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Cellulase-Producing Bacteria from Thai Higher Termites, *Microcerotermes* sp.: Enzymatic Activities and Ionic Liquid Tolerance

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Abstract The three highest hydrolysis-capacity-value isolates of *Bacillus subtilis* (A 002, M 015, and F 018) obtained from Thai higher termites, *Microcerotermes* sp., under different isolation conditions (aerobic, anaerobic, and anaerobic/aerobic) were tested for cellulase activities—FPase, endoglucanase, and β-glucosidase—at 37 °C and pH 7.2 for 24 h. Their tolerance to an ionic liquid, 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), was also investigated. The results showed that the isolate M 015 provided the highest endoglucanase activity whereas the highest FPase and β-glucosidase activities were observed for the isolate F 018. The isolate F 018 also showed the highest tolerance to [BMIM]Cl in the range of 0.1–1.0 vol.%. In contrast, the isolate A 002 exhibited growth retardation in the presence of 0.5–1.0 vol.% [BMIM]Cl.

Keywords Cellulose · Cellulase-producing bacteria · *Microcerotermes* sp. · *Bacillus subtilis* · Ionic liquid · 1-butyl-3-methylimidazolium chloride

Introduction

Bioethahol is considered to be a cleaner energy than fossil fuel because it does not produce undesirable end products containing sulfur, nitrogen, and aromatic contents, which are normally

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generated from the combustion of conventional fuels [1]. Bioethanol can be used as a pure fuel (100%) or blended with gasoline or diesel with suitable blending ratios [2]. Commercially, bioethanol is produced by the fermentation of sugar-containing feedstocks, such as sugarcane, corn, sugar beet, wheat, and barley, by yeast [3]. Increasing bioethanol demand causes a competitiveness in using these feedstocks for energy and food. To avoid these problems and improve the sustainability of bioethanol production, a new renewable resource must be used to replace those mentioned raw materials. Cellulose, comprising glucose units, which is a main component of most agricultural residues, can be used as a promising feedstock for the future production of chemicals and fuels, including ethanol. However, direct conversion of cellulose to ethanol is not economically feasible due to the rigidity of the crystalline structure of cellulose, which results from both intra- to inter-hydrogen bondings between the cellulose chains, and so cellulose can resist chemicals and microbial enzymes [4]. Hence, pretreatment steps are required to enhance the hydrolysis rate by increasing the adsorption of cellulase on the cellulose surface area, which is defined by particle size and porosity [5]. The degradability of cellulose at the surface may be again limited by crystallinity, which prevents certain enzymatic hydrolysis activities [6, 7]. The crystalline structure of cellulose has to be destroyed preliminarily in order to increase the accessibility of cellulase toward the treated cellulose. There are several pretreatment methods available: using acids, alkalis, and heat. Cellulose pretreatment by chemical processes has been widely studied using dilute-acid pretreatment, alkaline pretreatment, and ammonia fiber explosion [8–10]. However, all of these chemical pretreatment methods produce by-products generated from the cellulose degradation, which afterwards inhibit the subsequent step of hydrolysis, or fermentation. In addition, these pretreatment processes require high temperature and/or high pressure, as well as high chemical consumption, which, in turn, can affect the environment apart from the high treatment cost [11].

Apart from the mentioned pretreatment processes, enzymatic hydrolysis is of great interest since it can be carried out at ambient temperature and pressure. Cellulase, which is secreted extracellularly by several microbes, including bacteria from higher termites [12–14], has been widely investigated to hydrolyze cellulose. Cellulases comprise of at least three different enzymes with different specificities, i.e., endo-acting endoglucanase that cleaves chains in the cellulose interior, exo-acting exoglucanases or cellobiohydrolases that cleaves cellobiose units from the cellulose chain ends, and β-glucosidase that cleaves cellobiose to two glucose molecules. All of these enzymes can cleave cellulose and hemicellulose to simple sugars, including glucose; however, the enzymatic hydrolysis rate of the cellulose is relatively low because of its high crystallinity.

Recently, a new interesting pretreatment method, which can enhance the sequential cellulose hydrolysis rate (and is more environmentally friendly), is solvent pretreatment using ionic liquids (ILs) to reduce the crystallinity of the cellulose [15, 16]. It was reported that 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), one type of effective ILs, could dissolve up to 25 wt.% of the cellulose without any degradation products. The precipitation of [BMIM]Cl-dissolved cellulose could be achieved by adding water or alcohol. After the IL-treatment step, the cellulose structure was found to change from crystalline to amorphous, which resulted in a significant increase in the enzymatic hydrolysis rate and cellulose conversion [17]. It was also mentioned that a trace amount of IL was found in the regenerated cellulose. Hence, a toxicity test of IL is required.

The objective of this work, therefore, was, for the first time, to isolate cellulase-producing bacteria from Thai termites (lower termites: *Schedorhinotermes* sp.; and higher termites: *Microcerotermes* sp.) under various isolation conditions. All isolates were preliminarily screened for selecting effective cellulase-producing bacteria by using the Congo red–polysaccharide interaction technique. The most effective cellulase-producing bacteria,

including their tolerance to [BMIM]Cl, were selected and preliminarily tested for possibility of using their end products, glucose, as a substrate for ethanol fermentation in next step.

