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Thermostable, haloalkaline cellulase from *Bacillus halodurans* CAS 1 by conversion of lignocellulosic wastes

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ABSTRACT

An extracellular thermostable, haloalkaline cellulase by bioconversion of lignocellulosic wastes from *Bacillus halodurans* CAS 1 was purified to homogeneity with recovery of 12.54% and purity fold 7.96 with the molecular weight of 44 kDa. The optimum temperature, pH and NaCl for enzyme activity was determined as 60 °C, 9.0 and 30% and it retained 80% of activity even at 80 °C, 12 and 35% respectively. The activity was greatly inhibited by EDTA, indicating that it was a metalloenzyme and significant inhibition by PMSF revealed that serine residue was essential for catalytic activity. The purified cellulase hydrolyzed CMC, cellobiose and xylan, but not avicel, cellulose and PNPG. Furthermore, the cellulase was highly stable in the presence of detergents and organic solvents such as acetone, n-hexane and acetonitrile. Thus, the purified cellulase from *B. halodurans* utilizing lignocellulosic biomass could be greatly useful to develop industrial processes.

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1. Introduction

Cellulose is the most abundant renewable biopolymer which considered as most important reservoir of carbons to convert glucose residues (Back & Kwon, 2007; Delmer & Haigler, 2002). Enormous amounts of agricultural, industrial and municipal cellulosic wastes are being accumulated or used inefficiently due to their cost involved in utilization processes (Sukumaran, Singhania, Mathew, & Pandey, 2009). Lignocellulosic materials are ubiquitous and consist mainly of cellulose (40–60%) with lesser, but significant, amounts of hemicellulose (20–30%) and lignin (15–30%) (Saha & Cotta, 2008). Ethanol production from lignocellulosic biomass is emerging as one of the most important technologies for sustainable transportation fuels and major constrain in enzymatic saccharification of cellulosic materials for the production of fermentable sugars is low productivity and cost of cellulases (Sukumaran et al., 2009). Bioconversion of cellulose into fermentable sugars is a biorefining area that has invested enormous research efforts, as it is a prerequisite for the subsequent production of bio-energy (Kumar, Singh, & Singh, 2008). Bioconversion of cellulose containing raw materials is of considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as an inexpensive carbon sources for cellulase production (Ballesteros, Oliva, Negro, Manzannares, & Ballesteros, 2004).

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials (Lee & Koo, 2001). The complete enzymatic hydrolysis of cellulosic materials needs at least three different types of cellulases; endoglucanase (1,4-β-D-glucan-4-glucanohydrolase; carboxymethylcellulase), exocellobiohydrolase (1,4-β-D-glucanglucohydrolase; avicelase), and β-glucosidase (β-D-glucosideglucohydrolase) (Yi, Sandra, John, & Shu, 1999). Recently the potential of cellulases was revealed in various industries, such as food, textiles and laundry, pulp and paper and agricultural as well as research and development (Bhat, 2000).

Application of thermostable and alkaline cellulases found to be an ideal for the enhancement of the efficiency of laundry detergents, deinking of papers, biological wood pulping and synthesis of chiromolecules (Horikoshi, 1999). Marine microorganisms had received more attention as a resource for newer enzymes, because of relatively more stable and active than the corresponding enzymes derived from plants or animals (Annamalai et al., 2012; Kin, 2006; Lee, Kim, Lee, Chung, & Lee, 2010).

However many studies have been made on production and purification of cellulase, no attempt has been made on cellulase from *Bacillus halodurans* CAS 1 through bioconversion of

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lignocellulosic wastes. Hence in the present study, an attempt was made on production, purification and characterization of cellulase from *B. halodurans* CAS 1 to enhance the utilization of abundant cellulosic biomass.

2. Materials and methods

2.1. Materials

Rice bran (RB) and wheat bran (WB) were purchased from local market and sun dried. The dried materials were milled and sieved (100 μm) to get a uniform fine powder and used as sole carbon sources for cellulase production. Bovine serum albumin (BSA), carboxymethyl cellulose (CMC), reagents for protein estimation and SDS-PAGE, DEAE-cellulose and Sephadex G-50 and other analytical grade chemicals were purchased from Merck, Mumbai.

2.2. Microorganism

To isolate cellulase producing microorganisms, marine sediments collected from Parangipettai coast, Tamilnadu, India was suspended with 0.85% (w/v) NaCl and then plated on marine agar plate (pH 9) at 55 °C for 48 h. Further, isolated colonies were screened for cellulase production on agar plates contained 0.5% yeast extract, 0.5% casamino acid, 0.5% $MgSO_4 \cdot 7H_2O$, 0.3% tri-sodium citrate, 0.2% KCl and 5% sodium chloride, supplemented with 0.5% (w/v) carboxymethyl-cellulose (CMC) (pH 9) and flooded with Congo red followed by washing with 0.1% NaCl. The strain which produced highest clear hydrolysis zone was consider as potential strain and designated as CAS 1.

