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ORIGINAL PAPER

Purification and characterization of an acidothermophilic cellulase enzyme produced by *Bacillus subtilis* strain LFS3

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Abstract In the present investigation, a microorganism hydrolyzing carboxymethylcellulose (CMC) was isolated and identified as *Bacillus subtilis* strain LFS3 by 16S rDNA sequence analysis. The carboxymethylcellulase (CMCase) enzyme produced by the *B. subtilis* strain LFS3 was purified by $(NH_4)_2SO_4$ precipitation, ion exchange and gel filtration chromatography, with an overall recovery of 15 %. Native-PAGE analysis of purified CMCase revealed the molecular weight of enzyme to be about 185 kDa. The activity profile of CMCase enzyme showed the optimum activity at temperature 60 °C and pH 4.0, respectively. The enzyme activity was induced by Na^+, Mg^{2+}, NH_4^+, and EDTA, whereas strongly inhibited by Hg^{2+} and Fe^{3+}. The purified enzyme hydrolyzed CMC, filter paper, and xylan, but not *p*-nitrophenyl *β*-D-glucopyranoside and cellulose. Kinetic analysis of purified enzyme showed the K_m value of 2.2 mg/ml. Thus, acidophilic as well as thermophilic nature makes this cellulase a suitable candidate for current mainstream biomass conversion into fuel and other industrial processes.

Keywords Carboxymethylcellulase · Acidophilic · Purification · Characterization

Introduction

In recent years, cellulases have attracted much interest because of their applications in various industrial processes, including food, textiles, laundry, pulp and paper as well as in agriculture (Bhat and Bhat 1997). The rising concerns about the scarcity of fossil fuels, the emission of green house gasses and air pollution by incomplete combustion of fossil fuel have also resulted in an increasing focus on the use of cellulases to perform enzymatic hydrolysis of the lignocellulosic materials for the production of bioethanol (Zaldivar et al. 2001; Sun and Cheng 2002). Cellulases contain a group of three enzymes namely endo-1,4-*β*-glucanase (also referred to as carboxymethylcellulase or CMCase; EC 3.2.1.4), exo-1,4-*β*-glucanase (EC 3.2.1.91) and *β*-glucosidase (EC 3.2.1.21) that synergistically convert cellulose into soluble sugars and glucose (Lynd et al. 2002). Endoglucanases attack the cellulose crystalline structure at random points, breaking the linear chains of glucose molecules to produce shorter chains. Each break produces two new chain ends. Exoglucanases attach to these exposed ends of the chains and, working down the chains, release cellobiose and some glucose. Finally, *β*-glucosidases completes the saccharification by splitting cellobiose and small cello-oligosaccharides into glucose molecules (Henrissat et al. 1998).

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials. Several types of microorganisms can produce cellulase enzyme including fungi, actinomycetes and bacteria. Currently, most of the commercial and laboratory cellulases are obtained by fungi due to their high enzyme activity, but several factors suggest that bacteria may have greater potential (Nagendran et al. 2009). Bacteria often have a higher growth rate than fungi allowing for higher rate of enzyme production. Most importantly, they show a tendency to be more heat stable and are easier for genetic work. Several bacterial genera reported for cellulolytic activities include *Bacillus*, *Clostridium*, *Cellulomonas*,

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Rumminococcus, *Alteromonas*, *Acetivibrio* etc. Among bacteria, Bacillus species are well known for production of CMCase under a liquid culture medium (Jo et al. 2008; Mayende et al. 2006).

The enzymatic saccharification of cellulosic materials performed by commercial cellulases contains the CMCase as the major component of enzyme (Ballesteros et al. 2004). Thus, in the present investigation, the microorganism hydrolyzing wheat bran, a major cellulosic waste material, was isolated from soil and identified as *Bacillus* sp. However, several reports are available on characterization of alkalophilic CMCase, but information on acidophilic CMCase is still scarce. Here, we describe the purification and characterization of the acidothermophilic CMCase produced by *Bacillus* sp. for the exploitation of abundant cellulosic biomass.