Methods

Culture Media and Chemicals

National Institute of Health (NIH) thioglycollate broth, for culturing anaerobic bacteria, and tryptic soy broth were purchased from Difco Laboratories (USA). Carboxymethycellulose (CMC) with 99.5% purity and 1-butyl-3-methylimidazolium chloride, [BMIM]Cl, (Basionics®ST 70) with 99% purity were obtained from Sigma (USA) and Fluka (Germany), respectively. Biochemical test kits (API 20 E and API 50 CHB) were purchased from Biomérieux (France). A Genome DNA Simax kit for DNA extraction was purchased from Beijing SBS Genetech Co., Ltd. (China).

Termite Collection

Lower termites (*Schedorhinotermes* sp.) and higher termites (*Microcerotermes* sp.) were obtained from Nakhon Pathom province located in the central part of Thailand. Random sampling of lower termites from an ant hill to higher termites from coconut wood were collected as sources of cellulase-producing bacteria. Before the enrichment and isolation steps, these termites were surface-sterilized in 70 vol.% ethanol, and then washed in a sterile normal saline solution.

Enrichment and Isolation of Cellulase-Producing Bacteria from Termites

Cellulase-producing bacteria from the lower termites and from the higher termites were isolated under three different conditions: aerobic, anaerobic, and anaerobic/aerobic. The enrichment/isolation procedures are described below.

Aerobic conditions One hundred surface-sterile termites were ground and transferred into 20 test tubes, which contained 10 mL of a 65 modified Deutsche Sammlung von Mikroor Ganismen und Zellkulturen (DSMZ) medium 1 [14], supplemented with different cellulose sources (5 gL^{-1} of CMC or 5 gL^{-1} of Whatman filter paper No.1, FP). After that, the inoculum tubes, which had different cellulose sources, were divided into two groups and incubated in aerobic conditions at 37 °C and room temperature for 1 month.

Anaerobic conditions Twenty surface-sterile termites were ground and added to 9 mL of a sterile normal saline solution at ambient conditions for 1 h. Then, 0.5 mL of the solution was transferred to a test tube containing 7 mL of an NIH thioglycollate broth. The test tube was then incubated in anaerobic conditions at 37 °C for 1 day. After that, 0.5 mL of the culture medium was transferred to a fresh NIH thioglycollate broth and incubated at the same conditions. This step was repeated six times. Next, 0.5 mL from all dilution tubes were transferred to 7 mL of a fresh NIH thioglycollate broth, which contained 5 gL^{-1} of both CMC and FP. All of the culture media were anaerobically incubated at 37 °C for 1 month.

Anaerobic/aerobic conditions Twenty surface-sterile termites were prepared with six dilutions using a similar method to the anaerobic conditions. After that, 0.5 mL of each dilution was

transferred to 7 mL of fresh 65 modified DSMZ medium 1, which also contained 5 gL^{-1} of both CMC and FP. The culture medium was incubated in aerobic conditions at 37 °C for 1 month.

Afterwards, the enriched culture from each isolation condition (aerobic, anaerobic, and anaerobic/aerobic) was sampled every 5 days for four times and 10 days for the last time to streak on the 65 modified DSMZ medium 3 [14]. All bacterial isolates from each isolation condition were purified by re-streaking on the plate until all pure isolates were obtained.

Preliminary Screening of Cellulase-Producing Bacteria

All of the bacterial isolates were single-spotted on a CMC agar or 65 modified DSMZ medium 3, stained by a 0.1 wt./vol.% Congo red solution for 10 min, and then flooded with a 0.1 M NaCl solution on each individual colony. A clear zone on the culture plate, indicating the cellulase activity, was used to identify the cellulase-producing bacteria [18]. All of the cellulase-producing bacteria were preliminarily investigated for their potential to degrade cellulose by using the hydrolysis capacity (HC) value, which is calculated by the following equation:

$$\text{Hydrolysis capacity value} = \frac{\text{Diameter of clear zone (cm)}}{\text{Diameter of bacterial colony (cm)}} \quad (1)$$

The cellulase-producing bacteria obtained from the lower and higher termites under three different isolation conditions, which had the highest HC values, were selected as effective isolates for further investigation.

Cellulase Activity Assay

To prepare the crude enzyme for quantitative assay, each effective isolate was grown in 50 ml of the 65 modified DSMZ medium 3 (production medium), and all of the culture media were incubated in an orbital shaker at 37 °C and 200 rpm for 24 h. The samples taken from the production medium at different times (4, 8, 10, 12, 16, 20, and 24 h) of bacterial growth were centrifuged at 9,828×g for 10 min at 4 °C to separate the bacterial cells. These cultured supernatants were used as the crude enzyme quantitative assay. The experiments were also performed at other incubation temperatures—40, 45, and 50 °C—in order to investigate the effects of incubation time and temperature on the cellulase activities and bacterial growth.

FPase activity testing was performed by the method described by Ghose [19], with some modifications. Endoglucanase activity testing was performed by the method described by Mandels et al. [20]. β-glucosidase activity testing was conducted by the method suggested by Kubicek [21]. All data obtained from at least triplicate trials were averaged and used to assess the enzymatic activities of the test isolates.

The amount of released reducing sugar, which refers to the cellulase activities, was measured by the dinitrosalicylic acid method [22], using glucose as the standard. One unit (U) of cellulase activity is defined as the amount of enzyme yielding 1 μmol of glucose equivalent within 1 min under the assay conditions. The amount of protein from cultured supernatant from each bacterial isolate was determined by using the method reported by Lowry et al. [23] with triplicate trials.