The cellulase producing potential strain was identified by employing standard morphological, physiological and biochemical identification schemes (Garrity, Boone, & Castenholz, 2001) and confirmed by 16S rRNA gene sequencing. Briefly, the DNA was isolated by phenol chloroform method (Marmur, 1961). The primer sequences were selected from the conserved regions as previously reported for the bacterial 16S rRNA gene (Marchesi et al., 1998). Sequencing was done using forward primer (5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer (5′-GGGCGGTGTGTACAAGGC-3′). PCR reactions were performed with the following conditions: 35 cycles consisting of 95 °C for 1 min and 72 °C for 5 min, followed by final extension of 5 min at 72 °C. The 16S rRNA gene sequences were obtained by an automated DNA sequencer (Megabace, GE) and homology with those sequences in the GenBank was analyzed using CLUSTAL X software.

2.3. Effect of lignocellulosic wastes on cellulase production

To study the effect of lignocellulosic wastes for cellulase production, growth was carried out in 250 ml Erlenmeyer flasks in 50 ml of basal medium contained; 0.5% yeast extract, 0.5% casamino acid, 0.5% $MgSO_4 \cdot 7H_2O$, 0.3% tri-sodium citrate, 0.2% KCl and 5% NaCl (pH 9) supplemented with 1% (w/v) lignocellulosic wastes to be investigated such as rice bran (RB), wheat bran (WB) and rice and wheat bran at various proportion (RB:WB – 1:3, 1:1 and 3:1). The cultures were incubated aerobically at 55 °C for 60 h and the cellulase production was estimated by reducing sugar method (Miller, 1959).

2.4. Production and purification of cellulase

2.4.1. Enzyme production

For cellulase production, *B. halodurans* CAS 1 was grown in an Erlenmeyer flask (500 ml) containing 100 ml of basal medium supplemented with 1% rice bran (pH 9) was seeded with 1% inoculum (~10^5 cells/ml) and incubated (150 × *g*) for 60 h at 55 °C. The kinetics of cell growth and cellulase production were studied from aliquots withdrawn aseptically at every 6 h. Cell growth was estimated by measuring the viable cell count by serial dilution plating technique (log CFU/ml) and enzyme activity was measured by standard DNS assay. After incubation, culture broth was centrifuged (4 °C and 12,000 × *g* for 20 min), and the supernatant was used as crude enzyme for further purification.

2.4.2. Purification of cellulase

For purification of cellulase, ammonium sulfate was added to the culture supernatant to obtain 60% saturation (w/v) and allowed to stand overnight at 4 °C. The precipitate collected through centrifugation at 6000 × *g* for 15 min was dissolved in 50 mM Tris–HCl buffer (pH 9) and dialyzed against the same buffer (4 °C). The dialysate was loaded on DEAE-cellulose column (5 cm × 25 cm) and eluted with linear gradient of NaCl (0–1 M) at a flow rate of 0.5 ml/min. Fractions were collected and assayed for enzyme activity and fractions which exhibited enzyme activity were pooled together and concentrated by ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 50 mM Tris–HCl buffer (pH 9). Concentrated fractions were loaded onto a Sephadex G-50 column (2.5 cm × 25 cm) equilibrated with 50 mM Tris–HCl buffer (pH 9) and eluted with same buffer at a flow rate of 15 ml/h. Fractions exhibiting cellulase activity were pooled together and used as a purified enzyme for further characterization study.

2.5. Analytical methods

The cellulase activity was measured by mixing 100 μl of enzyme solution with 100 μl of 1% (w/v) CMC in 50 mM Tris–HCl buffer (pH 9) at 50 °C for 20 min. The reaction was stopped by adding the 3,5-dinitrosalicylic acid (DNS) regent. The mixture was boiled for 10 min, cooled in ice and amount of reducing sugars liberated was measured at 550 nm (Miller, 1959). One unit of CMCase was defined as the amount of enzyme required to liberate 1 μmol of glucose per min.

Protein content in the crude and purified cellulase was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin (BSA) as reference.

The molecular weight of the purified cellulase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using 4% stacking and 12% resolving gel. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie blue, 30% (v/v) methanol, and 10% (v/v) acetic acid. The standard molecular weight markers used for calibration were phosphorylase b (97.4 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.3 kDa).

2.6. Substrate specificity

The substrate specificity of the purified cellulase was determined by performing the assay with different substrates; avicel (microcrystalline cellulose), carboxymethylcellulose (CMC), cellobiose, β-glucan, *p*-nitrophenyl-β-D-glucopyranoside (PNPG) and xylan. Cellulase activity was measured by incubating 200 μl of diluted enzyme solution (10 μl of enzyme + 190 μl of 50 mM Tris–HCl buffer, pH 9) and 100 μl of substrate solution at 50 °C for 1 h and the amount of reducing sugar produced was measured by DNS method. β-Glucosidase activity was determined by hydrolysis of PNPG and the resulting *p*-nitrophenol was measured at 400 nm with *p*-nitrophenol (*p*NP) as reference (Riou, Salmon, Vallier, Gunata, & Barre, 1998).

