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Original Research Article

Purification and Characterization of Thermostable Cellulase from Consortium XM70 in Terrestrial Hot Spring with Sugarcane Bagasse

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Abstract

Purpose: *To better understand the sugarcane bagasse (SCB) degradation process and obtain thermostable cellulase from terrestrial hot spring.*
Methods: *Molecular community structure of the newly selected thermophilic bacterial consortium XM70 was determined using the method of the full-length 16S rRNA library-based analysis. The thermostable CMCase was purified with ion-exchange and gel filtration chromatography.*
Results: *Sequence-based identification of species belonging to the genera, Geobacillus, Desulfotomaculum, Bacillus, Exiguobacterium, Paenibacillus and Enterobacter were identified. The maximal activities of carboxymethyl cellulase (CMCase), filter paper cellulase and β-glucosidase of the consortium XM70 were obtained after incubation at 60 °C and pH 6.0 (4 days), 80 °C and pH 7.0 (2 days) and 70 °C and pH 8.0 (4 days), respectively. The yield of reducing sugars in the culture broth achieved 0.11 $g.g^{-1}$ dry SCB. An extracellular CMCase from consortium XM70 (XM70-CMCase) was purified 7.9-fold to apparent homogeneity with a recovery of 65.41 % and its molecular mass was about 31.0 kDa. Maximum CMCase activity of the purified XM70-CMCase was 3.77 U/mg at 70 °C and pH 7.0. CMCase activity maintained about its maximal value of 70 % after incubation at 80 °C for 60 min.*
Conclusion: *Due to its high temperature stability, the purified XM70-CMCase may be useful for industrial application such as biofuel, animal feed industry, paper industry and clarification of fruit juices.*

Keywords: *Thermostable cellulase, Sugarcane bagasse, Purification, Characterization, Hot spring*

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INTRODUCTION

The unstable and uncertain petroleum sources and the rising cost of fuels have created concern when it comes to burning fossil fuels [1]. These concerns have shifted global efforts to utilize biomass as an alternative resource, which is considered as being a 'greener' energy source and can also meet the high energy demand.

Sugarcane bagasse (SCB) is one of the most abundant byproducts of agro-industry in the world, creating 540 million tons of residues per year [2,3]. It is a porous residue of cane stalks left over after the crushing and extraction of the juice from sugarcane, and is composed of 19 - 24 % lignin, 27 - 32 % hemicellulose, 32 - 44 % cellulose and 4.5 - 9 % ashes [4,5]. SCB has been used extensively because of its availability and high volume/low cost production potential. One of the key steps to utilizing SCB is the

efficient hydrolytic conversion of cellulose into soluble sugar [2]. There has been vast interest in bacterial consortium for converting cellulosic biomass to glucose. Compared to a single bacterium, bacterial consortium can produce more efficient and novel enzymes, which have great potential for bioenergy.

Cellulases are used in the agri-food, brewery wine, animal feed, textile laundry and paper industries [6]. Wide ranges of applications have made cellulase one of the most desirable enzyme systems. However, the mesophilic enzymatic hydrolysis technologies exhibit slow enzymatic hydrolysis rates, generate low yields of sugars, require high dosages of enzymes and are prone to microbial contamination problems [7]. These limitations could be overcome by using thermophilic bacteria and thermostable enzymes [8]. Highly thermostable cellulases are not only accompanied with less viscosity and microbial contamination [9], but also with increased reaction rate and reduced hydrolysis time [10]. Thermophilic cellulose-degrading bacteria have been isolated from various environments [11]. Researches have focused on enhancing thermostable cellulase activity to improve the yield and rate of enzymatic hydrolysis [12-14].

An efficient conversion of the SCB to soluble sugar requires synergistic action of various enzymes. The thermal and pH stability affect the potential application of the enzymes in large-scale cellulose hydrolysis process. Currently, one of the major bottlenecks is that the thermostable cellulase which can work efficiently and inexpensively at high temperature condition is insufficient, although much attention has been paid to the thermophilic bioprocessing of cellulosic biomass to glucose [15,16]. In this study, we identified the selected efficient thermophilic SCB-degrading consortium XM70 and described the purification and characterization of the purified thermostable cellulase. Cultural factors affecting cellulase production were also investigated in order to optimize the fermentation conditions for production maximization.

EXPERIMENTAL

Sample collection and consortium enrichment

The water column and sediment samples were aseptically obtained from a terrestrial hot spring in Xiamen, China (where the temperature was above 70 °C) using sterile equipment. The samples were transported to the laboratory in sterile polypropylene tubes in ice and stored at -20 °C until analyzed. The medium used for the culture contained (per liter): 10 g pretreated SCB, 1 g peptone, 10 g yeast extract, 2 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4$, 1 g KH_2PO_4, 0.5 g NaCl, 1 mL $MnSO_4•H_2O$ (0.25 %) and 1 mL $FeSO_4•7H_2O$ (0.75 %). The SCB was dried and chopped into small pieces by a hammer mill and finally separated by 40 mesh sieve. The pre-treatment of the SCB was carried out with 2 % (w/v) NaOH at 85 °C for 75 min. The pre-treated residues were washed extensively to neutral pH and dried to constant weight at 65 °C [3]. Two grams of samples were inoculated in 250-mL conical flasks containing 100-mL of sterilized minimal growth medium. The culture was performed by incubating the conical flasks at 70 °C in an incubator shaker (120 rpm) for 6 days. Cultures showing growth were subcultured ten times into fresh medium to obtain the stable thermophilic bacterial consortium.

Structure dynamics of thermophilic bacterial consortium

For molecular characterization of the bacterial consortium, 16S rRNA gene cloning and sequencing analyses were performed. The genomic DNA was extracted using the Bacterial DNA Isolation Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. The 16S rRNA genes of the bacterial consortium were amplified by PCR using primers 27F and 1492R [17]. The process of PCR was under the following conditions: 94 °C, 5 min; 30 cycles of 94 °C, 45 s; 55 °C, 45 s; and 72 °C, 90 s; 1 cycle of 72 °C, 10 min; and then 4 °C continuously. The 16S rRNA genes with the expected size (about 1500 bp) were purified using a DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China) and cloned into pMD18-T vector (Takara, Kyoto, Japan) followed by sequencing. After that, sequence analysis was performed using the BLAST algorithm. To test the evolutionary relationships, phylogenetic analysis was performed with the program MEGA 4.0 [18]. Multiple alignments of the sequences were performed using CLUSTAL W. Bootstrap values were determined based on 1,000 replications.

Enzyme assays and sugar yield analysis

The major cellulase component activities were characterized by assays of carboxymethyl cellulase (CMCase), filter paper cellulase (FPase) and β-glucosidase [19]. The CMCase and FPase activities were determined using the IUPAC standard procedure [20]. The β-

glucosidase activity was assessed using salicin as substrate by Chahal's method [21]. The reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid method [22]. One unit (U) of the activity was defined as the amount of enzyme releasing 1 µM reducing sugar per minute under the assay conditions. The specific activity was expressed in µmol/min/mg protein. The culture was centrifuged at 7000 rpm in 4 °C for 20 min and then the supernatant was used as crude enzyme in the determination of cellulase activities and sugar yield.

Quantification of cellulase activities in different cultural conditions

In order to examine the effects of temperature and pH on enzyme activities, the bacterial consortium was grown in a 100-mL of medium containing 1 g pre-treated SCB. The temperature of the culture varied from 50 °C to 90 °C with increments of ten degrees unit and the pH of the growth medium varied from 5.0 to 9.0 with increments of one pH unit using sodium citrate buffer (50 mM, pH 5.0-6.0), sodium phosphate buffer (50 mM, pH 7.0 - 8.0) and glycine-NaOH buffer (50 mM, pH 9.0). The enzyme assay was carried out as described in the preceding section after 2 days of active growth. The enzyme activities obtained at the optimal medium temperature and pH were used to calculate the relative percentage enzyme activity produced at other temperatures and pH values. In order to determine the optimal incubation time for cellulase activities and sugar yield, the inoculated flasks were incubated at 70 °C and pH 7.0 for a period of 8 days. Culture samples were collected on 0, 1, 2, 4, 6 and 8 days during the cultivation period. The supernatants were analyzed for cellulase activities and sugar yield as described previously. The maximum enzyme activities obtained at a particular incubation time were considered as 100 % and used to calculate the relative enzyme activity in presence of other incubation times.