Effect of [BMIM]Cl on Toxic Tolerance and Specific Growth Rate of Cellulase-Producing Bacteria

Each selected effective isolate obtained from the three isolation conditions was grown in 3 ml of the 65 modified DSMZ broth medium 3, which contained various concentrations of

[BMIM]Cl ionic liquid (0.1, 0.5, 1.0, 5.0, and 10.0 vol.%), at 37 °C in an orbital shaker at 200 rpm for 24 h. Each test strain without [BMIM]Cl was used as a control. The samples taken at different growth times were analyzed for the bacterial concentration by measuring the optical density in terms of light absorbance at 550 nm, and the growth curves were obtained by plotting the bacterial concentration versus time. The effect of [BMIM]Cl concentration on the growth rate of each strain was determined by calculating the specific growth rate (μ, h^{-1}) from the exponential phase of the growth curve.

Characterization and Identification of Isolated Bacteria

Bacterial isolates on the 65 modified DSMZ agar medium 3 were examined for their microbiological and physical properties: colonial appearance, pigmentation, cell shape, Gram's staining, and spore forming under microscopic examination, including oxidase and catalase tests. The biochemical properties of the bacterial isolates were obtained using biochemical test kits (API 50 CHB and API 20 E), according to the manufacturer's manual. The results were analyzed with APILAB Plus software.

The genomic DNA of the effective isolates was extracted by using the Genome DNA Simax Kit for determination of nucleotide sequence of 16S rRNA gene. The extraction method was used according to the manufacturer's instructions. The polymerase chain reaction (PCR) analysis followed the procedure described by Horn et al. [24] with some modifications. The PCR amplification was carried out in a 10-μL mixture in a DNA thermocycler TP 600 (TaKaRa Bio Inc., Otsu, Shiga, Japan). The conditions consisted of 35 cycles of initial denaturation at 94 °C for 1 min, denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min [25]. The forward and reverse primers were as follows [26]:

Primer 1 16F27: 5′-AGAGTTTGATCCTGGCTCAG-3′
Primer 2 16R1522: 5′-AAGGAGGTGATCCAGCCGCA-3′

The PCR products were visualized using a gel electrophoresis apparatus (Mupid-ex, Bruker BioSpin Inc., Fällanden, Switzerland) with 10 μL of the reaction mixture in a 2 wt./vol.% agarose gel in a Trizma-EDTA buffer, or TE buffer. DNA was made visible by 0.5 μg ml^{-1} of Ethidium bromide staining and ultraviolet transillumination. After that, the PCR products were purified and sequenced for the 16S rRNA gene (Macrogen Inc., Seoul, South Korea). The obtained 16S rRNA gene was compared with the 16S rDNA sequences available in the Blast database.

Results and Discussion

Isolation Results

Fifteen bacterial isolates from the lower termites, *Schedorhinotermes* sp., did not produce any clear zone on the culture plates, indicating that the test isolates could not produce cellulase (data not shown). These results of undetectable cellulase-producing bacteria from the Thai lower termites agree well with the previous reports that the lower termites do not use bacteria for degrading cellulose, but rather rely on protozoa to degrade cellulose to glucose [27–29]. Forty-seven from 57 of the bacterial isolates from the higher termites, *Microcerotermes* sp., showed clear zones on the culture plates, suggesting that cellulase-producing bacteria presumably inhabit in termite guts as flora. Table 1 shows the number of

Table 1 Number of cellulase-producing bacteria and effective isolates from higher termites, *Microcerotermes* sp.

Isolation conditions	No. of isolates	No. of cellulase-producing isolates	Effective isolates[a]
Aerobic	13	7	A 002
Anaerobic	20	19	M 015
Anaerobic/aerobic	24	21	F 018
Total	57	47	3

[a] Cellulase-producing bacteria that gave the highest HC value of >2.0

isolates, the number of cellulase-producing bacteria, and the most effective isolates under different isolation conditions. The present results also confirm that cellulose-degrading bacteria are general residents in the higher termites' guts [12–14], and they play a major role in degrading cellulose in the higher termite guts. Based on the highest HC value (>2) under different isolation conditions, only A 002, M 015, and F 018 were selected as the effective isolates for further investigation.

Effect of Incubation Temperature on Cellulase Activities

Figure 1 shows the cellulase activity (FPase, endoglucanase, and β-glucosidase) profiles of the isolate A 002 at different incubation temperatures. For any given temperature, the FPase activity profile was found to increase with time and reach a maximum (Fig. 1a). Beyond the optimum time (~10 h), the FPase activity declined. For any given incubation time, the FPase activity decreased with increasing temperature in the studied range of 37 to 50 °C. The highest FPase activity of the isolate A 002 at about 10 h of incubation time was found to be 0.626, 0.529, 0.497, and 0.445 U mg protein^{-1} at 37, 40, 45, and 50 °C, respectively. The results indicate the significant influence of incubation temperature on the FPase activity. The incubation temperature also greatly affected the endoglucanase activity profile of the isolate A 002 when the temperature was below 40 °C, whereas at the temperature higher than 40 °C, only slight variation of the endoglucanase activity was observed (Fig. 1b), resulting in almost the same activity at different temperatures. For any given incubation temperature, the endoglucanase activity increased with time until reaching a maximum, and then gradually decreased. The highest endoglucanase activity of the isolate A 002 was found to be 0.814, 0.598, 0.625, and 0.598 U mg protein^{-1} at 37, 40, 45, and 50 °C, respectively. As shown in Fig. 1c, the β-glucosidase activity profile of the isolate A 002 at each temperature was found to be quite similar to the FPase activity profile (Fig. 1a). For any given incubation time, the β-glucosidase activity decreased with increasing temperature, as also observed for the FPase activity. The highest β-glucosidase activity of the isolate A 002 was 0.620, 0.555, 0.523, and 0.421 U mg protein^{-1} at 37, 40, 45, and 50 °C, respectively. It can be seen that among the three cellulase activities of the isolate A 002, the endoglucanase activity was higher than the FPase and β-glucosidase activities at the incubation temperature of 37 °C.