2.7. Effect of temperature on activity and stability of the purified cellulase

The optimum temperature for enzyme activity was determined by incubating the reaction mixture (200 μl diluted enzyme solution + 200 μl of 1% CMC) with for 1 h at various temperatures ranged between 30 and 95 °C. Thermal stability study was performed by incubating the purified enzyme in 50 mM Tris–HCl buffer (pH 9) at different temperatures ranging from 30 to 95 °C for 24 h and the residual activity was assayed by standard DNS method.

2.8. Effect of pH on activity and stability of purified cellulases

The optimal pH for the activity purified cellulase was evaluated by incubating the reaction mixture at 50 °C for 1 h with different pH buffers. Buffers used were; 50 mM sodium citrate (pH 3–6), 50 mM sodium phosphate (pH 6–8), 50 mM glycine–NaOH (9–11), and 50 mM dilute NaOH for pH 12–13. The pH stability of the purified enzyme was determined by incubating 10 μl of purified cellulase + 190 μl of above mentioned buffer solutions for 24 h and the residual activity were assayed by standard DNS method.

2.9. Effect of NaCl on activity and stability of purified cellulase

The optimum NaCl concentration for cellulase activity was determined by incubating the purified enzyme with substrate solution (1% CMC) for 1 h at different NaCl concentrations (0–35%). The halostability of purified enzyme was determined by incubating the purified enzyme in 50 mM Tris–HCl buffer (pH 9) at various NaCl concentrations (0–35%) for 24 h and the residual activity were assayed by standard DNS method.

2.10. Effect of metals, surfactants (ionic, non-ionic) and commercial detergents on activity of purified cellulase

Effect of metal ions such as Mn^{2+}, Fe^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+} and Hg^{2+} on purified cellulase activity was investigated at 0.1 and 1 mM concentrations. The influence of surfactants (ionic and non-ionic detergents) was evaluated using SDS, Triton X-100, Tween 80 and sodium deoxycholate (1%, v/v). Effect of commercial detergents on stability of purified cellulase was studied with 1% (w/v) of commercial detergents such as Ariel, Rin, Henko and Tide. The purified cellulase (10 μl enzyme + 190 μl 50 mM Tris–HCl buffer) was preincubated with aforementioned metal ions, surfactants and detergents at 50 °C for 1 h and then assayed for enzyme activity. The enzyme without addition of any metal and detergents was assayed (control) and its activity was considered as 100%.

2.11. Effect of organic solvents on cellulase activity and stability

The organic solvents stability on purified cellulase was investigated by incubating with various organic solvents such as methanol, ethanol, acetone, isopropanol, n-hexane, benzene, acetonitrile, ethyl ether, toluene and n-butanol (25%, v/v) for 4 h at 50 °C and the residual activity was determined by standard DNS assay.

3. Results and discussion

3.1. Microorganisms

A cellulase producing microorganism by utilizing lignocellulosic biomass was isolated from marine sediments of Parangipettai coast, Tamilnadu, India and designated as CAS 1. On the basis of the morphological, physiological, and biochemical characteristics, CAS 1 is a Gram-positive and endospore-forming bacillus, with catalase but without oxidase, which grows in both aerobic and anaerobic environments. The 16S rRNA gene sequencing analysis evidenced that this strain exhibited highest homology (99%) with *B. halodurans* strain US193 (Fig. 1). Based on the evolution distance and the phylogenetic tree, this strain was identified as *B. halodurans* and designated as *B. halodurans* CAS 1 (GenBank accession no. HQ116805).

3.2. Effect of various lignocellulosic wastes on cellulase production

The effect of lignocellulosic wastes as carbon source on cell growth and production of cellulase by *B. halodurans* CAS 1 was investigated with RB, WB and various proportion RB:WB. Results from this study revealed that 1% RB (3424 U/ml) was found as suitable substrate for cellulase production than other substrates used such as 1:1 RB:WB (3268 U/ml) and WB (3016 U/ml) (Fig. 2). Although, Lee et al. (2010) found that rice bran was the best carbon sources for cell growth and cellulase production by *Bacillus subtilis* subsp. *subtlis* A-53, the resultant cellulase production (89.6 U/ml) was comparatively lower than the cellulase produced by *B. halodurans* CAS 1. Jo et al. (2008) and Mayende, Wilhelmi, and Pletschke (2006) were also found that rice hulls and rice bran were the best carbon source for production of CMCase produced by *Bacillus amyloliquefaciens* DL-3 and *Bacillus* sp. CH 43 and HR 68 respectively.