Purification and characterization of XM70-CMCase

Bacterial cells were removed from culture broth by centrifugation at 7000 rpm for 20 min at 4 °C. First, the cell free supernatant was overnight precipitated to 80 % saturation with $(NH_4)_2SO_4$ at 4 °C and then the precipitated proteins were recovered by centrifugation [23]. The precipitate was dissolved in 20 mM sodium phosphate buffer pH 7.0. Then, for ion exchange chromatography, the sample was applied to DEAE Sephadex A-25 column equilibrated with a linear gradient of 0.1 - 1.0 M NaCl in the same buffer. At last, the fraction with the highest CMCase activity was further purified by gel filtration on Sephadex G-75 column with same buffer. The fractions were eluted at a flow rate of 1 mL/min. A total of 20 fractions were collected and assayed for CMCase activity. Fraction showing the maximum activity was selected for studying the purification and characteristics of the enzyme (XM70-CMCase).

Protein concentration of the XM70-CMCase enzyme was measured by using the Bradford method and the standard curve was prepared by using bovine serum albumin (BSA) as standard [24]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [25]. After electrophoresis, the gels were stained by a solution of 0.1 % (w/v) Commassie blue, 30 % (v/v) methanol and 10 % (v/v) acetic acid. Enzyme assay for XM70-CMCase were conducted in 3-mL reaction volumes containing 1 mL of an appropriate dilution of enzyme and 2 mL of 1 % (w/v) CMC solubilized in 200 mM sodium phosphate buffer (pH 7.0). The optimal temperature of XM70-CMCase was determined at different temperature ranging from 30 to 90 °C after incubation for 30 min. The optimum pH of XM70-CMCase was estimated in the pH range of 4.0 - 10.0 using different assay buffers at the optimum temperature as described above. The enzyme activities obtained at the optimal temperature and pH were used to calculate the relative percentage enzyme activity produced at other temperatures and pH values. The thermal stability of CMCase was tested by determining the enzyme activity remaining after incubation of the enzyme at different temperatures (70 - 100 °C with increments of 10 °C) for a period of 60 min. The residual activities were determined under optimum pH and temperature conditions. In all cases the initial activity was assumed to be 100 % and used to calculate the enzyme activities as percentages of the initial activity during the incubation period. Each experiment was repeated three times and the mean values were plotted for each experiment.

RESULTS

Isolation and analysis of thermophilic bacteria consortium

After subcultivation for 10 times, the thermophilic bacteria consortium, which demonstrated high cellulase activity, was obtained and designated as XM70. A total of twenty-one 16S rRNA gene sequences were deposited in GenBank with

accession numbers JQ041736-JQ041756 and generated 10 operational taxonomic units (OTUs). Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolates belonged to *Firmicutes* and *Proteobacteria* phyla (Fig 1). Among the detected bacteria in the consortium XM70, the following genera occurred: *Geobacillus, Desulfotomaculum, Bacillus, Exiguobacterium, Paenibacillus* and *Enterobacter*, respectively.

Optimization of cellulase and sugar production

The effects of various culture temperatures on cellulase production are presented in Fig 2a. The results reveal that consortium XM70 produced maximum CMCase, FPase and β-glucosidase at 60 °C, 80 °C and 70 °C, respectively. The optimum pH for CMCase, FPase and β-glucosidase are found to be 6.0, 7.0 and 8.0, respectively (Fig 2b). The time courses of CMCase, FPase and β-glucosidase activities are shown in Fig. 2c. The cellulase showed very high activities on CMC (0.052 U/mL), filter paper (0.044 U/mL), and salicin (0.046 U/mL) after incubation for 4, 2 and 4 days, respectively. SCB was tried as feed stock for production of reducing sugar. Fig 2d shows the content of residual reducing sugar in the culture broth within 8 days of incubation. The content of residual reducing sugar increased dramatically in the first 2 days of incubation, and then further increases in incubation time did not favor any obvious increase in sugar production. The maximum amount of residual reducing sugar in the culture broth released from 1 g of dry SCB was 0.11 g.