The three cellulase activity profiles of the isolate M 015 at different incubation temperatures are illustrated in Fig. 2. The FPase activity profiles at the temperature of 37, 40, and 45 °C slightly increased with incubation time to the maximum values at approximately 10 h of incubation and then decreased; however at 50 °C, the activity profile dropped with increasing incubation time (Fig. 2a). The highest FPase activity of the isolate

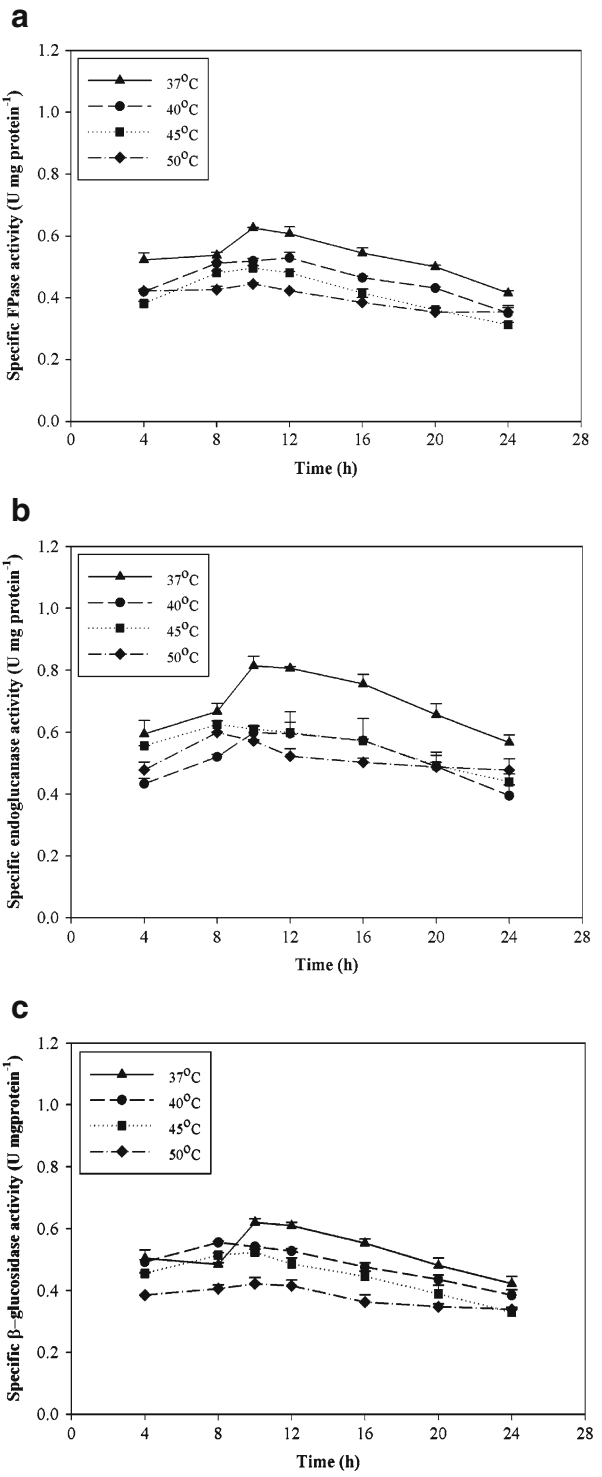

Fig. 1 Specific enzyme activities (U mg protein^{-1}) of isolate A 002 at various incubation temperatures (37, 40, 45, and 50 °C): **a** FPase, **b** endoglucanase, and **c** β-glucosidase