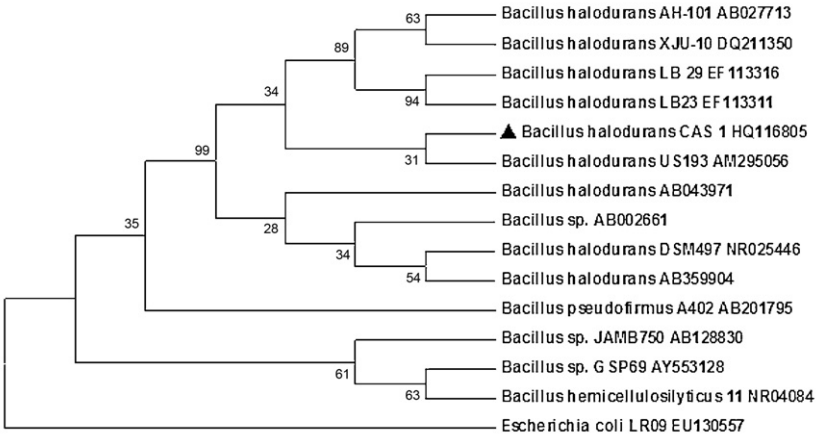

Fig. 1. Phylogenetic tree of *Bacillus halodurans* CAS 1 strain 16S rRNA gene sequence with other *Bacillus* species/strains.

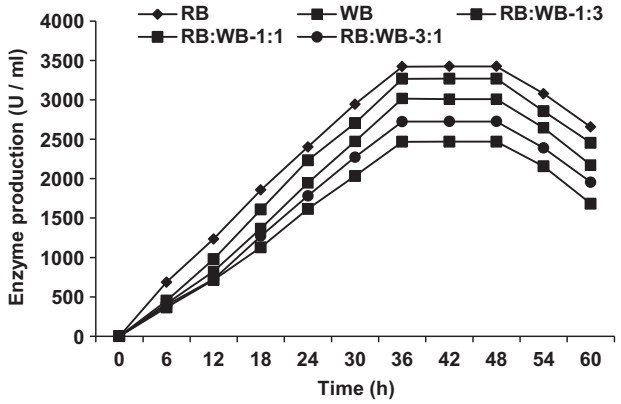

Fig. 2. Effect of various lignocellulosic wastes (carbon sources) on cellulase production by *B. halodurans* CAS 1 [RB: rice bran, WB: wheat bran, RB:WB (rice bran:wheat bran) ratio: RB:WB 1:3, RB:WB 1:1 and RB:WB 3:1].

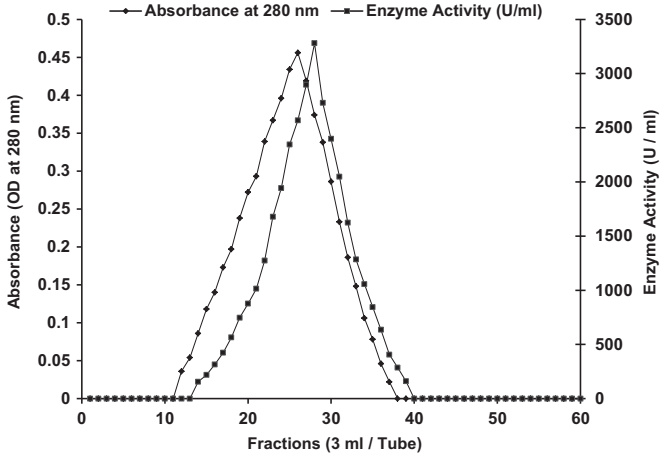

Fig. 4. Elution profile of cellulase from *B. halodurans* CAS 1on Sephadex G-50 gel filtration chromatography.

Rice bran and rice hull generates one of the largest sources of cellulosic waste when paddy is processed into edible rice (Sharma, Khare, & Gupta, 2001). The cellulosic waste materials such as rice bran, wheat bran and rice hulls were used as best carbon sources for cellulase production due to their inducible nature (Lee et al., 2010). The cellulase produced by the hydrolysis of cellulosic biomass by *B. halodurans* CAS 1 could be useful for the production of ethanol, single cell protein and other industrially important chemicals.

3.3. Production and purification of cellulase by B. halodurans CAS 1

Cellulase production was carried out in 500 ml Erlenmeyer flask containing 100 ml of basal medium supplemented with 1% rice bran. The cell growth and cellulase production were started parallely from the log phase itself (6 h) and reached maximum (3274 U/ml) at 36 h and it started decrease gradually afterwards (Fig. 3). It seems that the cellulase production (3274 U/ml) in this study was comparatively higher than the earlier reports. Lee et al. (2008) reported that highest activity of cellulase *B. amyloliquefaciens* DL-3 was about 153 U/ml with 2% rice hull. Thus, results obtained from the present study indicated that the marine bacterium *B. halodurans* CAS 1 efficiently utilized agricultural waste (rice bran) with less incubation time and higher cellulase production. For industrial applications *B. halodurans* CAS 1 could be an ideal candidate for the development of more efficient and cost-effective forms of the fermentation process to convert lignocellulosic biomass.