Materials and methods

Isolation and screening of cellulolytic bacteria

Soil samples were collected from different sites of paper and pulp industry and screened for the isolation of cellulose degrading bacteria. Different bacterial strains were isolated as pure culture on nutrient agar plates after incubation at 30 ± 2 °C. The purified colonies were screened for their cellulase activity on carboxymethylcellulose (CMC) agar containing (g/l) CMC sodium salt 10.0; yeast extract 5.0; $(NH_4)_2SO_4$ 0.5; KH_2PO_4 2.66; Na_2HPO_4 4.32; agar 20.0. After incubation, the plates were flooded with 1 % Congo red for 15–20 min followed by destaining with 1 M NaCl for 15–20 min (Teather and Wood 1982). The qualitative measure of extracellular cellulase activity is the presence of clear zone around the growing colony against the dark red background.

Culture conditions for enzyme production

The medium used for the production of cellulase enzyme contained the following components (g/l): wheat bran 30.0, yeast extract 3.0, $(NH_4)_2SO_4$ 1.0, K_2HPO_4 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, KH_2PO_4 0.5, NaCl 0.6, $FeCl_3$ 0.004, $CaCl_2{\cdot}2H_2O$ 0.002, Tween 80 1.0 ml, and pH 6.0. The inoculum of bacterial culture was prepared by overnight incubation of cells at 30 ± 2 °C and at 120 rpm. The resulting culture (OD = 0.5) was transferred to the production medium and incubated at 30 ± 2 °C on a rotary shaker (120 rpm) for 48 h.

Identification of the bacterial strain

Biochemical and morphological analysis were done according to the Bergey's Manual of Systematic Bacteriology. Genomic DNA for molecular identification of the selected bacterial strain was extracted using a Chromous Genomic DNA isolation Kit. The PCR amplification of 16S rRNA gene was carried out using universal forward primer pF (5′-AGAGTRTGATCMTYGCTWAC-3′) and reverse primer pR (3′-CGYTAMCTTWTTACGRCT-5′). The process of PCR was done under the following conditions: 94 °C, 5 min; 35 cycles of 94 °C, 30 s; 55 °C, 30 s; and 72 °C, 2 min; 1 cycle of 72 °C, 5 min; and then 4 °C forever. PCR amplified products were then purified, sequenced and compared with sequences in nucleotide database (NCBI) using the BLAST algorithm. Multiple sequence alignment was carried out with CLUSTAL W (Thompson et al. 1994). The neighbour-joining phylogenetic analysis was carried out with MEGA programme (Tamura et al. 2007).

Purification of CMCase

All the steps of purification were performed at 4 °C. After the cultivation, bacterial culture was centrifuged at 12,000×*g* for 10 min and supernatant was concentrated by ultrafiltration. Concentrated supernatant was precipitated overnight with $(NH_4)_2SO_4$ (80 % saturation) and the pellet was recovered by centrifugation at 12,000×*g* for 10 min. The pellet was resuspended in a small amount of 50 mM acetate buffer, pH 4.0, and dialysed overnight against the same buffer. The dialysed sample was applied to a DEAE Sephadex A-50 column previously equilibrated with the acetate buffer (pH 4.0). The adsorbed material was eluted with a linear gradient of sodium chloride in the range of 0–0.5 M in the same buffer at a flow rate of 1.0 ml/min. The active fractions were collected and dialysed against acetate buffer (pH 4.0). The dialysed sample was further purified by gel filtration on Sephadex G-100 column pre-equilibrated with the same buffer. The fractions were eluted at a flow rate of 1.0 ml/min. Total of 30 fractions were collected and assayed for CMCase activity. Fractions showing maximum activity were analysed for purity and other characterization studies.