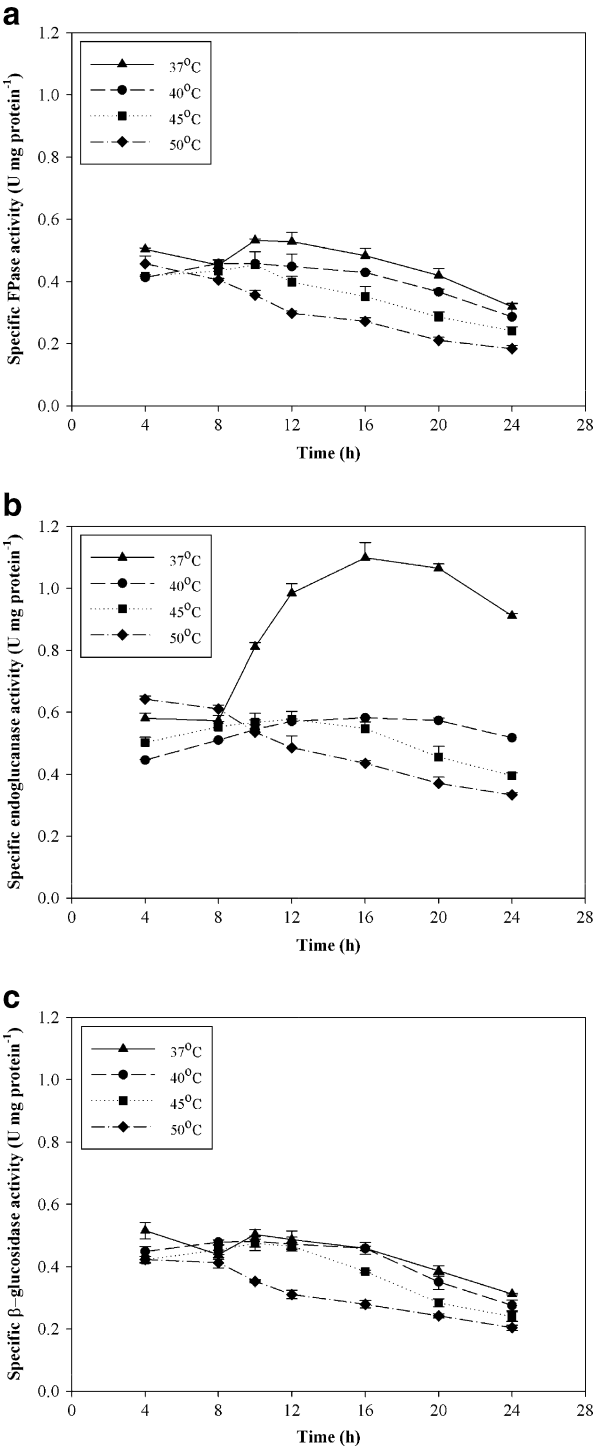

Fig. 2 Specific enzyme activities (U mg protein^{-1}) of isolate M 015 at various incubation temperatures (37, 40, 45, and 50 °C): **a** FPase, **b** endoglucanase, and **c** β-glucosidase

M 015 was 0.532, 0.458, 0.454, and 0.457 Umg protein^{-1}at 37, 40, 45, and 50 °C, respectively. In contrast to the FPase activity, largely different endoglucanase activities of this isolate (M 015) at different temperatures were observed (Fig. 2b). At 37 °C, the endoglucanase activity profile was found to be almost constant in the first 8 h. After that, the activity sharply increased to a maximum at 16 h and then gradually declined with time. At 40 and 45 °C, the increases in the endoglucanase activity were observed within 16 and 12 h, respectively, and after that, the activity for both temperatures slightly decreased. The endoglucanase activity profile at 50 °C was quite different from the profiles at the other temperatures; the activity decreased with increasing incubation time, without showing a maximum at any intermediate time. The highest endoglucanase activity of the isolate M 015 was 1.098, 0.581, 0.577, and 0.642 Umg protein^{-1} at 37, 40, 45, and 50 °C, respectively. For the β-glucosidase activity of this isolate (Fig. 2c), the activity profiles appeared to decrease with incubation time and temperature. The maximum β-glucosidase activity of the isolate M 015 was 0.516, 0.479, 0.475, and 0.423 Umg protein^{-1} at 37, 40, 45, and 50 °C, respectively. It can be observed for all three cellulase activities of the isolate M 015 that for any given incubation time below the optimum time, the three activities were quite comparable among the different incubation temperatures; however, beyond the optimum time, the higher the temperature, the lower the three activities. In addition, this isolate showed a higher endoglucanase activity than the other two activities at the incubation temperature of 37 °C, as similarly observed for the isolate A 002.

Figure 3 depicts the three cellulase activity profiles of the isolate F 018 at different incubation temperatures. The FPase activity at 37, 40, and 45 °C, shown in Fig. 3a, slightly increased to reach the highest value after 10 h, and then it decreased with increasing incubation time, as also observed for the isolate M 015; however, at 50 °C, the activity profile was nearly constant up to the first 10 h. The highest FPase activity of the isolate F 018 was 0.684, 0.557, 0.501, and 0.497 Umg protein^{-1} at 37, 40, 45, and 50 °C, respectively. For the endoglucanase activity of this isolate (Fig. 3b), the activity profiles in the temperature range of 40 to 50 °C remained relatively constant in the first 8 h, and then gradually increased until reaching the maximum values. In contrast, at 37 °C, the endoglucanase activity profile sharply increased to reach a maximum, and dropped after 20 h. The highest endoglucanase activity of the isolate F 018 was 0.900, 0.582, 0.551, and 0.658 Umg protein^{-1} at 37, 40, 45, and 50 °C, respectively. The β-glucosidase activity profiles of this isolate were also somewhat different in the studied temperature range (Fig. 3c). At 37 °C, the activity profile slightly dropped with increasing incubation time from 4 to 8 h prior to a sharp increase to a maximum whereas at 40 °C, a sharp increase in the activity within the first 10 h was observed before reaching a maximum. Nevertheless, at 45 and 50 °C, the activity profiles slightly increased within the first 12 h before decreasing with time. The highest β-glucosidase activity of the isolate F 018 was 0.673, 0.606, 0.540, and 0.473 Umg protein^{-1}at 37, 40, 45, and 50 °C, respectively. It can be seen comparatively that the incubation temperature of 37 °C provided the highest cellulase activities for the isolate F 018, while exhibiting a higher endoglucanase activity than the other two activities, as also observed for the above two isolates.

