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An alkali-halotolerant cellulase from *Bacillus flexus* isolated from green seaweed *Ulva lactuca*

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ABSTRACT

An extracellular alkali-halotolerant cellulase from the strain *Bacillus flexus* NT isolated from *Ulva lactuca* was purified to homogeneity with a recovery of 25.03% and purity fold of 22.31. The molecular weight of the enzyme was about 97 kDa and the V_{max} and K_m was 370.17 U/ml/min and 6.18 mg/ml respectively. The optimum pH and temperature for enzyme activity was 10 and 45 °C respectively. The enzymatic hydrolysis of the CMC was confirmed with GPC and GC-MS analysis. The stabilized activity of the enzyme even at high pH of 9.0–12.0 and residual activity of about 70% at salt concentration (NaCl 15%) revealed for its alkali-halotolerance nature. The metal ions Cd^{2+} and Li^{1+} were found as inducers while Cr^{2+}, Co^{2+}, Zn^{2+} and metal chelator EDTA have significantly inhibited the enzyme activity. Enzyme activity was insensitive to ethanol and isopropanol while partially inhibited by acetone, cyclohexane and benzene.

1. Introduction

The industrial and agriculture wastes contain considerable amounts of cellulose that can effectively be utilized either as a major source of energy feedstock or as a raw material for production of high value chemicals (Cherry & Fidantsef, 2003; Kim, Yoo, Oh, & Kim, 2003). Cellulose, the most abundant carbohydrate in nature, is a linear polysaccharide of repeating units of glucose linked with 1,4 β-acetal bond and mainly forms the primary structural cell wall component in both the lower and higher plants (Saha, Roy, Sen, & Ray, 2006). The herbaceous and woody plants are the primary sources of cellulose intact with complex hemicelluloses, lignin and pectin. Conversely, seaweeds contain mainly α-cellulose without much complex lignin thus differentiating them those of terrestrial plants and make them preferential source of cellulose (Siddhanta et al., 2009). The efficient hydrolytic conversion of cellulose into its monomers, i.e. glucose as source for high-energy molecule will facilitate to meet the future energy need and also will be an alternate to starch. Chemical hydrolysis (acid hydrolysis) is one of the viable methods currently being employed as a promising means of producing sugar from cellulose. The combination of high temperatures and strong acids in acid hydrolysis leads to the degradation of products, accumulation of non-sugar byproducts (such as inhibitors to subsequent chemical and biological conversion), and also pose problem of recovery of reaction agents and resulting saccharides (Sasaki et al., 1998).

The microorganisms with potential cellulolytic activities could provide unique opportunity towards the biodegradation of cellulosic matter through efficient enzymatic conversion into high energetic molecules (Wen, Liao, & Chen, 2005). Cellulases are inducible enzymes synthesized by microorganisms during their growth on the media containing cellulose as a sole source for carbon (Lee & Koo, 2001). At commercial scale, cellulases have been obtained mainly from fungal species of A*spergillus* and *Trichoderma* due to their high activity but at moderate temperature (Nandakumar, Thankur, Raghavarao, & Ghildyal, 1994). Several bacterial genera reported for cellulolytic activities include *Bacillus*, *Clostridium*, *Cellomonas*, *Rumminococcus*, *Alteromonas*, *Acetivibrio*, *Bacteriodes* (Roboson & Chambliss, 1989). Industrial applications of cellulases have been potentially utilized in leather, textile, agriculture, food, paper and pulp industries (Bhat, 2000; Kim, Hur, & Hong, 2005). The industrial utility of cellulase enzymes can further be improved by investigating the functional efficiency of these enzymes under extreme conditions of temperature and pH. In comparison to terrestrial environment, marine habitat with hyper variable conditions could represent the novel functional abilities of the microbes that can be further elucidated for their potential as source of extracellular enzymes.

This study describes the potential of marine bacteria *B. flexus* as a source of extracellular cellulase with promising applications in various industries. The strain was studied for salt tolerance with the

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sustainability of enzyme functionality and stability under extremities of pH, temperature and salinity. The influence of various solvents on the enzymatic activity was further enumerated to bring the industrial potential of enzyme.

2. Materials and methods

2.1. Materials

All analytical chemicals, media components and reagents used in these work were purchased from Sigma–Aldrich (U.S.A.) and Himedia laboratories (Mumbai, India).

2.2. Isolation and screening of cellulose degrading bacteria

Degraded marine algae *Ulva lactuca* maintained in the laboratory condition was used to screen out cellulose degrading bacteria. Different bacterial strains were isolated as pure culture on Marine agar Plates 2216 (Zobell marine agar) after incubation at 30 ± 2 °C. The cellulose degrading bacteria were screened by zone of clearance as qualitative measure of extracellular cellulase activity (Kasana, Salwan, Dhar, