Journal of Genetic Engineering and Biotechnology (2017) **15**, 187–196

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Journal of Genetic Engineering and Biotechnology

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ORIGINAL ARTICLE

Production and characterization of an acido-thermophilic, organic solvent stable cellulase from *Bacillus sonorensis* HSC7 by conversion of lignocellulosic wastes

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Received 17 July 2016; revised 15 November 2016; accepted 19 December 2016
Available online 11 January 2017

KEYWORDS

Acidophilic;
CMCase;
Hot spring;
Lignocellulosic biomass;
Thermophilic

Abstract The acidophilic and thermophilic cellulase would facilitate the conversion of lignocellulosic biomass to biofuel. In this study, *Bacillus sonorensis* HSC7 isolated as the best thermophilic cellulose degrading bacterium from Gorooh hot spring. 16S rRNA gene sequencing showed that, this strain closely related to the *B. sonorensis*. CMCase production was considered under varying environmental parameters. Results showed that, sucrose and $(NH_4)_2SO_4$ were obtained as the best carbon and nitrogen sources for CMCase production. *B. sonorensis* HSC7 produced CMCase during the growth in optimized medium supplemented with agricultural wastes as sole carbon sources. The enzyme was active with optimum temperature of 70 °C and the optimum CMCase activity and stability observed at pH 4.0 and 5.0, respectively. These are characteristics indicating that, this enzyme could be an acidophilic and thermophilic CMCase. Furthermore, the CMCase activity improved by methanol (166%), chloroform (152%), while it was inhibited by DMF (61%). The CMCase activity was enhanced in the presence of Mg^{+2} (110%), Cu^{+2} (116%), Triton X-100 (118%) and it retained 57% of its activity at 30% NaCl. The compatibility of HSC7 CMCase varied for each laundry detergent, with higher stability being observed in the presence of Taj® and darya®. This enzyme, that is able to work under extreme conditions, has potential applications in various industries.

1. Introduction

Cellulose, a polymer of B (1–4) linked glucose units, is the most abundant, renewable energy source in the natural environment [1]. The industrial and agricultural wastes have been accumulating or are used inefficiently due to the high cost of

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Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2016.12.005

their utilization processes [2]. In the technologies for effectively converting cellulosic biomass into bioethanol and other chemicals, cellulases provide a key opportunity [3]. The complete enzymatic hydrolysis of cellulosic materials needs at least three types of cellulase including; endo (1, 4) b-D-glucanase [EC 3.2.1.4] (Carboxymethyl cellulase or CMCase), exo (1, 4) b-D-glucanase [EC 3.2.1.91] (cellobiohydrolase, avicelase, microcrystalline cellulase, b-exoglucanase) and b-glucosidase [EC 3.2.1.21] [4]. These three enzymes act synergistically to degrade cellulose materials [5]. Microorganisms from various environments had received more attention as a resource for newer enzymes. Bacteria have a high growth rate as compared to fungi; they have good potential to be used in cellulase production [6]. Several bacterial genera reported for cellulolytic activities include Erwinia, cellomonase, pseudomonas, Thermomonospora, Bacillus, Micrococcus, Bacteroides, Acetivibrio. Clostridium and Ruminococcus [7–9]. However, research on cellulase has addressed thermophilic enzymes only to a very limited extent. Thermophilic enzymes are usually optimally active between 60 and 80 °C. With their intrinsical stability and activities at high temperatures, thermophilic enzymes have major biotechnological advantages over mesophilic enzymes [10]. The potential of cellulases has been revealed in various industrial processes, as food, feed, textile and detergent industries. In particular, cellulase systems can transform cellulose into glucose, which can be fermented to fuel ethanol [11]. This study involves the purification and characterization of a novel cellulase from *Bacillus sonorensis* strain HSC7, which was isolated from Kerman hot spring. The optimal cultivation condition for cellulase production was explored.

2. Materials and methods

2.1. Materials

Carboxymethylcellulose (CMC), reagents for enzyme assay, and all analytical chemicals used in this study were purchased from Sigma–Aldrich. Samples of agriculture wastes were collected from different parts of wood yards in Kerman and were ground into fine particles and used for CMCase production.

2.2. Isolation and screening of thermophilic bacteria

The samples were isolated from Gorooh hot spring located in Jiroft, Kerman. The samples were transported to the laboratory in sterile tubes. A carboxymethylcellulose (CMC) medium that contained 0.05% yeast extract, 0.05% $MgSO_4{\cdot}7H_2O$, 0.1% KCl, 0.1% K_2HPO_4, 0.1% $NaNO_3$ supplement with 0.5% (w/v) CMC was used. 1 ml of the water sample was added to 25 ml CMC media. The enrichment was performed by incubation in Erlenmeyer flasks at 60 °C in an incubator shaker (150 rpm) for 6 days. 100 µl of each Erlenmeyer flask was spread on CMC agar plate and incubated at 60 °C. After 48 h incubation, the plates were flooded by 0.5% (w/v) Congo red solution for 20 min and then washed with a 0.1 M NaCl solution. The cellulose degrading bacteria were screened by a zone of clearance around the colonies. The isolate was identified based on different biochemical characteristics like catalase, citrate utilization, Voges-Proskauer, methyl red, indole, gelatin hydrolysis, and hydrogen sulfide production tests [12].

2.3. 16S rRNA sequencing for strain identification

Genomic DNA was extracted by phenol–chloroform method [13]. PCR was conducted using two universal primers for the bacterial 16S rRNA gene. The PCR amplification of 16S rRNA was carried out in 25 µl reaction mixture containing H_2O, 10X buffer (1X), dNTP (200 µM), $MgCl_2$ (0.2 mM), Taq DNA polymerase (0.05 units/µL), template DNA (200 pg/µL) and 0.2 µM of universal forward primer (5-AGTTTGATCCTGGCTCAG-3) and reverse primer (5-GGCACCTTGTTACGACTT-3). The process of PCR was conducted using a denaturation step of 5 min at 94 °C, 35 cycle of 94 °C for 30′, annealing temperature at 55 °C for 30′, extension at 72 °C for 1 min with a final extension at 72 °C, 10 min. The PCR products were separated by agarose gel electrophoresis (1%). The 16S rRNA sequence of the isolate was sequenced (Bioneer Company, South Korea) and compared to the other sequences in the National Center for Biotechnology Information (NCBI) database using the BLAST algorithm. Multiple sequence alignment was carried out with Clustal W and the neighbor-joining phylogenetic analysis was carried out with MEGA 4.0 program [14].

2.4. Enzyme assay

CMCase activity was measured by the 3, 5 dinitrosalicylic acid (DNS) method [15]. CMCase activity was determined by incubation 500 µl of 1% CMC in 50 mM sodium phosphate buffer (pH 7.5) with 500 µl cell free culture for 30 min at 50 °C. The reaction was stopped by adding the 1 ml 3, 5 dinitrosalicylic acid (DNS) reagent and boiled in a water bath for 10 min. After cooling at room temperature, the amount of glucose released was determined by measuring absorbance at 540 nm. CMCase activity was determined using a calibration curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that releases 1 µmol reducing sugars per min.