Comparison of Cellulase Activities and Protein Production of Three Effective Isolates

The comparative FPase activity profiles of all the isolates at 37 °C revealed a similar trend (Figs. 1a, 2a, and 3a). The FPase activities of the isolates F 018 and M 015 were almost constant from 4 to 8 h and then sharply increased to maximum values of 0.684 and 0.532 U mg protein^{-1}, respectively. After that, they decreased significantly with increasing

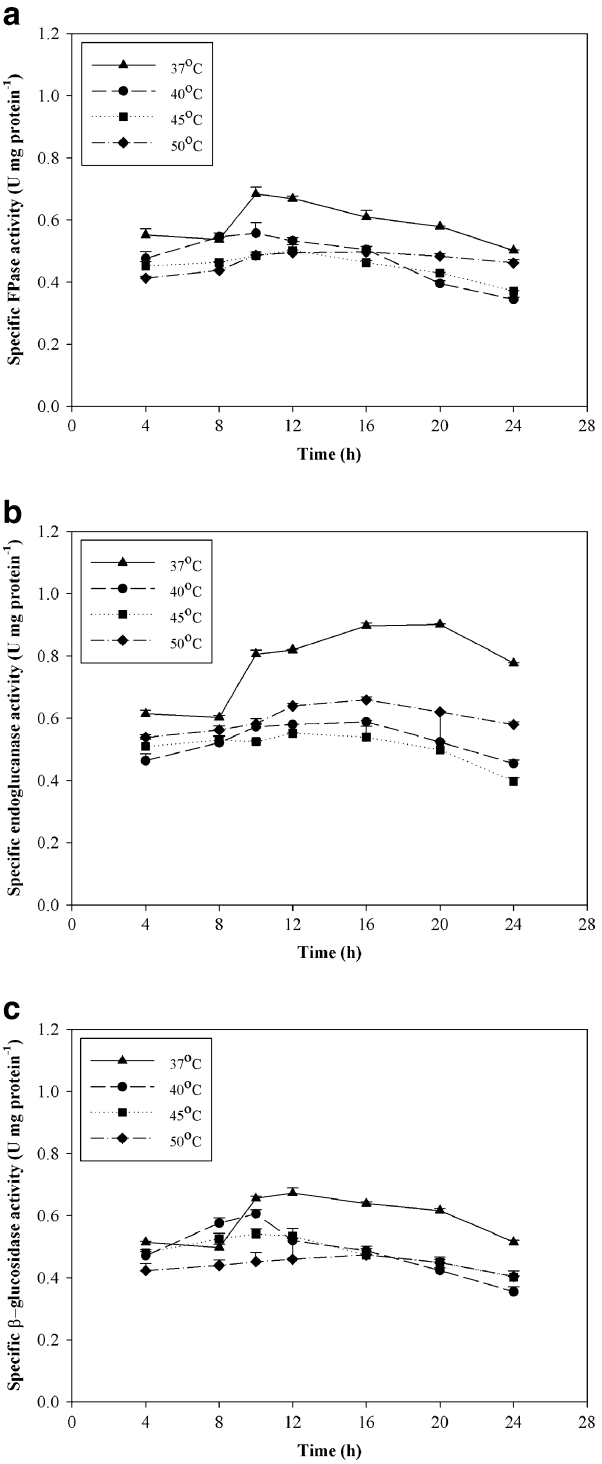

Fig. 3 Specific enzyme activities (U mg protein^{-1}) of isolate F 018 at various incubation temperatures (37, 40, 45, and 50 °C): **a** FPase, **b** endoglucanase, and **c** β-glucosidase

incubation time whereas for the isolate A 002, the FPase activity gradually increased with increasing incubation time from 4 to 10 h and reached a maximum activity of 0.626 U mg protein^{-1}. After that, the activity also decreased with increasing incubation time. The results show that the maximum FPase activities of the three isolates were in the following order: F 018>A 002>M 015.

Figures 1b, 2b, and 3b show comparative endoglucanase activity profiles of the three isolates. The activity profile of the isolate M 015 was relatively constant in the first period (4 to 8 h) and then sharply increased after 8 h. Its profile reached the maximum activity of 1.098 U mg protein^{-1} at 16 h before gradually decreasing with incubation time. The activity profile of the isolate F 018 showed a similar trend to that of the isolate M 015, but with a lower maximum activity (0.900 U mg protein^{-1}) at a longer incubation time (20 h). However, the activity profile of the isolate A 002 was different from those of the other two isolates. The activity increased from 4 to 10 h until reaching the maximum value of 0.814 U mg protein^{-1} at 10 h and then steadily decreased with increasing time. The results show that the isolate M 015 had a higher maximum endoglucanase activity than the other two isolates, and the maximum activities were in the following order: M 015>F 018>A 002.

As shown in Figs. 1c, 2c, and 3c, the profile characteristics of the β-glucosidase activity of all the isolates had a similar trend to those of the FPase activity. In the first period (4 to 8 h), the activities of all the isolates slightly decreased, but they rapidly increased to the maximum values at 10 h for the isolates M 015 and A 002 and at 12 h for the isolate F 018. From the maximum β-glucosidase activities of 0.620, 0.516, and 0.673 U mg protein^{-1} for the isolates A 002, M 015, and F 018, respectively, the maximum β-glucosidase activities were in the following order: F 018>A 002>M 015.

The comparative protein production results of the three effective isolates at different incubation temperatures are given in Fig. 4. It can be clearly seen that the protein production either tended to only slightly decrease or remained nearly invariant with time. The increase in incubation temperature from 37 to 50 °C was found to increase the protein production, but not considerably for each isolate. Even though the lowest investigated incubation temperature of 37 °C exhibited a more significant effect on the protein production as compared with higher temperatures, each effective isolate could produce protein in the same range of 1.2 to 2.2 mg.