Enzyme assay

CMCase activity was determined by measuring the amount of reducing sugar liberated from CMC using 3,5-dinitrosalicylic acid (DNS) method (Miller 1959). The reaction mixture was prepared by mixing 0.5 ml of appropriately diluted enzyme solution with 0.5 ml of 2 % CMC dissolved in 50 mM acetate buffer (pH 4.0). This mixture was incubated at 60 °C for 30 min and the reaction was stopped by adding 3 ml of DNS reagent. The resulting samples were boiled for 5 min, cooled in water for colour stabilization, and the optical density was measured at 540 nm.

One unit (U) of the enzyme activity was defined as the amount of enzyme releasing 1 µmol reducing sugar per min using glucose as a standard. Protein concentration was determined by Lowry's method (Lowry et al. 1959), using bovine serum albumin as a standard.

Native PAGE and zymogram analysis

To determine the apparent molecular weight of purified enzyme, native polyacrylamide gel electrophoresis (Native-PAGE) was carried out at 4 °C and the bands were visualized by Coomassie Brilliant Blue staining. For zymogram analysis, samples were applied to 8 % Native-PAGE gel containing 0.5 % (w/v) CMC incorporated directly into the resolving gel at 4 °C. The gel was incubated at 60 °C in sodium acetate buffer (pH 4.0) for 1 h, stained with Congo red (1 %) for 30 min, and destained with 1 M NaCl until the CMCase activity was visualized as clear band against the red background.

Influence of temperature and pH on the enzyme activity and stability

The temperature and pH profile of purified enzyme was evaluated by measuring the enzyme activity at different temperatures (40–80 °C with an interval of 10 °C) and pH (3–12 with an interval of 1.0) using following buffers: 0.05 M acetate buffer (pH 3.0–5.0), 0.05 M phosphate buffer (pH 6.0–7.0), 0.05 M Tris–HCl buffer (pH 8.0–10.0), and 0.05 M glycine NaOH (pH 11.0–12.0). Thermal stability of the enzyme was determined at respective temperatures with the pre-incubation of enzyme for 30 and 60 min and pH stability was determined at respective pH with pre-incubation of enzyme for 60 min. The residual activity of each sample for hydrolysis of CMC was then quantified under the optimized condition of enzyme assay.

Effect of additives on enzyme activity

The effect of various additives on enzyme activity was also examined. The additives used in this study were the salts of Cu^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, Na^{+}, K^{+}, NH^{4+}, and EDTA (5 mM each). In all the cases, initial activity was assumed to be 100 % and used to calculate the enzyme activities as percentages of the initial activity (relative activities) during the incubation period.

Enzyme kinetics

The kinetics of CMCase enzyme was characterized in terms of Michaelis–Menten kinetic constants (K_m and V_{max}) using the Lineweaver–Burk plots by assaying the enzyme activity at CMC concentrations ranging from 0.25 to 3.0 mg/ml in 50 mM acetate buffer (pH 4.0) at 60 °C for 30 min. The study of enzyme kinetics was done using Graph Pad software.

Substrate specificity

The substrate specificity of the purified enzyme was determined by performing the assay with different substrates: CMC, cellulose, filter paper, *p*-nitrophenyl *β*-D-glucopyranoside (PNPG) and xylan. The filter paper cellulase (FPase) and CMCase activities were determined using the IUPAC standard procedure (Ghose 1987).The reducing sugar liberated in the reaction mixture was measured by DNS method at 540 nm (Miller 1959). One unit (U) of the enzyme activity was defined as the amount of enzyme releasing 1 µmol reducing sugar per min using glucose as a standard. *β*-glucosidase activity was measured as the hydrolysis of para nitrophenyl-D-glucopyranoside (pNPG) at 405 nm. One enzyme unit was defined as "the µmoles of *p*-nitrophenol released per min upon hydrolysis of pNPG under standard assay conditions". Xylanase activity was determined based on the amount of reducing sugars released from birchwood xylan by the DNS method using xylose as standard (Bailey et al. 1992). One unit of xylanase activity was defined as the amount of enzyme that liberated reducing sugar at the rate of 1 µmol/min.