2.5. Optimization of CMCase production

Various carbon sources such as CMC, glucose, galactose, sucrose, starch and avicel at 0.5 gL^{-1} (w/v) were separately added as carbon source to the culture medium. After 48 h of growth, the various culture broths were centrifuged at 4 °C and 5000 rpm for 10 min. The CMCase production was estimated by the reducing sugar method [15]. Different concentrations of CMC in the range of 0.0–15 gL^{-1} were also used for determining the optimum concentration for maximum enzyme yield by this strain. Similarly, the effect of nitrogen sources was studied. Various inorganic and organic nitrogen sources such as $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$, peptone, yeast extract, tryptone at 2.5 gL^{-1} (w/v) were examined for the production of CMCase. For determining the optimum pH, medium pH adjusted between pH 4.0 and 9.0 with 1 N HCl and 1 N NaOH and incubated at 50 °C for 48 h. Finally different ion sources such as, $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CaCl_2$ and KCl at 0.05 gL^{-1} were also investigated to find the best ion source for bacterial growth and enzyme production.

2.6. Effect of lignocellulosic wastes on CMCase production

Various lignocellulosic wastes such as corn stover, filter paper, wheat bran, newspaper, alfalfa straw and rice bran were collected from local farms in Kerman and ground to fine powder. Bacterial growth was done in 500 ml Erlenmeyer flasks in 100 ml of basal medium contained; 0.025% yeast extract, 0.05% $MnSO_4{\cdot}7H_2O$, 0.025% $(NH_4)_2SO_4$ and 0.05% KCl (pH 6.0) supplemented with 1% (w/v) lignocellulosic wastes. The cultures were incubated aerobically at 55 °C for 96 h and the CMCase production was measured by reducing sugar method [15]. The enzyme extract was centrifuged (10,000 rpm, 5 min, 4 °C) and the clear supernatant obtained was used as crude enzyme for purification and enzymatic measurements.

2.7. CMCase production and purification

For CMCase production, B. *sonorensis* HSC7 was grown in an Erlenmeyer flask (500 ml) containing 150 ml of optimum medium supplemented with 1% alfalfa straw (pH 6.0) and was seeded with 5% 16 h old pre-culture and incubated (160 rpm) for 72 h at 60 °C. The kinetics of CMCase production was considered from aliquots withdrawn aseptically at every 12 h and CMCase activity was measured by DNS method. After incubation, culture broth was centrifuged (4 °C and 10,000×*g* for 15 min), and the supernatant was used as crude enzyme for further purification. The supernatant was precipitated with ammonium sulfate (85%) at 4 °C. The precipitates were collected through centrifugation at 12,000*g* for 10 min. The pellets were dissolved in a minimum volume of 50 mM sodium phosphate buffer (pH 7.5) and were dialyzed against the same buffer at 4 °C. The dialysate enzyme was subjected to Q-Sepharose column chromatography (5 cm × 20 cm) which equilibrated with 50 mM sodium phosphate buffer (pH 7.5) at flow rate 0.5 ml/min. The bound proteins were eluted with a linear gradient of NaCl (0.1–0.5 M) in the equilibration buffer. Fractions which displayed CMCase activity were pooled together and concentrated by ammonium sulfate precipitation with the same procedure. The resulting precipitate was collected by centrifugation and dissolved in 50 mM Tris–HCl buffer (pH 7.5). Concentrated fractions were loaded onto a Sephadex G-100 column (2.5 cm × 50 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.5) and eluted with the same buffer at a flow rate of 0.2 ml/min. Fractions exhibiting CMCase activity were pooled and used as a purified enzyme for the following studies.

SDS–PAGE was done on 10% polyacrylamide gel according to the method of Laemmli [16]. The samples were dissolved in 10 µl of sample buffer and heated in boiling water for 5 min. After electrophoresis, the protein bands were stained with silver nitrate staining method. For staining, the gel was incubated in fixer (40% methanol, 10% acetic acid, 50% H_2O) for 1 h. After several washings with H_2O for at least 30 min, the gel was laid on sensitize solution (0.02% $Na_2S_2O_3$) for only 1 min, and washed with H_2O three times for 20 s. Then, the gel was stained with silver nitrate solution for 20 min (0.2% $AgNO_3$. 200 ml H_2O, 0.02% formaldehyde). After washing with H_2O, the gel was placed in a 3% sodium carbonate and 0.05% formaldehyde. The cellulase band was visualized when the solution turned yellow. Finally, the reaction was stopped by adding 5% acetic acid for 5 min.

2.8. Biochemical characterization of the enzyme

2.8.1. Effect of temperature on activity and stability of CMCase

The effect of temperature on CMCase activity was determined at different temperatures ranging from 30 to 90 °C. For enzyme activity, the enzyme was assayed in the presence of 1% CMC at each temperature. Thermal stability was carried out by incubating of the enzyme at 30–90 °C for 60 min in the absence of CMC. Then, the remaining activity was measured in the presence of 1% CMC. The irreversible thermal inactivation was determined by incubating the enzyme solution (in the absence of CMC) at different temperatures (50, 60 and 70 °C) and samples were picked up at different time intervals, and then the residual CMCase activity was measured as standard method. An enzyme which incubated on ice was considered as control.

2.8.2. Effect of pH on the activity and stability of CMCase

The optimum pH for CMCase activity was estimated in the range of 3.0–12.0 using appropriate buffers. The buffers used were 50 mM of sodium acetate (pH 3.0–6.0), sodium phosphate (pH 7.0–8.0), Tris-base (9.0–10.0) and glycine-NaOH (pH 11.0–12.0). pH stability was determined by the incubation of 500 µl enzyme and 500 µl buffer solution for 60 min at room temperature. After adding 1% CMC, the reaction mixture was incubated for 30 min at 50 °C and releasing glucose was measured by DNS method.

2.8.3. Effect of NaCl on the CMCase activity and stability

The enzyme activity was assayed in the presence of different NaCl concentration ranging from 0 to 30% as standard DNS method. The stability of CMCase was determined by incubating enzyme with various NaCl concentrations (0–30%) for 24 h and then the residual activity was measured as standard DNS method.

2.8.4. Kinetic determination

Kinetic studies were determined at different concentrations of CMC as substrate. The Michaelis–Menten kinetic parameters (K_m and V_{max}) were estimated using linear Lineweaver–Burk plot.

2.8.5. Effect of organic solvents on CMCase activity and stability

The effects of different solvents, such as methanol, chloroform, toluene, DMF, diethyl ether, n-butanol, DMSO and cyclohexane, on CMCase activity were examined by incubating the solvents at 5% (v/v) concentrations in the presence of 1% CMC as substrate, for 30 min at 50 °C. For solvent stability, the enzyme solution was incubated with various organic solvents (40%, v/v) for 60 min (in the absence of substrate). After incubation, the residual enzymatic activity was assayed by the standard DNS method.