Toxicity Results of [BMIM]Cl

In order to monitor the effect of [BMIM]Cl on bacterial growth during the in situ treatment of cellulose, the toxic tolerance to [BMIM]Cl of the three effective isolates was studied by observing the change in growth curves at different concentrations of [BMIM]Cl within 24 h. As shown in Fig. 5, all the isolates could tolerate the [BMIM]Cl in the concentration range of 0.1 to 1.0 vol.%, but they completely died when the [BMIM]Cl concentration exceeded 5.0 vol.%. In addition, the growth retardation, as observed by the presence of the lag phase, was found only in the case of the isolate A 002 when the [BMIM]Cl concentration was in the range of 0.5 to 1.0 vol.%. The lag phases were changed from 2 h, which is a normally observed lag phase for the control experiment (with 0 vol.% [BMIM]Cl), to 6 and 11 h when the [BMIM]Cl concentration increased to 0.5 and 1.0 vol.%, respectively. However, the lag phases of the isolates A 002 and M 015, even though the [BMIM]Cl concentration increased up to 1.0 vol.%, were still found to be the same as that of the control experiment (2 h).

Since the [BMIM]Cl tolerable range of all the three effective isolates was found to be 0.1 to 1.0 vol.%, the specific growth rate (μ) of each isolate was determined in this tolerable

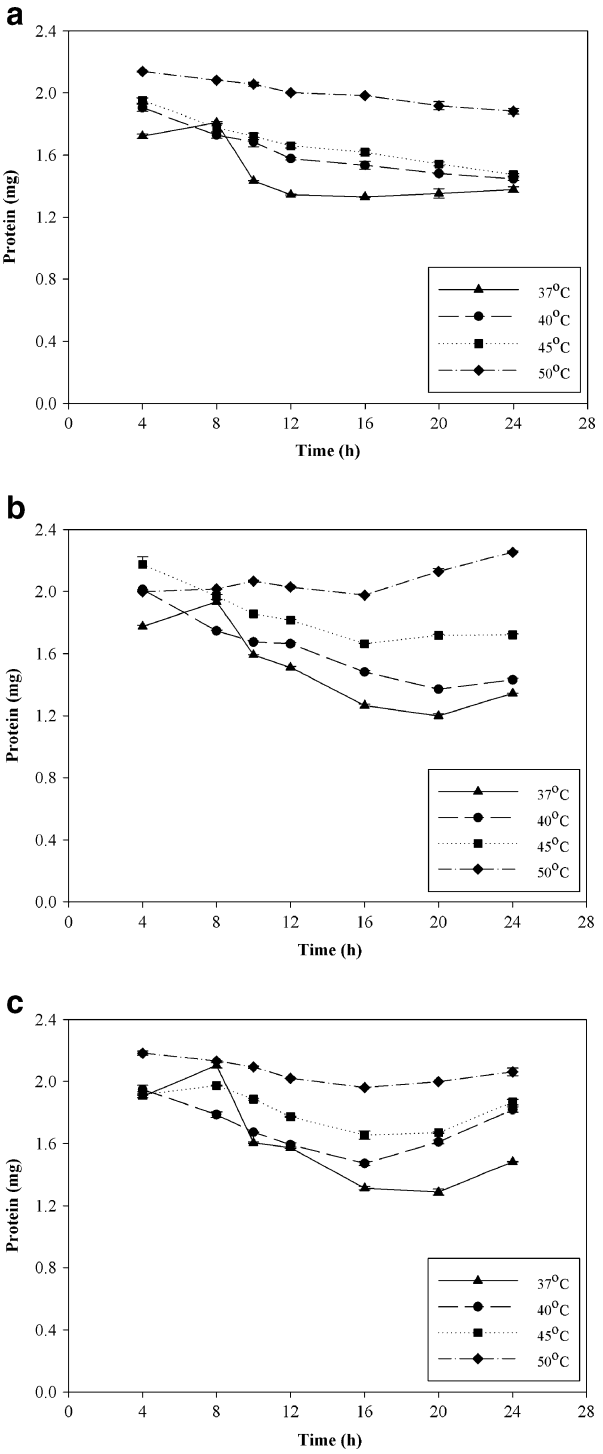

Fig. 4 Total cultured supernatant protein production (mg) of the three effective isolates **a** A 002, **b** M 015, and **c** F 018 at various incubation temperatures (37, 40, 45, and 50 °C)