Statistical analysis

Analysis of variance (ANOVA) was done with Statistical software using the program Stpr2 and Stpr3. All the experiments were conducted in triplicates, and the results have been reported in terms of critical difference (CD).

Results and discussion

Identification of cellulolytic strain

A number of microorganisms hydrolyzing CMC were isolated and screened for their cellulolytic potential on CMC agar plates. Of them, isolate LFS3 was selected for further studies based on its maximum zone of clearance and the highest activity in liquid fermentation medium (Fig. 1a). The strain was found to be gram positive, rod shaped, and aerobic in nature. The phylogenetic analysis based on BLAST search using 16S rDNA gene sequence exhibited its maximum homology (98 %) with *Bacillus subtilis* strain BSX5 and thus designated as *B. subtilis* strain LFS3 (Fig. 1b). In previous studies, diverse types of genera have been reported for producing the cellulase enzyme including *B. subtilis* (Kim et al. 2009), *Marinobacter* (Shanmughapriya et al. 2010), *Penicillium* (Jeya et al.

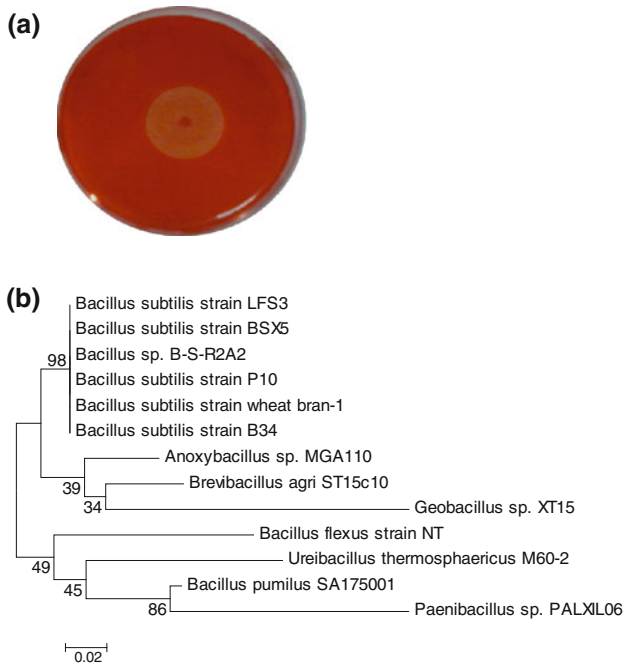

Fig. 1 Screening for cellulolytic activity of *B. subtilis* LFS3 **a** zone of clearance after staining with Congo red on CMC agar plate; **b** Phylogenetic tree of *B. subtilis* LFS3 associated with other members of the genus *Bacillus* using 16S rDNA sequence retrieved from the database using neighbour-joining method. The bootstrap values were generated from 1,000 replicates

Table 1 Summary of purification of the CMCase produced by *B. subtilis* strain LFS3

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield
Crude enzyme	2,500	3,500	0.714	1	100
$(NH_4)_2SO_4$ precipitation	1,120	658	1.7	2.3	45
DEAE Sephadex A-50	825	70	11.78	16	33
Sephadex G-100	375	26	14.42	20	15

2010), *Aspergillus* (Tao et al. 2010), and *Thermomonospora* (George et al. 2001).