2.8.6. Effect of additives on CMCase activity

The effect of various metal ions, such as Co^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, Ca^{2+} and as well as SDS, EDTA and Triton X-100, on CMCase activity was examined. The CMCase activity was assayed by incubating the enzyme with various additives at a concentration of 5 mM for 30 min at 50 °C and enzyme activity was measured by DNS method.

2.8.7. Effect of commercial detergents on activity of CMCase

The influence of commercial detergent such as Dioxygene, Shooma, Banoo, Barf, Darya, Kaf and Taj was investigated on the CMCase activity. The reaction mixture was incubated at 50 °C for 30 min and enzyme activity was measured by DNS method.

3. Results and discussion

3.1. Isolation and identification of cellulolytic strain

A CMCase producing bacterium by utilizing lignocellulosic biomass was isolated from water samples of Gorooh hot spring, Jiroft and designated as HSC7. This isolate exhibited maximum zone of clearance around the colonies after staining with 1% congo red solution. Biochemical and morphological characterization of the HSC7 strain revealed that this strain is gram positive and rod shaped. HSC7 isolate was positive for production of catalase, Voges-Proskauer/methyl red test, utilization of citrate and hydrolysis of casein, gelatin hydrolysis, while H_2S and indole production were negative. The 16S rRNA gene sequencing analysis showed 99% homology with *B. sonorensis*. Evolution distance and the phylogenetic tree for this strain confirmed the identity of the isolate as *B. sonorensis* (Fig. 1).

3.2. Optimization of CMCase production

The production of enzymes by microorganisms was dependent upon the carbon source used in the culture medium. The effect of various polysaccharides, as a carbon source for enzyme production was evaluated (Table 1). The strain utilized a variety of carbon sources such as glucose, galactose, starch, etc., but sucrose was the best substrate for CMCase production as reported in *Trichoderma viride* [17]. Previously it was reported that, sucrose, glucose and mannitol as the substrates significantly increased optimum levels of cellulase production by *Acetobacter xylinum* [18].

Among different concentrations of CMC, maximum enzyme production by *B. sonorensis* HSC7 was obtained at 1% CMC (Table 1). It was recently reported that *Bacillus* sp. produced maximum enzyme production in the presence of CMC as a carbon source [19]. In addition, the optimum CMC concentration for enzyme production was achieved at 1%. Similar results were obtained from *B. thuringiensis* [20] While *Achromobacter xylosoxidans* BSS4 and *Paenibacillus barcinonensis* MG7 showed maximum cellulase activity at 0.5 and 2% concentration of CMC, respectively [21,22].

Organic and inorganic nitrogen sources are important factors which support bacterial growth and enzyme production. Among different organic and inorganic nitrogen sources, $(NH_4)_2SO_4$ was found to be the appropriate nitrogen source for the production of *CMCase* by *B. sonorensis* HSC7, the same as cellulase production by *B. subtilis* [23]. Yeast extract was also reported as a suitable nitrogen source for cellulase production by *B. cereus* [24] and *P. barcinonensis* MG7 [22]. Another report suggested that, NH_4Cl induced cellulase production in *Bacillus* sp. [19].

pH is a very essential parameter which influences cellulase production. Results showed that, HSC7 strain displayed the highest CMCase production at pH 6.0, the same as cellulase

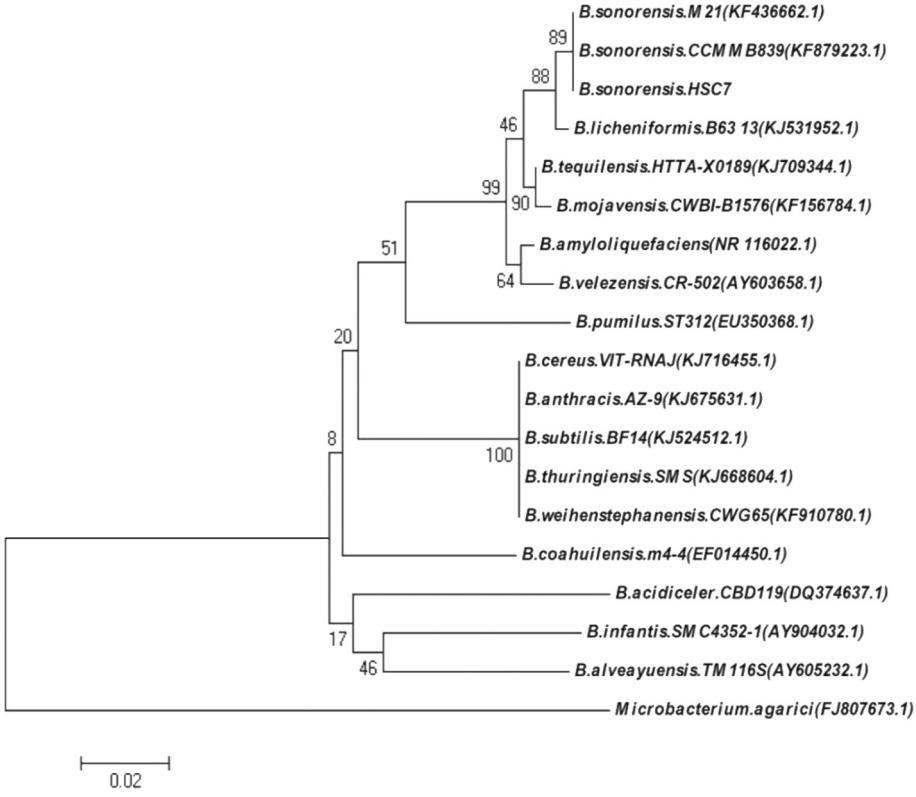

Fig. 1 The phylogenetic tree for *Bacillus sonorensis* HSC7 and related strains based on the 16 S rRNA sequence.

Table 1 Impact of various factors on CMCase production.

Variouse factor ($g\ L^{-1}$)	Enzyme activity (%)	Dry cell mass ($mg\ ml^{-1}$)
Carbon sources		
Galactose	8 ± 0.04	10
Glucose	78 ± 0.08	13
Sucrose	100 ± 0.011	18
CMC	45 ± 0.09	17
Avicel	10 ± 0.03	20
Starch	32 ± 0.01	15
Carboxymethyl cellulose (CMC)		
0	6 ± 0.03	5
0.2	42 ± 0.5	10
0.5	51 ± 0.01	15
1	100 ± 0.02	17
1.5	23 ± 0.013	18
Nitrogen sources		
$NaNO_3$	12 ± 0.09	12
Peptone	84 ± 0.07	10
NH_4Cl	56 ± 0.09	10
Yeast extract	91 ± 0.04	17
$NH_4)_2SO_4)$	100 ± 0.02	5
Tryptone	75 ± 0.03	22
pH		
4	18 ± 0.12	14
5	70 ± 0.0.6	10
6	100 ± 0.11	11
7	42 ± 0.07	26
8	35 ± 0.04	16
9	34 ± 0.03	
Ione sources		
Control	38 ± 0.00	14
$MgSO_4$	88 ± 0.09	11
$CaCl_2$	31 ± 0.04	11
$MnSO_4$	100 ± 0.01	9
$ZnSO_4$	53 ± 0.05	16
KCl	68 ± 0.05	15

Carbon source: 0.05 gL^{-1}, nitrogen source: 0.025 gL^{-1}, Ione sources: 0.05 gL^{-1}, Carboxymethyl cellulose (CMC): %

production by *Bacillus* sp. [25]. It is mentioned that, cellulase production by *P. barcinonensis* MG7 increased from pH 6.0 to 8.0 and maximum was observed at pH 7.0 [22].