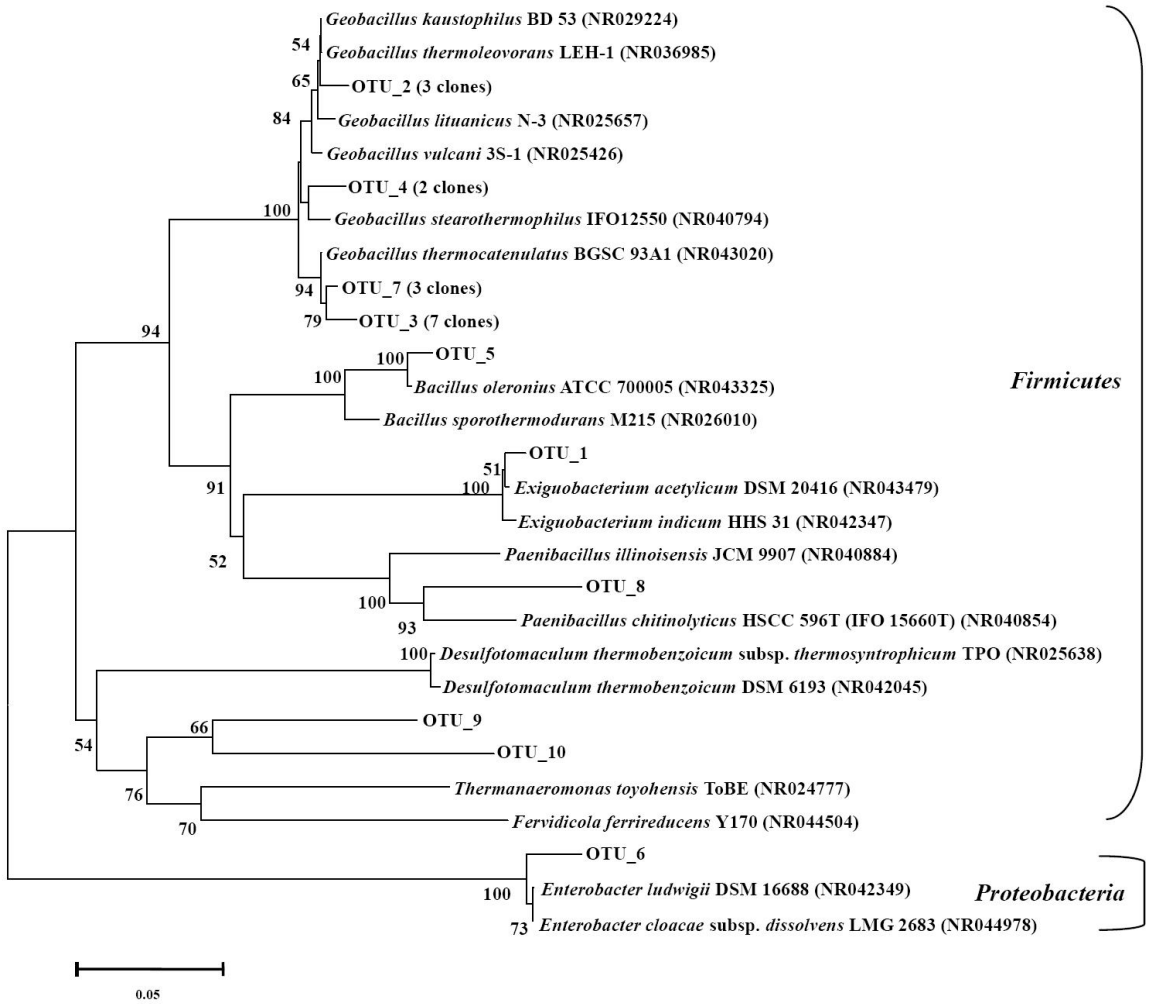

Fig 1: The phylogenetic tree of consortium XM70 (OUT_1 – OUT_10) and related strains based on the 16S rRNA gene sequences. The tree was based on Jukes-Cantor distance and constructed using a neighbor-joining algorithm using MEGA 4.0 software. Reliability of the predicted tree was tested using bootstrapping with 1000 replicates. The scale bar represents 0.05 substitutions per nucleotide position. Numbers at the node are the bootstrap values (%)

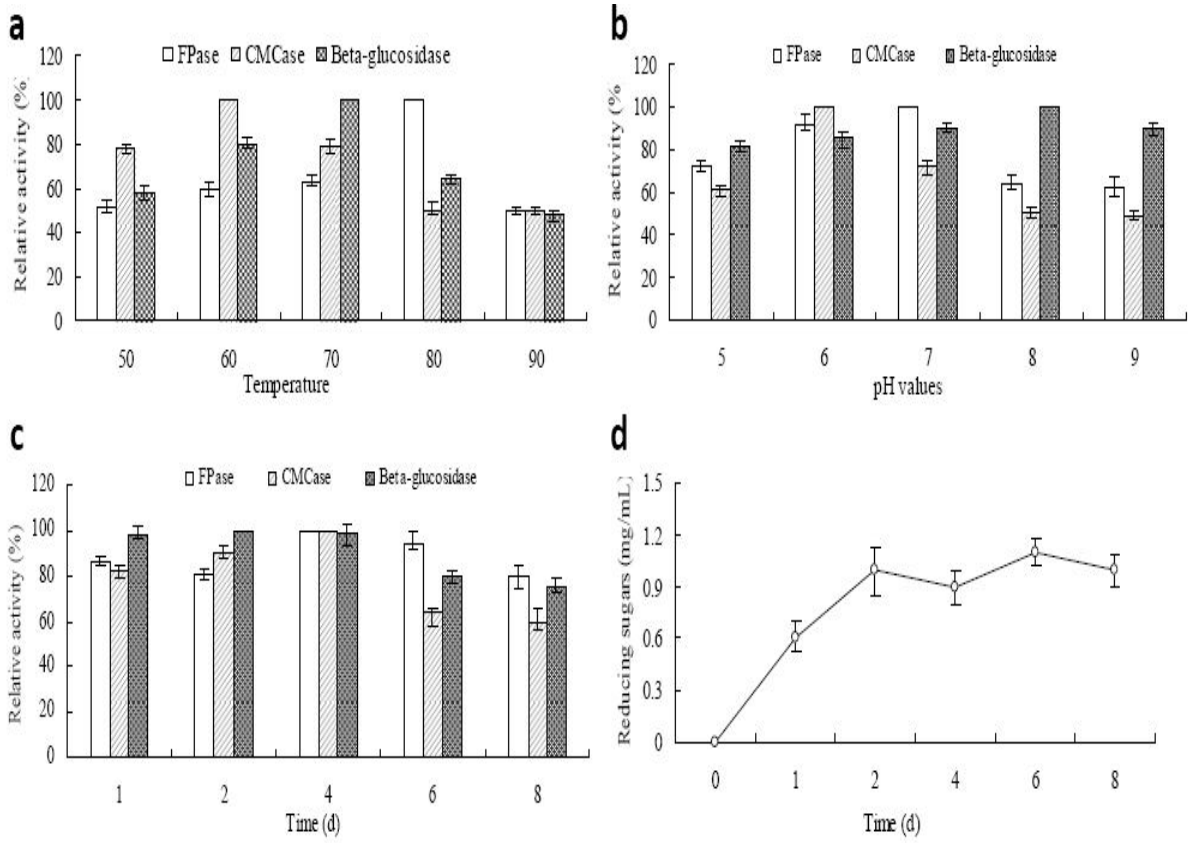

Fig 2: Effects of temperature (a) and pH (b) on the production of CMCase, FPase and β-glucosidase from consortium XM70 with pretreated bagasse. Time courses of cellulase production (c) and sugar yield (d) by consortium XM70 in defined medium (pH 7.0, 70 °C)

Purification and characterization of XM70-CMCase

XM70-CMCase was purified by three step procedure including ammonium sulphate precipitation, ion exchange on a DEAE Sephadex A-25 column and gel filtration on a Sephadex G-75 column. The purified XM70-CMCase from the culture broth of consortium XM70 following the steps indicated in Table 1 and showed 7.9-fold increase in activity with a final yield of 65.41 %. The specific activity which used CMC as the substrate was 3.77 U/mg after three steps of purification.

The protein purity was confirmed by the presence of a single band on SDS-PAGE and its relative molecular mass was 31.0 kDa (Fig 3). The effect of temperature on the CMCase activity was shown in Fig 4a. The optimal temperature of the purified XM70-CMCase was 70 °C. As shown in Fig 4b, the optimal pH for the CMCase activity was 7.0. The CMCase thermostability was assessed by incubating the enzyme at different temperatures (70 - 100 °C) for different times (0 - 60 min). Results shown in Fig. 4c indicated that CMCase was optimally stable at 70 °C and about 90 % activity was retained after incubation for 60 min. The enzyme was found to retain about 70 % of its maximal activity for 60 min at 80 °C. However, when at 90 and 100 °C, almost all activities were lost after 40 and 30 min of incubation, respectively.

Table 1: Summary of the purification of XM70-CMCase from consortium XM70

Purification steps	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	109.09	52.04	0.48	-	-
$(NH_4)_2SO_4$ precipitation	37.65	44.01	1.17	2.45	84.57
DEAE-Sephadex A-25	16.34	36.99	2.26	4.75	71.08
Sephadex G-75	9.03	34.04	3.77	7.90	65.41

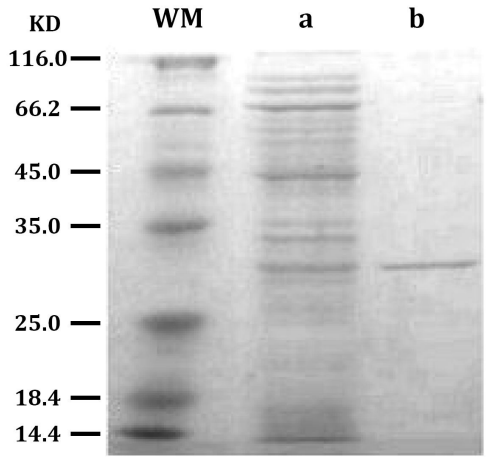

Fig 3: SDS-polyacrylamide gel electrophoresis of the purified XM70-CMCase. Protein molecular weight makers (WM), crude cellulose (a) and purified cellulose (b)

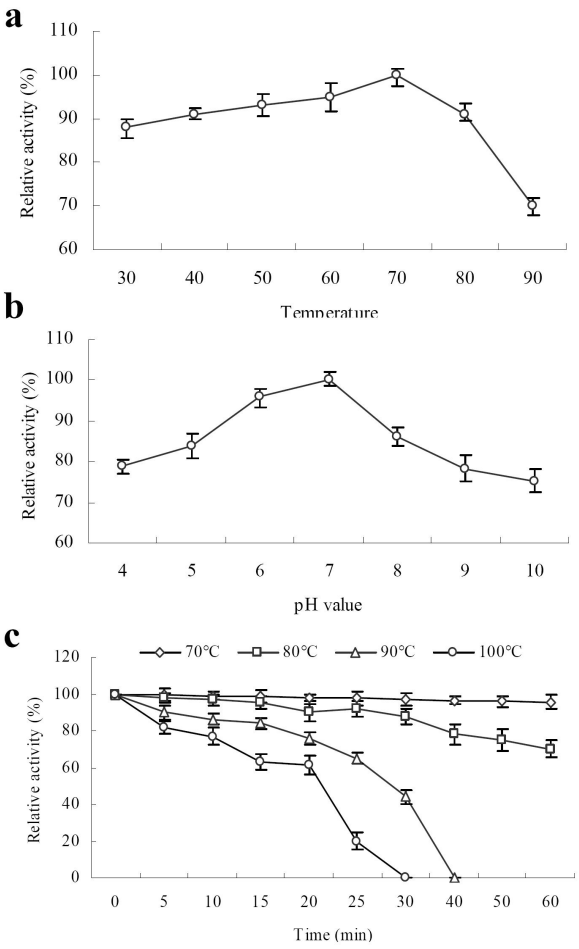

Fig 4: Effects of temperature (a), pH (b) and thermal stability (c) on CMCase activity of the purified XM70-CMCase enzyme