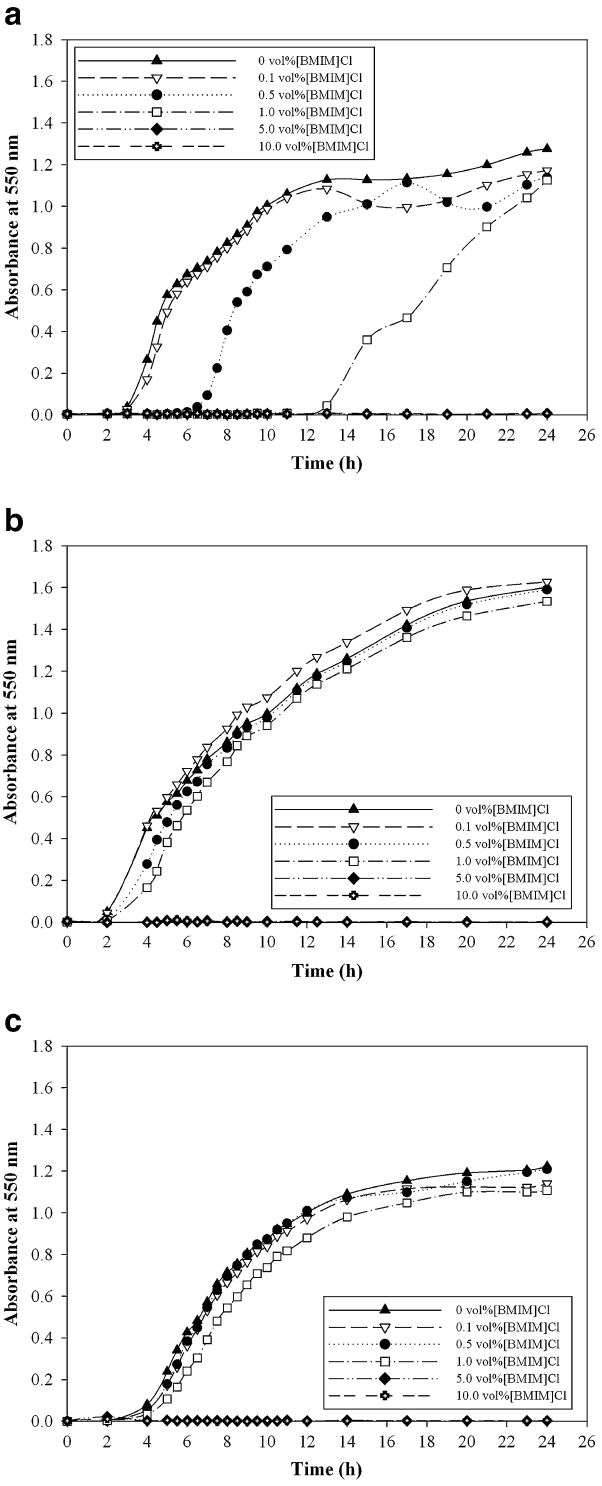

Fig. 5 Growth profiles of the three effective isolates: **a** A 002, **b** M 015, and **c** F 018 in the presence of various concentrations of [BMIM]Cl at 37 °C

Table 2 Average specific growth rate (μ, h^{-1}) of isolates A 002, M 015, and F 018 in the [BMIM]Cl tolerable range of 0.1 to 1.0 vol.% at 37 °C

Isolate	Average specific growth rate (μ, h^{-1})
A 002	0.111
M 015	0.221
F 018	0.288

range from the exponential phase of the growth curve (Fig. 5). It was found that the specific growth rates of each isolate at various [BMIM]Cl concentrations in the tolerable range were insignificantly different. Therefore, the specific growth rates were averaged and used for a growth comparison (Table 2). It is clearly seen that the specific growth rates of all isolates were in the following order: F 018>M 015>A 002. These results indicate that the isolation of cellulase-producing bacteria from the higher termites by the anaerobic/aerobic condition provided an effective isolate (F 018) that possessed the highest specific growth rate.

Bacteria Identification

The microbiological characteristics of the three effective isolates are summarized in Table 3. Slight differences in the colonial appearance and pigmentation for the three isolates were observed possibly due to their unique physical characteristics; however, their other microbiological properties were the same. With a combination of the biochemical tests and the 16S rRNA gene sequencing results (data not shown), the isolates A 002 and M 015 were identified as *Bacillus subtilis* with 99.9% identity. However, different identification results from the biochemical tests and the 16S rRNA gene sequencing for the isolate F 018 were obtained, i.e. as *Brevibacillus* non-reactive with 94.9% identity from the biochemical tests, but as *B. subtilis* from the 16S rRNA gene sequencing method with 99.9% identity. With the higher identity percentage from the 16S rRNA gene sequencing method, the isolate F 018 was more likely verified as *B. subtilis*.

Conclusions

In this work, 47 cellulase-producing bacteria were isolated from Thai higher termites, *Microcerotermes* sp., under three different isolation conditions. Only three effective isolates of A 002, M 015, and F 018 that possessed the highest HC value were tested comparatively for their enzymatic cellulase activities—Fpase, endoglucanase, and β-glucosidase— at 37 °

Table 3 Characteristics of isolates A 002, M 015, and F 018 by microbiological methods

Isolate	Colonial appearance	Pigmentation	Cell shape	Gram's staining	Spore forming	Oxidase test	Catalase test
A 002	Circular, flat, entire, rough, and membranous	Light brown cream	Rod	+	+	−	+
M 015	Spindle, raised, entire, glistering, and opaque	Light brown cream	Rod	+	+	−	+
F 018	Spindle, flat, filamentous, glistering, and opaque	Light green cream	Rod	+	+	−	+

C and pH 7.2. All the effective isolates were identified as *B. subtilis* by the 16S rRNA gene sequencing method. The results showed that the isolate M 015 exhibited the highest endoglucanase activity whereas the isolate F 018 gave the highest FPase and β-glucosidase activities. In addition, these effective isolates were tested for their toxic tolerance to [BMIM]Cl. All of the isolates were able to tolerate the [BMIM]Cl in the concentration range of 0.1 to 1.0 vol.%, and no growth retardation in the lag phases was observed, except that the isolate A 002 had a growth retardation in the [BMIM]Cl concentration range of 0.5 to 1.0 vol.%. Therefore, the cellulose-degrading ability of these isolates can potentially be used as a pretreatment step for alcohol fermentation.

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