The influence of different ions on cellulase production was investigated using the basal medium with the addition of $CaCl_2$, $MgSO_4$, $MnSO_4$, $ZnSO_4$, and KCl (Table 1). Results indicated that addition of $MnSO_4$ (0.05 gL^{-1}) improved CMCase productions about 2.6 folds compared to the control (Table 1). Karim et al. reported that $MgSO_4$ (1.0 gL^{-1}), and $CaCl_2$ (0.001 gL^{-1}) was also increased β-glucanase production [26]. Gao et al. reported that cellulase production increased by incorporating the ions in the production medium [27]. The ions play significant role in the enzyme activity as well as the stability of enzymes.

3.3. Effect of lignocellulosic wastes on CMCase production

The effect of lignocellulosic wastes as a carbon source on cell growth and CMCase production by *B. sonorensis* HSC7 was examined with corn stover, filter paper, wheat bran, newspaper, alfalfa straw and rice bran. Results showed that, *B. sonorensis* HSC7 produced CMCase and avicelase during the growth in agricultural wastes as a sole carbon sources. It has been shown that *B. sonorensis* HSC7 used a variety of inexpensive cellulosic substrates, but maximum CMCase and avicelase production was detected in the medium containing alfalfa straw (Fig. 2a). It has been reported that, rice bran was the best carbon source for cellulase production by *B. subtilis* subsp. *subtilis* A-53 and *B. halodurans* CAS 1 [28,29]. Bahaa et al. [30] also reported an increase in cellulase activity on rice straw and corn stalks as the substrate. Jo et al. and Mayende et al., also reported that rice hulls and rice bran were the best agricultural biomass for CMCase production by *B. amyloliquefaciens* DL-3 and *Bacillus* sp. CH 43, respectively [7,31].

Yang et al. also showed that, 1% corn flour was the best carbon source for fermentation by *B. subtilis* BY-2 [32].

In spite of that, it has shown that alfalfa is about three times more efficient than the other agricultural biomass sources such as soybean or corn owing to its high biomass

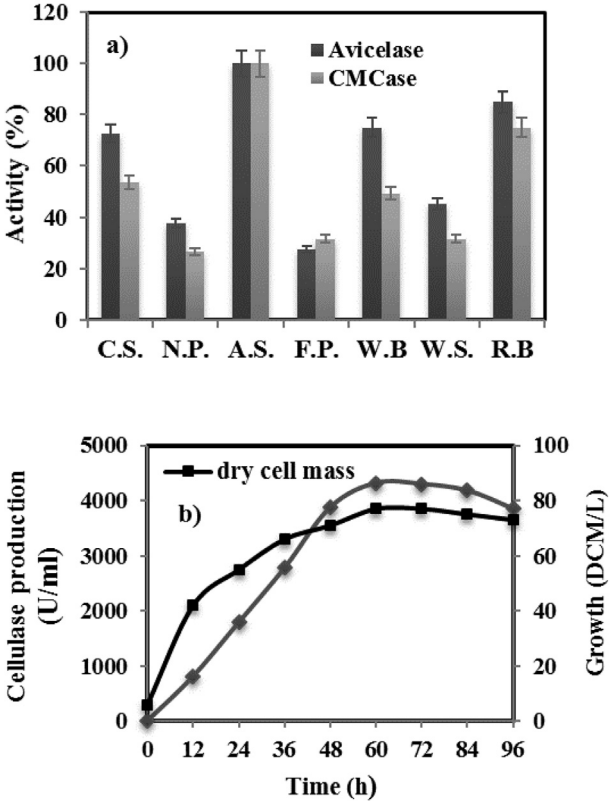

Fig. 2 (a) Effect of various agriculture wastes (1%, carbon sources) on CMCase production by *Bacillus sonorensis* HSC7 was examined with corn stover (C.S.), filter paper (F.P.), wheat bran (W.B.), newspaper (N.P.), alfalfa straw (A.S.) and rice bran (R. B.). CMCase production was assayed after 60 h of incubation at 55 °C. (b) Kinetics of cell growth (dcm/ml) and CMCase production (U/ml). For CMCase production, B. *sonorensis* HSC7 was grown in 100 ml of basal medium (0.025% yeast extract, 0.05% $MnSO_4.7H_2O$, 0.025% $(NH_4)_2SO_4$, and 0.05% KCl (pH 6.0) supplemented with 1% (w/v) lignocellulosic wastes containing 1% alfalfa straw in 500 ml of Erlenmeyer flask was incubated for 60 h at 55 °C.

yield, perennial nature, fixation of aerial nitrogen, and production of valued co-products, making it a model forage species for biofuel study [33]. However, the CMCase produced by the hydrolysis of cellulosic biomass by *B. sonorensis* HSC7 could be valuable for the production of ethanol and other industrially important chemicals.

3.4. CMCase production and purification

Production of CMCase by *B. sonorensis* HSC7 was done in 500 ml Erlenmeyer flask containing 150 ml of basal medium supplemented with 1% alfalfa straw. Results showed that, CMCase production reached maximum (4320 U/ml) at 60 h and it started to decrease slowly afterward (Fig. 2b). It seems that the cellulase production (4320 U/ml) by this strain was relatively higher than the previous reports. Annamalai et al., reported that the highest activity of cellulase *B. halodurans* CAS 1 was about 3274 U/ml at 36 h with 1% Rice bran [29]. Lee et al., reported that the highest activity of cellulase *B. amyloliquefaciens* DL-3 was about 153 U/ml with 2% rice hull [7]. Thus, our results indicated that the thermophilic *B. Sonorensis* HSC7 efficiently utilized agricultural waste (alfalfa straw) with higher cellulase production. So, it could be a perfect candidate to convert lignocellulosic biomass for industrial applications. The CMCase from the culture broth of *B. sonorensis* HSC7 was purified through multistep purification, ammonium sulfate precipitation, Q-Sepharose, and gel filtration chromatography. The overall purification fold of the enzyme was about 8.85 with the specific activity of 412.32 U/mg (Table 2). Cellulase obtained from an alkaliphilic strain, *Bacillus sphaericus* also has specific activity of 38.4 U/mg [37]. The specific activities of purified cellulase from various microorganisms vary from 3.8 to 71.0 U/mg of protein [28,50]. Annamalai et al., reported a yield of 12.54% with a purification of 8.5-fold during alkali halotolerant cellulase production from *B. halodurans* CAS 1 [29]. Likewise, a study showed 5.5-fold purification of cellulase from *Bacillus* sp. L1 [35]. The homogeneity of the purified CMCase was investigated and confirmed by the single band obtained in SDS–PAGE. The molecular weight of the purified enzyme was valued as 37 kDa (Fig. 3) which is smaller than cellulases from other *Bacillus* strains such as *B. circulans* (43 kDa) [34], *B. subtilis subsp. subtilis* A-53 (56 kDa) [28], *B. amyloliquefaciens* DL-3 (53 kDa) [7], *Bacillus* sp. L1 (45 kDa) [35] and *B. flexus* (97 kDa) [36], but higher than *B. sphaericus* JS1 (29 kDa) [37] and the same as cellulase from B. *licheniformis* AU01 (37 kDa) [38].

3.5. Biochemical characterization of the enzyme

3.5.1. Effect of temperature on CMCase activity and stability

The optimum temperature for the enzymatic activity was determined to be at 60 °C (Fig. 4a). A similar result was also found in *B. pumilus* EB3 [39] and *Bacillus* sp. L1 [35]. Maximum HSC7 CMCase stability was determined to be at 70 °C. It is mentioned that, CMCase from HSC7 strain retained more than 80% of its activity in a broad range of temperatures within 30 to 90 °C. (Fig 4a). Irreversible thermal inactivation results also showed that the CMCase half-life was about 30 min at 70 °C (Fig. 4b). Cellulase from *P. barcinonensis* MG7 was active in the temperature range 35–75 °C and maximum activity was observed at 65 °C [22]. A thermostable cellulase from *B. subtilis* DR showed an optimum activity at 50 °C, and retained 70% of its optimum activity after incubation at 75 °C for 30 min [40]. Furthermore, the optimal thermostability of a cellulase from *B. agaradhaerens* JAM-KU023 increased from 50 to 60 °C [41]. Thermostable CMCase are valuable for some applications, since the hydrolysis of cellulosic substrates can be done in faster rates at higher temperatures [42].

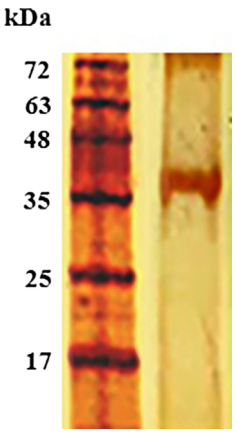

Fig. 3 SDS–page of CMCase produced by *B. sonorensis* HSC7. Separation was performed on a 10% (w/v) SDS–polyacrylamide gel and stained with silver stain.

3.5.2. Effect of pH on activity and stability of CMCase

CMCase activity was examined in a broad range of pH (3.0–12.0). Results showed that, the maximum HSC7 CMCase activity was observed at pH 4.0 and more than 68% of its activity still reserved even the pH dropped to 3.0 (Fig. 4c). These results characterize the acidophilic nature of enzyme. The cellulase activity was maximum at pH 6.0 in *B. pumilus* EB3 [39] and pH 6.5 in *B. subtilis* strain LF3 [43]. But, alkaline cellulase produced by *B. sphaericus* JS1 [37], *Vibrio* sp. G21 [27] and *Marinobacter* sp. MS 1032 [44].

The stability studies exhibited that the CMCase toward acidic pH (3.0–6.0) and maximum CMCase stability was found at pH 5.0 (Fig. 4c). Similar results have been reported in *B. subtilis* strain LFS3 [43], *Thermomonospora* [45], *Bacillus*

Table 2 Purification steps of cellulase enzyme isolated from *Bacillus sonorensis* HSC7.

Purification steps	Total protein (mg/ml)	Enzyme activity (U/ml)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	17.3	3212	185.66	1	100
Ammonium sulfate precipitation	7.1	1623	228.59	5	50.53
Dialysis	2.1	635	302.38	6.5	19.77
Ion exchange	0.9	371.09	412.32	8.85	11.55

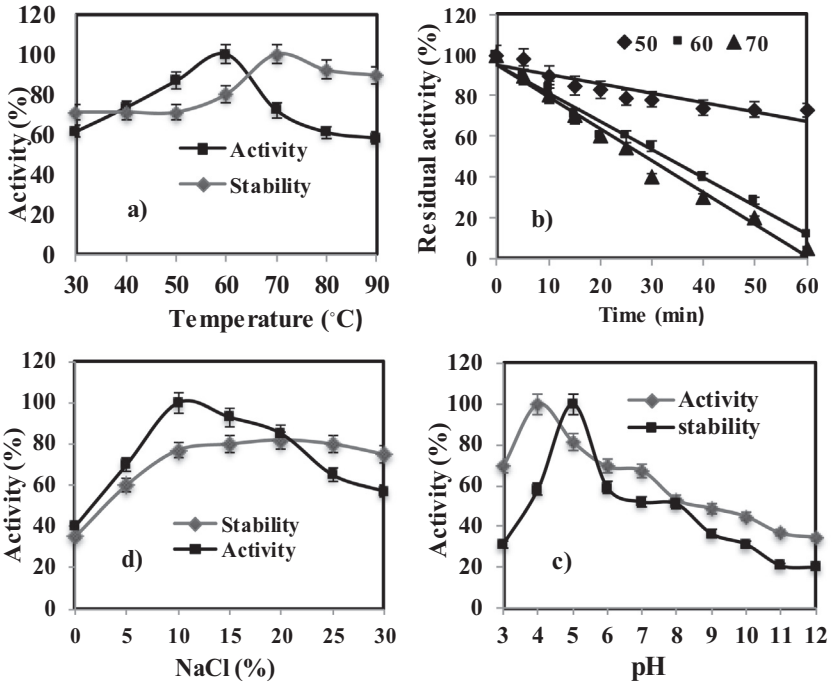

Fig. 4 (a) Effect of temperature on CMCase activity and stability. The enzyme activity was evaluated at different temperature ranging from 30–90 °C using sodium phosphate buffer (pH 7.5). For the stability the enzyme solution was incubated for 60 min at various temperatures. (b) Thermal stability of the CMCase *B. sonorensis* HSC7. The enzyme was incubated at 50 °C, 60 °C and 70 °C for various lengths of time and the residual activity was measured on CMC. (c) Effect of pH on the activity and stability of CMCase. The CMCase activity was assayed at 50 °C for 30 min by incubating reaction mixture in 50 mM of the following buffers: acetate buffer (pH 3.0–6.0), phosphate buffer (pH 7.0–8.0) and glycine NaOH (pH 9.0–12.0). For stability, the enzyme was incubated for 60 min at various pH buffers. d) Effect of NaCl concentrations on the activity and stability of CMCase.

sp. M-9 [46] and *B. licheniformis* [47]. But, optimum cellulase activity was reported in pH 7.0 by *P. fluorescence* [48], *B. amyoliquefaciens* DL3 [7]. Furthermore, cellulase was optimally active at alkaline pH in *Bacillus* sp. HSH-910 [49] and *B. sphaericus* JS1 [37].

