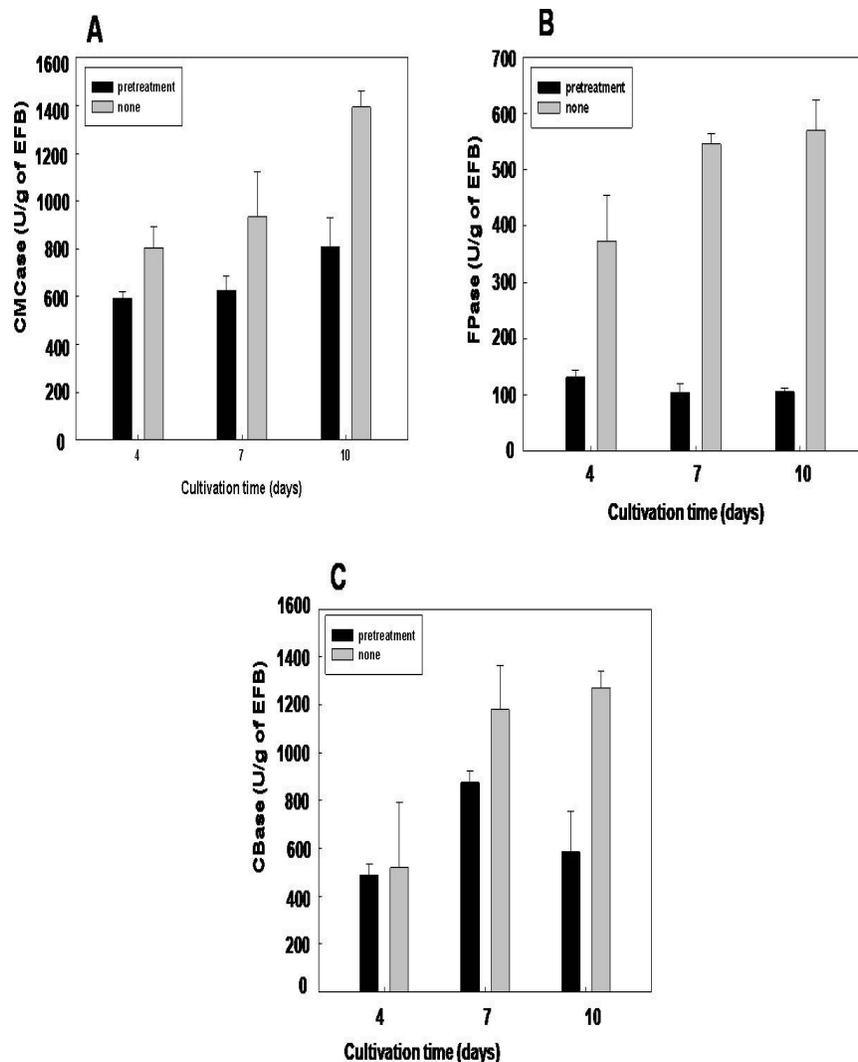


Figure 7. Effect of EFB pretreatment on cellulase activity. The EFB was treated with sodium hydroxide before being used in SSF. (A) CMCase, (B) FPase, (C) CBase. SSF conditions: pH 7; temperature, 30°C; moisture content, 50%; nitrogen source, 5% yeast extract; particle size, 2 mm; no pretreatment and alkali-pretreatment.

Saccharification using cellulases from *Penicillium* sp. GDX01

Cellulases from *Penicillium* sp. GDX01 and commercial cellulase (Celluclast 1.5L) were used in the saccharification of NaOH-pretreated EFB, and their hydrolytic capacities were compared. Celluclast 1.5L (Novozymes, Denmark) was used without the addition of β -glucosidase. The time-course experiment of enzymatic saccharification revealed that a higher yield of glucose was obtained with cellulases from *Penicillium* sp. GDX01 than from Celluclast 1.5L (Figure 9), with the difference more markedly observed at the initial stages of saccharification (Figure 9). The low β -glucosidase activity of Celluclast 1.5L may explain the difference in glucose yield of the 2 cellulases.

Hydrolysis yields of 81%, 71% obtained with 5 FPU GDX01 and 5FPU celluclast respectively. Compared with 10 FPU purified enzyme mixture, the saccharification yield of 5 FPU GDX01 was more than 15% higher than it (Anikó et al., 2010). These results demonstrated that the cellulases from *Penicillium* sp. GDX01 can be usefully applied to the saccharification of EFB to produce glucose, a key step in bioethanol production from EFB.