Molecular weight determination of purified enzyme

The cellulase enzyme was purified from the culture broth of *B. subtilis* strain LFS3 following the steps specified in Table 1. The molecular weight of the purified enzyme was estimated to be about 185 kDa as confirmed by the presence of single protein band in native gel. The result of activity staining has also shown the active band of CMCase enzyme corresponding to the size of about 185 kDa

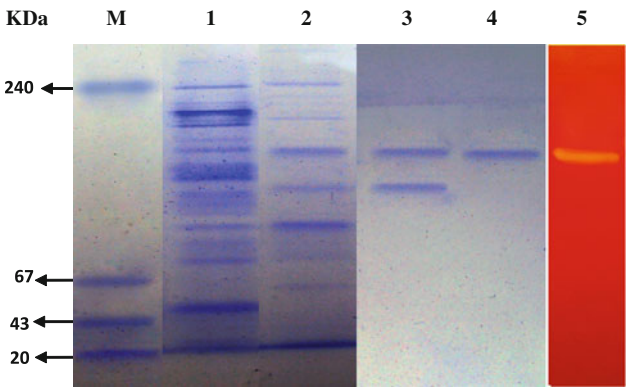

Fig. 2 Native PAGE analysis of CMCase produced by *B. subtilis* strain LFS3. *Lane 1* molecular mass markers, *Lane 2* crude extract, *Lane 3* ammonium sulphate fractionation, *Lane 4* active fractions of DEAE Sephadex A-50, *Lane 5* active fractions of DEAE Sephadex G100 chromatography, *Lane 6* activity staining of CMCase with Congo red

(Fig. 2). Our results are close to those of Kotchoni et al. (2006) and Singh et al. (2004) who have also reported the molecular mass of purified CMCase around 170 and 183 kDa, respectively, produced by *Bacillus* sp. The purified enzyme showed 20-fold increase in the activity with a recovery yield of 15 %. A previous study on purified CMCase enzyme from *B. subtilis* subsp. *subtilis* A-53 has found 5.7 times increase in activity with recovery yield of only 0.73 % (Kim et al. 2009).

Effect of pH on enzyme activity and stability

The effect of pH on the CMCase activity was examined at various pH values ranging from pH 3.0 to 12. Activity profile of purified enzyme showed its highest activity at pH 4.0 and more than 85 % of the activity still retained even the pH dropped to 3.0. These results represent the acidophilic nature of enzyme. On increasing the pH level from 4.0 to 12.0, the enzyme activity was reduced progressively (Fig. 3a). More than 50 % of the original CMCase activity of the purified cellulase enzyme was recorded between pH 3.0 and 8.0, whereas it was least at alkaline pH 12.0 with only 20 % activity. Several studies have been conducted on alkaline stable cellulases from *Bacillus* sp. (Tian and Wang 1998; Hakamada et al. 2000; Singh et al. 2004; Kim et al. 2005; Trivedi et al. 2011), *Marinobacter* sp. MS1032 (Shanmughapriya et al. 2010), and *Stachybotrys atra* BP-A (Picart, Diaz, and Pastor 2008). However, there are only a few reports on thermoacidstable cellulases from *Bacillus* species (Mawadza et al. 2000; Li et al. 2006).

The pH stability of the purified cellulase was also evaluated at different pH values as indicated above. The enzyme revealed good stability toward acidic (pH 3–5), while the stability toward highly alkaline (pH 11.0–12.0) conditions was found to be little. It is apparent from Fig. 3b