3.5.3. Effect of NaCl on activity and stability of CMCase

The CMCase from HSC7 strain was active at different NaCl concentrations (0–30%) and the highest activity was observed at 10% NaCl. It was also found that this enzyme retained 57% of its activity at 30% NaCl (Fig. 4d). It was previously reported that, the cellulase activity was higher with 30% NaCl concentration in *B. halodurans* CAS 1 [29]. The cellulase from *Bacillus* sp. L1 showed a high stability at NaCl concentration between 2.5 and 15% [35]. However, most of the cellulase described to date was stable only between 5 and 20% NaCl [36]. It is mentioned that, the HSC7 CMCase displayed a higher stability even at 25% NaCl. This is an important property in various biotechnological processes that depends on high salinity or osmotic pressures.

3.5.4. Kinetic determination

Kinetic study of the HSC7 CMCase exhibited enzymatic saturation above 0.5% (w/v) of CMC (Fig. 5a). The kinetic parameters (K_m and V_{max}) were calculated using Lineweaver–Burk plots. The results demonstrated that the K_m and V_{max} value of the enzyme was 0.186 mg/ml and 0.052 µmol min^{-1}, respectively (Fig. 5b). The K_m and V_{max} values of cellulase were found 3.03 mg/ml and 142.86 µmol/min in *Salinivibrio* sp. strain NTU-05 [50]. In case of *B. subtilis* strain LFS3, the K_m and *V*max of enzyme for the substrate CMC were 2.2 mg/ml and 699.0 U/ml, respectively [43]. Some recent papers reported that the *km* value in the range of 0.6–7.2 mg/ml for CMC [50]. These results indicated that HSC7 CMCase has the highest affinity to the CMC as substrate.

3.5.5. Effect of organic solvents on CMCase activity and stability

The organic solvent stable cellulases could be potentially useful for industrial processes such as bioremediation of chemically polluted salt marshes [51]. The CMCase activity was improved in the presence of some organic solvents such as methanol (166%), chloroform (152%), diethylether (113%), proposing the prospects of the cellulase for several industrial applications. But, enzyme activity was diminished in the presence of isopropanol (73%) and DMF (61%) (Fig. 6a). Cellulase from *B. aquimaris* showed 85% activity in the presence of methanol [52]. Furthermore, cellulases (CelA10, CelA20 and CelA24) characterized from metagenomic method have also been considered for their stability in the presence of diverse organic solvents [53]. No decrease in cellulase activity was observed in the presence of ethanol and isopropanol, but it was partially inhibited with DMSO (82%), toluene (87%), methanol (85%), whereas cellulase activity was decreased up to 32, 58 and 59% in the presence of acetone, benzene and cyclohexane, respectively [53].

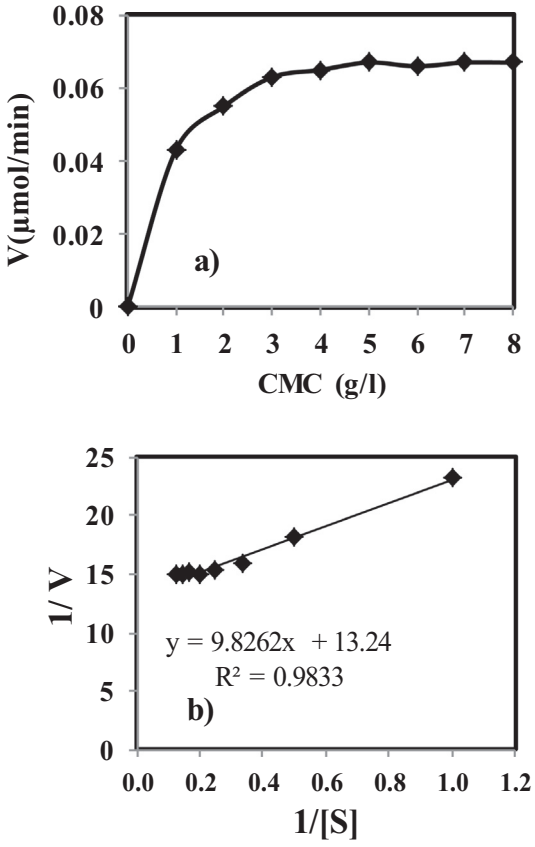

Fig. 5 (a) Michaelis–Menten and (b) lineweaver berg plots of CMCase produced by *B. sonorensis* HSC7 in the presence of different substrate concentration (0–25 mM). Kinetic parameters (K_m and V_{max}) were obtained from Lineweaver–Burk plot equation.

3.5.6. *Effect of additives on the activity of CMCase*

CMCase activity was examined at 5 mM concentration of various additives. In this study, the CMCase activity was enhanced in the presence of $MgSO_4$ and $CaCl_2$, while $FeSO_4$ ions could decrease the enzyme activity (Fig. 6b). Cellulase activity of *B. subtilis* was also increased in the presence of Mg^{2+} [43]. In addition, the activity of HSC7 cellulase was inhibited by $ZnSO_4$. Inhibition of the enzyme activity by Zn^{2+} was also reported from *B. flexus* [36], *B. subtilis* [54] and *P. barcinonensis* MG7 [22]. Inhibition by Zn^{2+} indicated the inhibitory effects of heavy metals on enzymes. Heavy metals can bind with the thiol group in the active site of the enzyme and thus, decrease the enzyme activity [55]. The CMCase from HSC7 strain showed partial inhibition in the presence of Hg^{2+}. A similar result has been reported for cellulase from *B. sphaericus* JS1, *P. barcinonensis* MG7 and *B. subtilis* strain LFS3 which was completely inhibited in the presence of Hg^{2+}. Inhibition by Hg^{2+} may be related to binding the thiol groups, and may be the results of interaction with tryptophan residue or the carboxyl group of amino acids in the enzyme [43]. The HSC7 CMCase activity was also inhibited by $Co^{2+} > Mn^{2+} > K^+$ as cellulase from *P. barcinonensis* MG7 [22] and *B. subtilis* strain LFS3 [43], while K^+ ion in *B. pumilus* [56], Co^{2+} and Ca^{2+} in *Geobacillus* sp. [57], Mn^{2+} in *B. mycoides* have been reported to increase the enzyme activity.