DISCUSSION

Lignocellulosic biomass is considered to be the most abundant renewable source of sugar. In order to make better use of it, more attention needs to be paid to the cellulase-producing bacteria. Over the past years, culturable cellulase-producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material from forestry or agricultural waste, the feces of ruminants, soil and organic matter, and extreme environments like hot-springs [26]. The bacteria consortium XM70 which isolated from terrestrial hot spring showed high cellulase activities and produced thermostable cellulases. Consortium XM70 included *Geobacillus, Desulfotomaculum, Bacillus, Paenibacillus, Exiguobacterium* and *Enterobacter* species, all of which were grouped in *Firmicutes* and *Proteobacteria*. Interestingly, both of them have been reported earlier after being discovered in a hot compost environment and their abilities to produce thermostable cellulases were also demonstrated [11]. Many researchers have purified and characterized cellulases isolated from different types of bacteria. However, bacteria consortium cellulases are often more complex and expressed in multi-enzyme complexes providing increased synergy and functions. A family of cellulases has at least three groups of enzymes (cellobiohydrolases, endo-glucanases and β-glucosidases) to complete the hydrolysis. Our results presented that production of cellulases requires the presence of the consortium and not a certain microorganism.

Nowadays, there is a great need to obtain sugars from cost-effective fermentation materials [27]. The SCB is a largely abundant and inexpensive fermentation material. However, most of them are not effectively utilized which results in a great waste. The consortium XM70 could hydrolyze pretreated SCB into soluble sugar. Moreover, it also had the ability to utilize SCB to produce the cellulase enzyme complex, which worked on the hydrolysis of cellulose. The total amount of reducing sugar released from 1 g of dry feed stock catalyzed by the crude enzyme was 0.11 g g-1 dry SCB. Deswal *et al* have isolated a brown rot fungus *Fomitopsis* sp. RCK2010 and used the crude enzymes for saccharification with alkali pre-treated wheat straw and rice straw [28]. The yields of reducing sugar from wheat and rice straw were 0.214 g g^{-1} dry mass and 0.157 g g^{-1} dry mass, respectively. Accumulation of 11 % sugar from delignified SCB in cultural broth showed consortium XM70 has industrial application potential.

Currently, much attention has been paid to the thermophilic bioprocessing of cellulosic biomass to biofuels which, due to the use of elevated temperatures [11]. Thermostable enzymes offer

several potential advantages in the hydrolysis of lignocellulosic materials [7]. There have been some reports about thermostable cellulase. The thermostable cellulase produced by *E. coli* TCP-1 retained more than 60 % of its maximal activity for at least 20 min at 50 - 70 °C and 10 min at 80 °C, respectively [29]. After 20 h incubation at temperatures ranged from 40 to 60 °C, the CMCase activity of the crude enzyme from *Aspergillus fumigatus* Z5 retained more than 80 % of the original CMCase activity, but at 80 and 90 °C, less than 10 % of the original CMCase activity was remained [30]. The FPase and CMCase activities of the crude enzymes from consortium XM70 were lower than plant cell wall-degrading fungi such as *Trichoderma* and *Aspergillus* [31]. But the thermal stability and broad pH-optimum were better.

Because the crude enzyme may have a lot of interfering substances for enzyme assays, Jantaporn and Haruyuki purified the CMCase from *Cryptococcus* sp. S-2 to homogeneity from the supernatant [32]. The optimum temperature for the purified enzyme was between 40 and 50 °C, and it retained approximately 50 % of its maximum activity after incubating at 90 °C for 1 h. In this paper, the maximum CMCase activity of the purified XM70-CMCase was measured at 70 °C and the specific activity which used CMC as the substrate was 3.77 U/mg. XM70-CMCase also retained approximately 90 % and 70 % of its maximal activity after incubating at 70 °C and 80 °C for 60 min. Compared with previous studies [33], the maximum CMCase activity of the purified XM70-CMCase is a little lower, but the thermal stability and broad pH-optimum of isolated CMCase at neutral and alkaline pH make this enzyme promising for application in detergents or denim wash.

CONCLUSION

Cellulase-secreted consortium XM70 in terrestrial hot spring has high thermo-tolerance. In particular, the purified enzyme has excellent thermal-resistance compared with those obtained previous studies. Their broad temperature and pH activity range as well as utilization of sugarcane bagasse constitute a contribution to current knowledge of bacterial consortium and their potential uses such as in the biofuel, animal feed and paper industries.

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