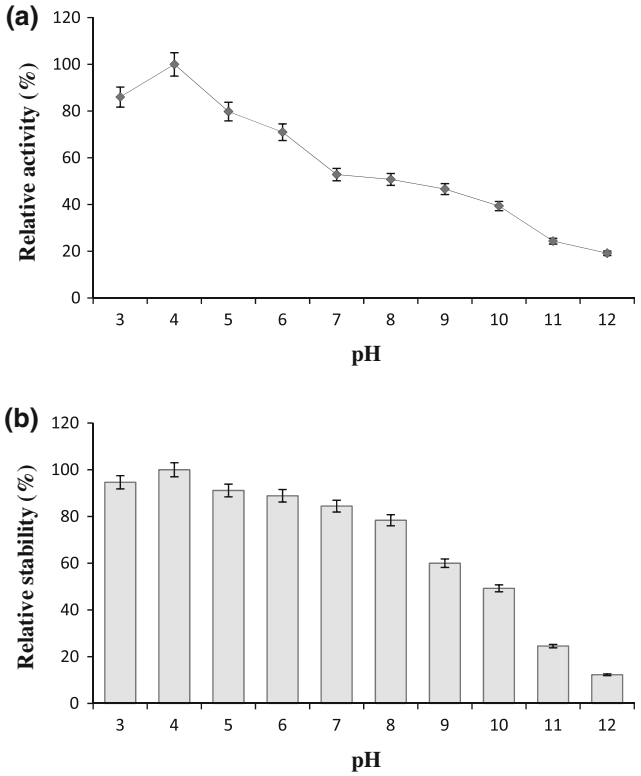

Fig. 3 Effect of pH on the enzyme activity (**a**) and stability (**b**) of purified cellulase produced by *B. subtilis* strain LFS3. For optimal enzyme activity, the enzyme was incubated at 50 °C for 30 min with 2 % CMC dissolved in different buffers (50 mM): acetate buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–7.0), Tris–HCl buffer (pH 8.0–10.0), and glycine NaOH (pH 11.0–12.0). For pH stability, the enzyme was incubated at room temperature for 1 h using different buffers as indicated above

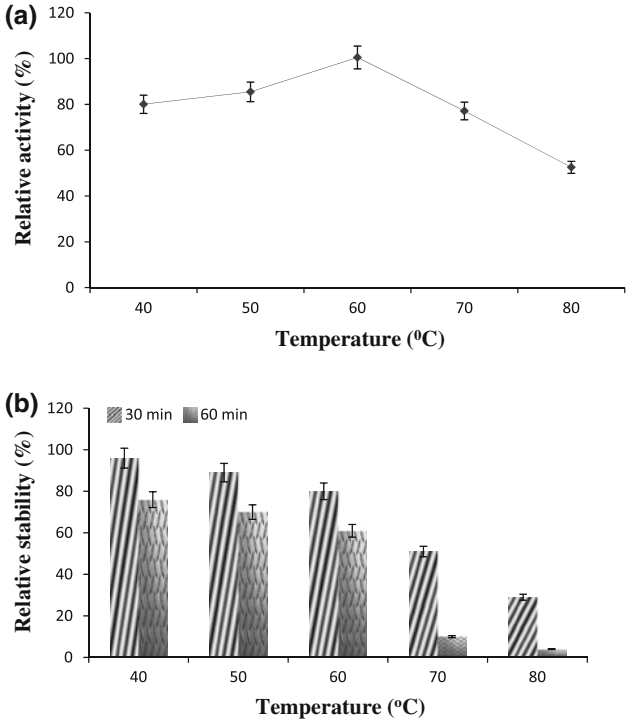

Fig. 4 Effect of temperature on the enzyme activity (**a**) and stability (**b**) of purified cellulase produced by *B. subtilis* strain LFS3. The enzyme activity was measured at temperatures ranging from 40 to 80 °C using acetate buffer (pH 4.0). For the thermal stability of cellulase, the enzyme was incubated at indicated temperatures for 30 min and 60 min

that the enzyme can retain >75 % of its maximum activity at broad pH values ranging from pH 3.0 to 8.0. The stability over a broad pH range seems to be characteristic of many *Bacillus* endoglucanases. Some previous workers have also reported that the cellulase enzymes produced by several *Bacillus* sp. are stable over a wide pH range (Mawadza et al. 2000; George et al. 2001; Lee et al. 2008).