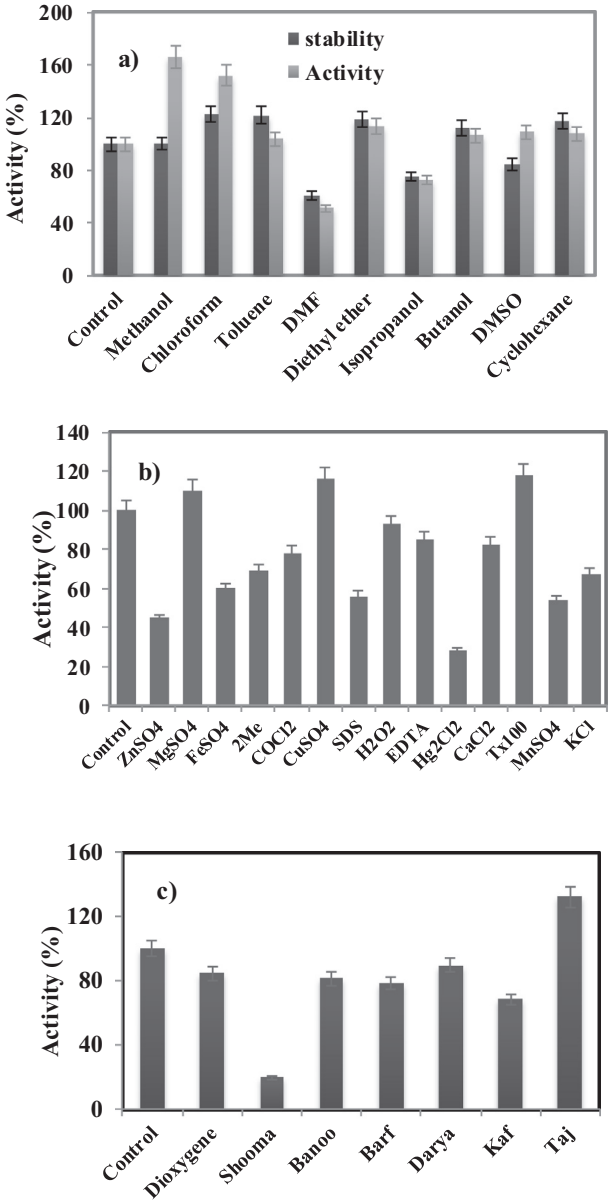

Fig. 6 (a) Effect of organic solvents on CMCase activity and stability. The effects of different solvents, such as methanol, chloroform, toluene, DMF, diethyl ether, n-butanol, DMSO and cyclohexane, on CMCase activity and stability were in the presence of 1% CMC as substrate, for 30 min at 50 °C. (b) Effect of additives on CMCase activity. (c) Effect of commercial detergents on CMCase activity.

Furthermore, the CMCase showed 118% activity in the presence of Triton X-100 (nonionic detergent), but activity was reduced in the presence of SDS (ionic detergent) about 56% (Fig. 6b). Usually, nonionic surfactant can modify the enzyme surface property and minimize the cellulase irreversible inactivation. Improved cellulase activity in the presence of nonionic detergents has greater advantages in industrial applications in the paper industry [22,58,59].

3.5.7. *Effect of commercial detergents on activity of CMCase*

The highest CMCase activity was observed in the presence of Taj® (132%) and darya® (90%), while the opposite result

was reported with Shooma® (20%) (Fig. 6c). Results indicated that, the compatibility of a cellulase diverse for each laundry detergent. The cellulase from *Bacillus* sp. SMIA-2 exhibited higher stability with Ultra Biz® and lower stability was also reported in the presence of Ariel® [60]. In addition, CMCase from *B. licheniformis* AMF-07 showed the highest activity in the presence of Dioxigene® about (122%) [61].

4. Conclusion

In spite of various reports on alkaline cellulase, there are just a few reports about acidic cellulase. In this study, a new acido-thermophilic, salt and solvent tolerant cellulase was purified and characterized from a novel thermophilic bacterium, *B. sonorensis*. HSC7. It is mentioned that, excellent cellulase production by this strain was achieved in optimized medium supplemented with alfalfa straw, suggesting the conversion of biomass into biofuels. Acido-thermophilic celluloses are commonly appropriate for biomass conversion of lignocellulosic waste. They can also be useful for industrial application such as pulp freeness and repulping efficiency, clarification of fruit juices, animal feed industry. Thus, the remarkable properties found with this cellulase could make this enzyme as an ideal candidate for real applications in biotechnological processes.