The cellulase from the culture broth of *B. halodurans* CAS 1 was purified through multistep purification (Fig. 4) and summary of the purification profile was presented in Table 1. The overall purification fold of cellulase was about 7.96 with the specific activity of 509.84 U/mg and 12.54% yield. The homogeneity of the purified enzyme was analyzed and confirmed by the single band obtained in SDS-PAGE. The molecular weight of the purified cellulase was estimated as 44 kDa (Fig. 5) which is much larger than cellulases from other *Bacillus* strains such as *Bacillus licheniformis* AU01 (37 kDa) (Annamalai et al., 2012), *Bacillus sphaericus* JS1 (29 kDa) (Singh, Batra, & Sobti, 2004) and *Bacillus circulans* (43 kDa) (Hakamada et al., 2002), but smaller than the cellulase from *B. subtilis* subsp.

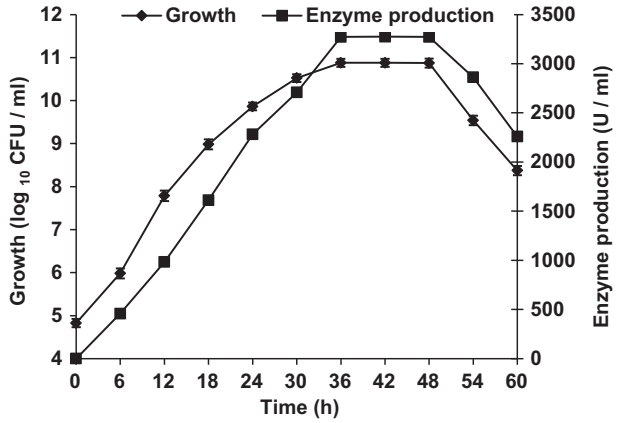

Fig. 3. Kinetics of cell growth ($\log_{10}$ CFU/ml) and cellulase production (U/ml). For cellulase production, *B. halodurans* CAS 1 was grown in 100 ml of basal medium (0.5% yeast extract, 0.5% casamino acid, 0.5% $MgSO_4 \cdot 7H_2O$, 0.3% tri-sodium citrate, 0.2% KCl and 5% NaCl, pH 9) containing 1% RB in 500 ml of Erlenmeyer flask was incubated for 60 h at 55 °C. The results were presented as mean ± SD, $n = 3$.

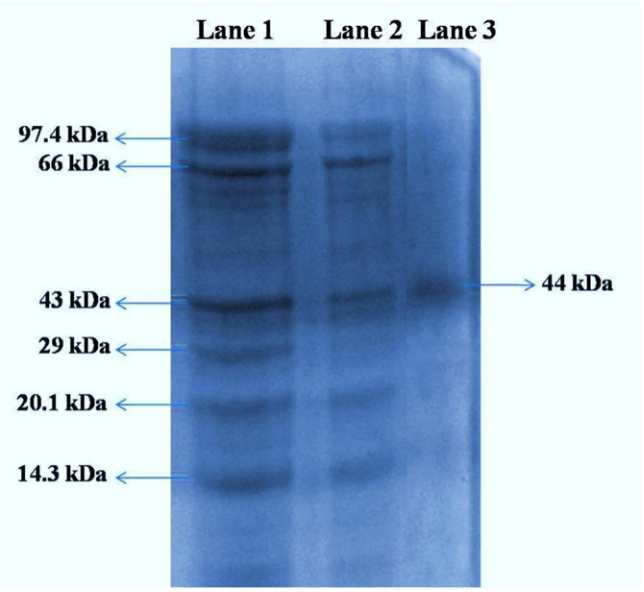

Fig. 5. SDS-PAGE analysis of cellulase from *B. halodurans* CAS 1. Lane 1: molecular markers [14.3 kDa – lysozyme, 20.1 kDa – trypsin inhibitor, 29 kDa – carbonic anhydrase, 43 kDa – ovalbumin, 66 kDa – bovine serum albumin, 97.4 kDa – phosphorylase 'B'], Lane 2: crude enzyme and Lane 3: purified cellulase (44 kDa).

Table 1
Summary of purification profile of cellulase from *B. halodurans* CAS 1.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Culture filtrate	31,237.2	163	191.63	1	100
$(NH_4)_2SO_4$ precipitate	15,324.6	71.7	213.73	2.03	49.05
DEAE-cellulose	6243.4	14.2	362.98	5.00	19.98
Sephadex G-50	3672.6	9.03	428.54	8.50	11.75

B. halodurans CAS 1 was grown in 100 ml of basal medium in an Erlenmeyer flask (500 ml) containing 1% RB, (pH 9.0) in a shaking incubator for 60 h at 55 °C. Culture broth from the resulting experiment was used for purification.

subtilis A-53 (56 kDa) (Kim et al., 2009), *B. amyloliquefaciens* DL-3 (53 kDa) (Lee et al., 2008), *Bacillus* sp. L1 (45 kDa) (Li & Yu, 2012) and *Bacillus flexus* (97 kDa) (Trivedi et al., 2011).