Effect of temperature on enzyme activity and stability

The impact of temperature on the CMCase activity was also determined at different temperatures ranging from 40 to 80 °C. Among the five different temperatures tested, 60 °C is the optimum temperature for maximum enzyme activity; on either side of this temperature there was a decline in activity. This value of temperature required for maximal activity is similar to those of commercial cellulase enzyme. Our findings are in agreement with those of Endo et al. (2001) and Hakamada et al. (2002) who have also found 60 °C as a most favourable temperature for CMCase activity. A closer look at Fig. 4a revealed that the enzyme activity was decreased rapidly above 60 °C, and a value of about 53 % was obtained at 80 °C. Relative activities of purified enzyme recorded at 40 and 50 °C were 80 and 85 %, respectively.

When the enzyme was studied for its stability at various temperatures as specified above, it was found that the enzyme was stable enough at 40–60 °C after a pre-incubation period of 30 min. The enzyme exhibited 96 % activity at 40 °C and declined to 29 %, when the enzyme was incubated at 80 °C for 30 min. On extending the incubation period from 30 min to 1 h, more than 61 % of the original CMCase activity of the purified enzyme was maintained at broad temperatures ranging from 40 to 60 °C, whereas <10 % of the original CMCase activity was observed at temperature higher than 60 °C (Fig. 4b). These results suggest that our enzyme was stable up to 60 °C and above this temperature; a rapid decrease in stability takes place particularly after incubation for 1 h. On the other hand, endoglucanase from *A. niger* (Parry et al. 1983) and *Bacillus* strains CH43 and HR68 (Mawadza et al. 2000) was reported to be stable up to 50 °C.

Effect of additives on enzyme activity

The influence of various additives on the purified cellulase was determined by performing the assay with additives.

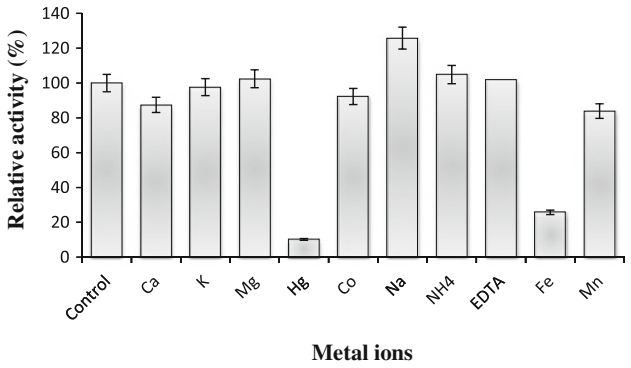

Fig. 5 CMCase enzyme activity affected by the presence of various additives with the final concentration of 5 mM dissolved in the acetate buffer (pH 4.0)

Majority of *Bacillus* spp. producing cellulase showed different type of inhibition as well as activation with different additives depending on the type of cations (Christakopoulos et al. 1999). Presence of Na^+ metal ions in the reaction mixture stimulated the enzyme activity largely, while the metal ions of Mg^{2+}, NH_4^+, and EDTA caused it to enhance moderately. It is clear from the Fig. 5 that the enzyme activity was strongly inhibited by Hg^{2+} and Fe^{3+}, while the partial inhibition was observed in case of Ca^{2+}, K^+, Co^{2+}, and Mn^{2+}. The inhibition by Hg^{2+} ions is not just related to binding the thiol groups but may be the result of interactions with tryptophan residue or the carboxyl group of amino acids in the enzyme (Lamed et al. 1994). Our results differ from some earlier studies in which Co^{2+} (Mawadza et al. 2000), Ca^{2+} (Lee et al. 2008), and K^+ (Kim et al. 2009) were reported as inducers of cellulase activity.

Kinetic analysis

Kinetic study of CMCase enzyme demonstrated that the enzyme was completely saturated at concentration of 0.25 % (w/v) CMC. The Michaelis–Menten kinetic parameters (V_{max} and K_m) of purified enzyme were calculated using Lineweaver–Burk double reciprocal plot. A closer look at Fig. 6 demonstrated the kinetic properties of the thermoacidophilic cellulase with K_m value 2.2 mg/ml and V_{max} with 699.0 U/ml. Some previous workers have also found K_m value in the range of 0.6–7.2 mg/ml for CMC (Kim 1995; Wang et al. 2009).

Substrate specificity

The relative hydrolytic activities of purified enzyme with different substrates were compared by measuring the amount of hydrolysed products. The enzyme degraded CMC, filter paper and xylan; however, almost negligible activity was observed in case of cellulose and pNPG as shown in Table 2. The purified enzyme exhibited significantly higher activity towards CMC, a soluble cellulosic substrate with β-1,4-linkage, than any other substrates. It could not hydrolyze crystalline cellulosic material such as cellulose powder which is possibly due to the low affinity of enzyme for crystalline cellulose. These results suggest the nature of our enzyme as an endo type of cellulase. Our results are similar to those of Kim et al. (2009) who has also found the same characteristics of endoglucanase enzyme produced by *Bacillus subtilis*.

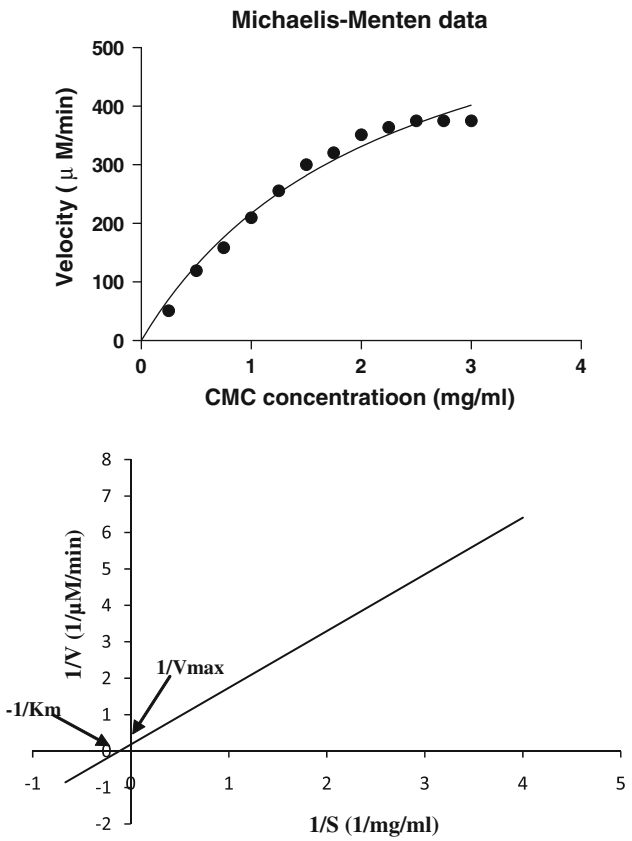

Fig. 6 Lineweaver–Burk double reciprocal plots of purified CMCase produced by *B. subtilis* strain LFS3

Table 2 Substrate specificity of the CMCase produced by *B. subtilis* strain LFS3

Substrate	Relative activity (%)
Control	ND
CMC	100
Cellulose	ND
Xylan	20.3
pNPG	ND
Filter paper	8.4

ND not detectable

Conclusion

The results of our study suggest that the cellulase secreted by *B. subtilis* LFS3 is acidophilic as well as thermophilic in nature. Enzymes that are active in acidic pH ranges and high temperature are usually desirable for biomass conversion of lignocellulosic waste. Acidic cellulases can also be useful for industrial application such as animal feed industry, clarification of fruit juices, and non-ionic surfactant-assisted acidic deinking of old news print (ONP) and old magazines (OMG). The use of acidic cellulases during deinking is advantageous as it improves pulp freeness and repulping efficiency. Cellulase enzymes rich in endoglucanases are best suited for biopolishing and biofinishing of cotton and other cellulosic fabrics. It enhances softness and water absorbance property of fibres, strongly reduces the tendency for pill formation, and provides a cleaner surface structure with less fuzz.

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