References

[1] S.C. Back, Y.J. Kwon, Biotechnol, Bioprocess Eng. 12 (2007) 404–409.
[2] R.K. Sukumaran, R.R. Singhania, G.M. Mathew, A. Pandey, Renew. Energy 34 (2009) 421–424.
[3] J.C. Yi, J.C. Sandra, A.B. John, T.C. Shu, Appl. Environ. Microbiol. 65 (1999), 553–559.3.
[4] L.R. Lynd, P.J. Weimer, W.H. Van-Zyl, I.S. Pretorius, Microbiol. Mol. Biol. Rec. 66 (2002) 506–577.
[5] H. Ariffin, N. Abdullah, M.S. Umi Kalsom, Y. Shirai, M.A. Hassan, J. Eng. Technol. 3 (2006) 47–53.
[6] M. Mandels, Biochem. Soc. Trans. 13 (1985) 414–416.
[7] K.I. Jo, Y.J. Lee, B.K. Kim, B.H. Lee, C.H. Chung, S.W. Nam, Biotechnol, Bioprocess Eng. 13 (2008) 182–188.
[8] K. Nakamura, K. Kitamura, J. Ferment. Tech. 60 (1982) 343–348.
[9] L.M. Robson, G.H. Chambliss, Enzyme Microb. Technol. 11 (1989) 626–644.
[10] C. Vieille, G.J. Zeikus, Microbiol. Mol. Biol. Rev. 65 (2001) 1–43.
[11] T.R. Dhiman, M.S. Zaman, R.R. Gimenez, J.L. Walters, R. Treacher, Anim. Feed Sci. Technol. 101 (2002) 115–125.
[12] J.G. Cappuccino, N. Serman, Microbiology a laboratory manual (six edition), Pearson education, 2005, pp. 116–119.***
[13] J.A. Marmur, Mol Biol. 3 (1961) 208–218.
[14] K. Tamura, J. Dudley, M. Ne, S. Kumar, Mol. Biol. Evol. 24 (2007) 1596–1599.
[15] G.L. Miller, R. Blum, W. Glennon, A.L. Burton, Anal. Biochem. 1 (1960) 127–132.
[16] U.K. Laemmli, Nature 227 (1970) 680–685.
[17] S.P. Gautam, P.S. Bundela, A.K. Pandey, M.K. Awassthi, S. Sarsaiya, Environ. 1 (2010) 656–665.
[18] K.V. Ramana, A. Tomar, L. Singh, J. Microbiol. Biotechnol. 16 (2010) 248–254.
[19] S. Sadhu, P.K. Ghosh, T.K. De, T.K. Maiti, J. Microbiol. 3 (2013) 280–288.
[20] L. Lin, X. Kan, H. Yan, D. Wang, Biotechnol. J. 15 (2012) 1–7.
[21] N.M. Shaikh, A.A. Patel, S.A. Mehta, N.D. Patel, Environ. Sci. Technol. 1 (2013) 39–49.
[22] B.M. Asha, M. Revathi, A. Yadav, N. Sakthivel, J. Microbiol. Biotechnol. 22 (11) (2012) 1501–1509.
[23] M.S. Shabeb, A.M. Younis, F.F. Hezayen, M.A. Nour-Eldien, World Appl. Sci. J. 8 (1) (2010) 35–42.
[24] D.J. Mukesh-Kumar, P.D. Poovai, C.L. Kumar, Y. Sushma-Saroja, A. Manimaran, P.T. Kalaichelvan, Der Pharmacia Lett. J. 4 (2012) 881–888.
[25] D. Gomathi, C. Muthulakshmi, D.G. Kumar, G. Ravikumar, M. Kalaiselvi, C. Uma, Trop. Biomed. 2 (2012) 567–573.
[26] A. Karim, M.A. Nawaz, A. Aman, S.A.U. Qader, J. Radiat. Res. Appl. Sci. 6 (2014) 1–6.
[27] Z. Gao, L. Ruan, X. Chen, Y. Zhang, X. Xu, J. Microbiol. Biotechnol. 87 (2010) 1373–1382.
[28] B.K. Kim, B.H. Lee, Y.J. Lee, I.H. Jin, C.H. Chung, J.W. Lee, Enzyme Microb. Technol. 44 (2009) 411–416.
[29] N. Annamalai, M.V. Rajeswari, S. Elayaraja, T. Baklasubramanian, Carbohydr. Polym. 94 (2013) 409–415.
[30] T.S. Bahaa, G.M. Manal, A.G. Eman, M.S.A. Mohsen, S.I. Gada, Genet. Eng. Biotechnol. J. 9 (2011) 59–63.
[31] L. Mayende, B.S. Wilhelmi, B.I. Pletschke, Soil Biol. Biochem. 38 (2006) 2963–2966.
[32] W. Yang, F. Meng, J. Peng, P. Han, F. Fang, L. Ma, B. Cao, Electron, J. Biotechnol. 17 (2014) 262–267.
[33] A. Badhan, Y. Wang, R. Gruninger, D. Patton, J. Powlowski, A. Tsang, BMC Biotechnol. 14 (2014) 31–45.
[34] Y. Hakamada, K. Endo, S. Takizawa, T. Kobayashi, T. Shirai, T. Yamane, Acta Biochim. Biophys. 1570 (2002) 174–180.
[35] X. Li, H.Y. Yu, J. Ind. Microbiol. Biotechnol. 39 (2012) 1117–1124.
[36] N. Trivedi, V. Gupta, M. Kumar, P. Kumari, C.R.K. Reddy, B. Jha, Carbohydr. Polym. 83 (2011) 891–897.
[37] J. Singh, N. Batra, R.C. Sobti, J. Ind. Microbiol. Biotechnol. 31 (2004) 51–56.
[38] N. Annamalai, R. Thavasi, S. Vijayalakshmi, T. Balasubramanian, J. Microbiol. Biotechnol. 27 (2011) 2111–2115.
[39] H. Ariffin, N. Abdullah, M.S. Umi-Kalsom, Y. Shirai, M.A. Hassan, Eng. Technol. 3 (2006) 47–53.
[40] W. Li, W.W. Zhang, M.M. Yang, Y.L. Chen, Mol. Biotechnol. 2 (2008) 195–201.
[41] K. Hirasawa, K. Uchimura, M. Kashiwa, W.D. Grant, S. Ito, T. Kobayashi, Antonie Van Leeuwenhoek 89 (2006) 211–219.
[42] G. Rastogi, A. Bhalla, A. Adhikari, K.M. Bischoff, S.R. Hughes, L.P. Christopher, Bioresour. Technol. 101 (2010) 8798.
[43] R. Rawat, L. Tewari, Extremophiles 16 (2012) 637–644.
[44] S. Shanmughapriya, G. Seghal-Kiran, J. Selvin, T.A. Thomas, C. Rani, Appl. Biochem. Biotechnol. 162 (2010) 625–640.
[45] S.P. George, A. Ahmad, M.B. Rao, Bioresour. Technol. 77 (2001) 171–175.
[46] B.K. Bajaj, P. Himani, A. Masood, P.S. Wani, A. Sharma, Indian J. Chem. Technol. 16 (2009) 382–387.
[47] K.M. Bischoff, A.P. Rooney, X. Li, S. Liu, S.R. Hughes, Biotechnol. Lett. 28 (2006) 1761–1765.
[48] M.K. Bakare, I.O. Adewale, A. Ajayi, O.O. Shonukan, Afr. J. Biotechnol. 9 (2005) 898.
[49] J.Y. Kim, S.H. Hur, J.H. Hong, Biotechnol. Lett. 27 (2005) 313–316.
[50] C. Wang, Y.R. Hsieh, C.C. Chan, H.T. Lin, W.S. Tzeng, Enzyme Microb. Technol. 44 (2009) 373–379.
[51] M. Shafiei, A.A. Ziaee, M.A. Amoozegar, J. Ind. Microbiol. Biotechnol. 38 (2) (2011) 275–281.
[52] N. Trivedi, V. Gupta, M. Kumar, P. Kumari, C.R. Reddy, B. Jha, Chemosphere 83 (5) (2011) 706–712.
[53] J. Pottkamper, P. Barthen, N. Ilmberger, U. Schwaneberg, A. Schenk, M. Schulte, Green Chem. 11 (2009) 1–7.
[54] B.M. Asha, N. Sakthivel, Ann. Microbiol. 64 (2014) 1839–1848.
[55] M.N. Kambourova, R. Krilova, D.A. Mandeva, J. Mol. Catal. B: Enz. 22 (2003) 307–313.

[56] A.O.S. Lima, M.C. Fungaro, F.D. Andreote, J.W. Maccheroni, W.L. Araujo, Appl. Microb. Biotechnoly. 68 (2005) 57–65.
[57] I.S. Ng, C.W. Li, Y.F. Yeh, P.T. Chen, J.L. Chir, C.H. Ma, Exthermophile 13 (2009) 425–435.
[58] J. Wu, L.K. Ju, Biotechnol. Prog. 14 (1998) 649–652.
[59] S. Ito, S. Shikata, K. Ozaki, S. Kawai, K. Okamoto, S. Inoue, Agric. Biol. Chem. 53 (1989) 1275–1281.
[60] S.A. Ladeira, A.B. Erica-Cruz, J.B. Delatorre, M.L. Leal Martins, Barbosa 18 (2) (2015) 110–115.
[61] F. Azadian, A. Badoei-dalfard, A. Namaki-Shoushtari, M. Hassanshahian, MBRC 5 (2016) 143–155.