3.4. Effect of temperature, pH and NaCl on enzyme activity and stability

The effect of temperature on activity and stability of cellulase was determined at various temperatures ranged between 30 and 95 °C. The optimum temperature for cellulase activity was found to be 60 °C at pH 9.0 and decreased rapidly as the temperature increased above 60 °C. The optimal temperature of cellulase produced by the marine bacterium, *B. halodurans* CAS 1 is higher than those produced by *B. licheniformis* AU 01 (Annamalai, Thavasi, Vijayalakshmi, & Balasubramanian, 2011), *B. amyloliquefaciens* DL-3 (Lee et al., 2008) and *B. subtilis* subsp. *subtilis* A-53 (Kim et al., 2009). Thermostability studies revealed that the purified enzyme was 100% stable up to 70 °C and it retained more than 90% of the original activity at 80 °C and 36% even at 95 °C (Fig. 6a). The thermostability of the cellulase purified from *B. halodurans* CAS 1 was higher than that those produced by *B. licheniformis* AU 01 (Annamalai et al., 2011), *B. amyloliquefaciens* DL-3 (Lee et al., 2008) and *B. subtilis* subsp. *subtilis* A-53 (Kim et al., 2009).

The effect of pH on activity of purified cellulase produced by *B. halodurans* CAS 1 was examined at various pHs ranging from pH 4.0 to 12.0. The optimal pH for cellulase activity was 9.0 and it exhibited more than 60% activity between pH 6.0 and 11.0. The optimum pH of the purified cellulase from *B. halodurans* CAS 1 was higher than the cellulases produced by *B. amyloliquefaciens* DL-3 (Lee et al., 2008) and *B. subtilis* subsp. *subtilis* A-53 (Kim et al., 2009) which exhibited optimum activity between pH 6 and 7.5. The pH stability studies revealed that the purified cellulase from *B. halodurans* CAS 1 was active over broad ranges of pH between 6 and 12. The purified enzyme was 100% stable at pH 9.0–11.0 and it retained 78% of its original activity even at pH 12.0 (Fig. 6b). The pH stability of the purified cellulase was higher that of cellulases produced by *B. licheniformis* AU 01 (Annamalai et al., 2012), *B. amyloliquefaciens* DL-3 (Lee et al., 2008) and *B. subtilis* subsp. *subtilis* A-53 (Kim et al., 2009) which were stable between pH 7.0 and 9.0. Recently, many researchers have been trying to exploit microbes for the isolation of thermostable, alkaline enzymes due to their tremendous industrial potential. In view of that, higher temperature and pH stability found with this purified cellulase could be useful for harsh industrial applications.

The effect of NaCl on activity of purified cellulase revealed that the activity was increased with increasing concentrations and optimum being at 30% whereas enzyme exhibits only 24% in the absence of NaCl. The halostability of the purified cellulase depicted that the enzyme was 100% stable up to 30% NaCl after 24 h and it retained 94% of its original activity even at 35% (Fig. 6c). But, the CMCase purified from *B. licheniformis* AU 01 retained only 34% of activity at 30% NaCl (Annamalai et al., 2011). However, most of the cellulases reported to date were stable only between 5% and 20% NaCl (Wang et al., 2009; Trivedi et al., 2011). The high salt-tolerance characteristic future of this enzyme is an important to the future application in various biotechnological processes that depends on high salinity or osmotic pressures.

Table 2
Substrate specificity of the purified cellulase from *B. halodurans* CAS 1 towards different substrate at pH 9.0 and 60 °C. The results were presented as mean ± SD, $n = 3$.

Substrate	Relative activity (%)
Avicel	–
CMC	100 ± 1.7
Cellobiose	24.6 ± 2.8
Cellulose	–
PNPG	–
Xylan	16.4 ± 1.3

3.5. Substrate specificity

Substrate specificity assay revealed that the purified cellulase exhibited significantly higher activity towards CMC (100 ± 1.7) followed by cellobiose (24.6 ± 2.8) and xylan (16.4 ± 1.3) (Table 2). The purified enzyme did not hydrolyzed avicel (crystalline cellulose) and cellulose (powder) due to the low affinity towards crystalline cellulose. Moreover, the purified enzyme was not degraded PNPG as the substrate for β-glucosidase. Similar findings were reported for other cellulases from *Bacillus* sp. BG-CS10 (Zhang, Li, Xue, Mao, & Ma, 2012) and *Bacillus* sp. L1 (Li & Yu, 2012). The results of the present study on substrate specificity revealed that this enzyme as an endo type of cellulase.

3.6. Effect of metals ions, surfactants (ionic, non-ionic) and commercial detergents on cellulase activity

The effect of metals on the activity of purified cellulase was summarized in Table 3. The activity of the cellulase was enhanced by

Table 3
Effect of metals and enzyme inhibitors on purified cellulase of *B. halodurans* CAS 1.