Conclusion

Penicillium sp. GDX01 isolated from moistened EFB was found to secrete cellulases under SSF using EFB. Optimal enzyme production was obtained with 5% yeast extract and 40-50% moisture. Analysis of the saccharification of pretreated EFB showed that the cellulases of *Penicillium*

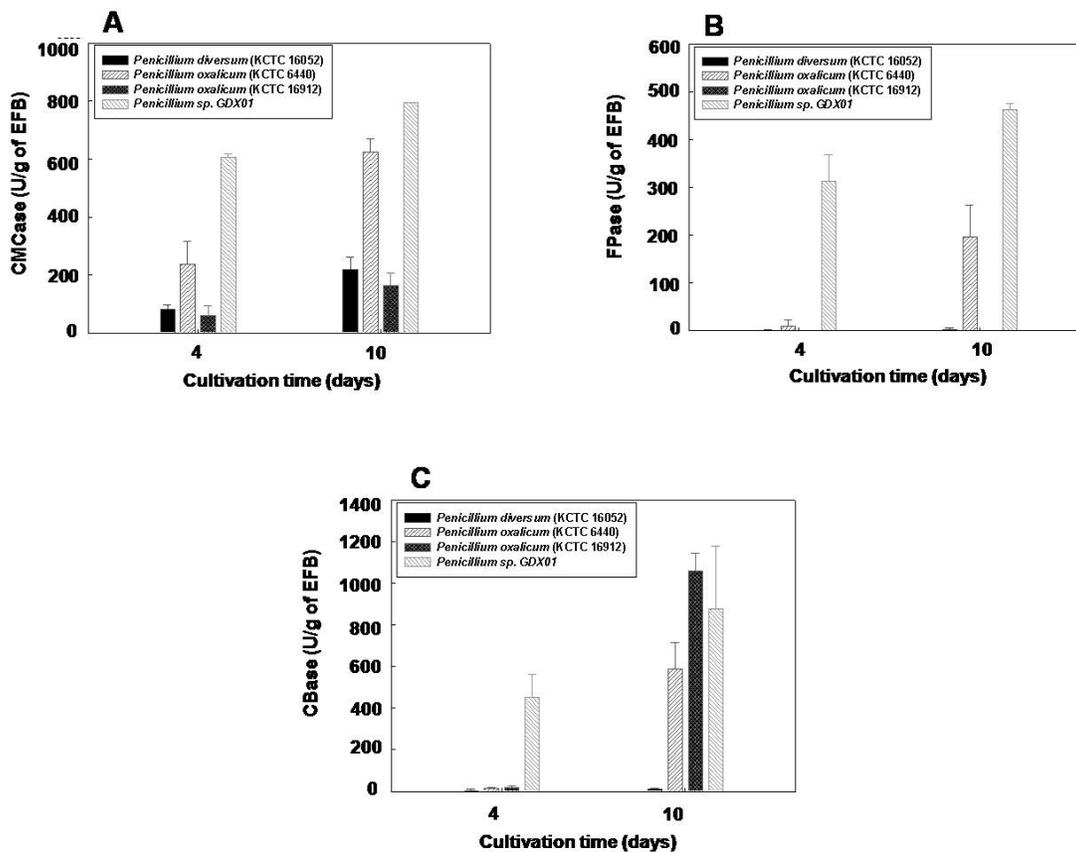


Figure 8. Comparison of cellulase activities of different *Penicillium* species. (A) CMCase, (B) FPase, (C) CBase. SF conditions: pH 7; temperature, 30°C; moisture content, 50%; nitrogen source: 5% yeast extract; particle size, 2 mm; no pretreatment.

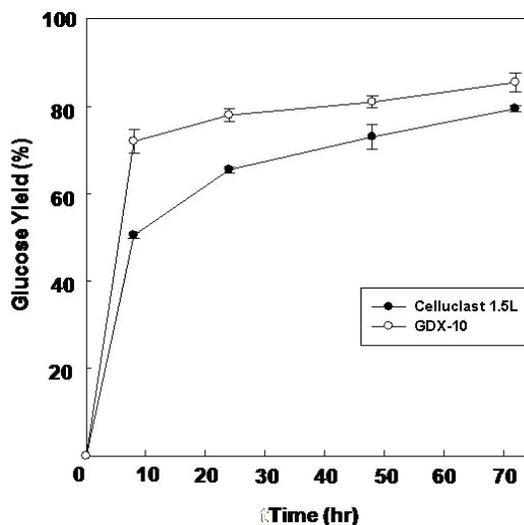


Figure 9. Saccharification of EFB by Celluclast 1.5L and GDX-10 (10-fold concentration of crude enzyme by SF). Pretreated EFB was subjected to saccharification by different cellulases. Y-axis shows the percentages of cellulose converted to glucose. Saccharification conditions: Enzyme loading 5 FUP/g glucan; sodium citrate pH 4.8; temperature 50°C; time 72 h; biomass loading 10%.

sp. GDx01 have a higher hydrolytic activity than those of commercial cellulases.

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