Metals	Concentrations (mM)	Residual activity (%)
Control	–	100
$MnSo_4$	1	113.3 ± 2.5
	5	127.5 ± 3.5
$FeSo_4$	1	81.3 ± 3.0
	5	60.6 ± 2.1
$CoCl_2$	1	65.3 ± 2.5
	5	49.3 ± 3.5
$MgSo_4$	1	123.6 ± 3.5
	5	134.4 ± 2.5
$CaCl_2$	1	115.6 ± 1.8
	5	134.3 ± 2.1
$HgCl_2$	1	18.6 ± 2.2
	5	27.3 ± 3.5
EDTA	1	26.6 ± 2.5
	5	15.2 ± 2.1
PMSF	10	23.2 ± 1.8
β-Mercaptoethanol	10	90.3 ± 2.5
DEPC	10	85.4 ± 2.2

The purified cellulase was preincubated with metals at 50 °C for 1 h and then assayed for enzyme activity. The results were presented as mean ± SD.

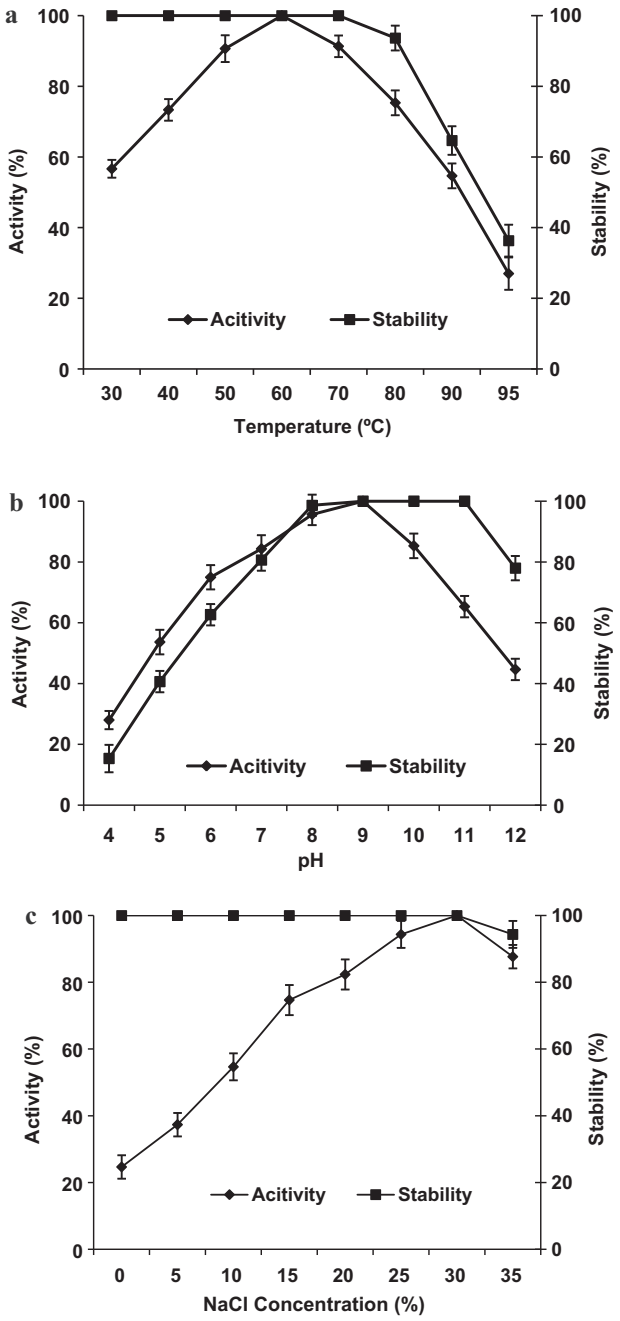

Fig. 6. (a) Effect of temperature on activity and stability of purified cellulase of *B. halodurans* CAS 1. Effect of temperature on enzyme activity was evaluated by incubating enzyme solution and substrate (1% CMC) for 1 h at different temperatures (30–95 °C) and stability was evaluated by incubating the enzyme solution for 24 h at various temperatures (30–95 °C) and the residual activity was measured. The results were presented as mean ± SD, $n = 3$. (b) Effect of pH on activity and stability of purified cellulase of *B. halodurans* CAS 1. Effect of pH on enzyme activity was evaluated by incubating reaction mixture (enzyme and substrate) for 1 h at different pH buffers (4–12) and stability was evaluated by incubating the enzyme solution for 24 h at various pH buffers and the residual activity was measured. The results were presented as mean ± SD, $n = 3$. (c) Effect of NaCl on activity and stability of purified cellulase of *B. halodurans* CAS 1. Effect of NaCl concentrations on enzyme activity was evaluated by incubating enzyme and substrate (1% CMC) for 1 h at different concentrations of NaCl (0–35%, w/v) and stability was evaluated by incubating the enzyme solution for 24 h at various NaCl concentrations (0–35%, w/v) and the residual activity was measured. The results were presented as mean ± SD, $n = 3$.

Table 4
Effect of anionic, non-ionic and commercial detergents on stability of purified cellulase from *B. halodurans* CAS 1.

	Residual activity (%)
Detergents	
SDS	84.3 ± 3.5
Tween 80	94.3 ± 2.0
Triton X-100	68.0 ± 3.0
Sodium deoxycholate	74.3 ± 2.5
Commercial detergents	
Rin	85.3 ± 2.5
Ariel	76.6 ± 1.8
Henko	64.6 ± 2.5
Tide	80.3 ± 3.5

The purified cellulase was preincubated with ionic, non-ionic and commercial detergents at 50 °C for 1 h and then assayed for enzyme activity. The results were presented as mean ± SD.

Mn^{2+}, Mg^{2+} and Ca^{2+} and inhibited with Fe^{2+}, Co^{2+} and Hg^{2+}. Similarly, activity of endoglucanase from *Bacillus* sp. AC-1 also inhibited by Fe^{2+}, Co^{2+} and Hg^{2+} (Li, Ding, Wang, Xu, & Zhao, 2006). Lamed, Tormo, Chirino, Morag, and Bayer (1994) suggested that 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.

The enzyme inhibitors such as EDTA (chelating agent) and PMSF (serine modifier) were significantly inhibited the enzyme activity, whereas no significant effects were found with β-mercaptoethanol and DEPC (Table 3). Thus, the activity was greatly inhibited by EDTA, indicated that the cellulase purified from this study was a metalloenzyme and inhibition by PMSF suggested that serine residue played an important role in its catalytic activity.

Regarding cellulase activity with surfactants, the enzyme retained 94.33% and 68% activity in the presence of Tween 80 and Triton X-100 (non-ionic surfactant) and 84.33% and 74.33% with SDS and sodium deoxycholate (anionic surfactant), respectively (Table 4). However, cellulase from a halotolerant isolate, *Bacillus* sp. L1 retained 91% activity towards Triton X-100, Tween 20, and Tween 80 and lost about 41.1% activity in the presence of SDS (Li & Yu, 2012). In general, cellulases used in detergent industry should not lose their activity in the presence of commercial detergents. The purified cellulase from *B. halodurans* CAS 1 retained its activity in the presence of some commercial detergents such as Rin (85.33%), Ariel (76.67%), Henko (64.67%) and Tide (80.33%). Thus, the stability of the purified cellulase towards surfactants and commercial detergents could be certainly useful in detergent industry dealing with cellulases.

3.7. *Effect of organic solvents on stability of purified cellulase*

The organic solvent stable cellulases were found to be quite attractive in recent days due its industrial applications such as bioremediation of carbohydrate-polluted salt marshes and industrial waste waters contaminated with organic solvents (Shafiei, Ziaee, & Amoozegar, 2011). In view of that, stability of the purified cellulase of *B. halodurans* CAS 1 was investigated and the results revealed that no complete inactivation was observed in the presence of the organic solvents. Furthermore, the enzyme activity was stimulated in the presence of some organic solvents such as acetone (114.3%), n-hexane (128.6%) and acetonitrile (105.2%) and no significant effect was found with methanol (83.2%), ethanol (68.3%), isopropanol (80.2%), ethyl ether (85.3%) and n-butanol (86.4%) (Table 5). Zaks and Klibanov (1988) suggested that stimulation of enzyme activity by organic solvents might be due to the residues of carried-over nonpolar hydrophobic solvent providing an interface, thereby keeping the enzyme in an open conformation

Table 5
Effect of organic solvents on stability of purified cellulase from *B. halodurans* CAS 1.

Organic solvents	Residual activity (%)
Methanol	83.2 ± 3.5
Ethanol	68.3 ± 2.5
Acetone	114.3 ± 2.5
Isopropanol	80.2 ± 1.8
n-Hexane	128.6 ± 2.5
Benzene	73.4 ± 2.5
Acetonitrile	105.2 ± 1.6
Ethyl ether	85.3 ± 2.2
Toluene	62.5 ± 1.8
n-Butanol	86.4 ± 2.2

The purified cellulase was incubated with organic solvents (25%, v/v) at 50 °C for 4 h and then assayed for enzyme activity. The results were presented as mean ± SD, $n = 3$.

which resulting stimulated activation. Hence, organic solvent stability of the purified cellulase indicates that this enzyme could be useful in reactions with both aqueous and organic solvents.

4. Conclusions

Considering the production cost and reutilization of cellulosic wastes, cellulase production through bioconversion in the present investigation seems to be promising approach which will also enhance its utilization. Moreover, the purified cellulase in this study exhibited higher thermostability (80 °C), alkaline tolerance (pH 12) and compatibility with ionic, non-ionic and commercial detergents and organic solvents. Thus, the interesting properties found with this purified cellulase will make this as potential candidate for practical applications in biotechnological processes and industrial applications.

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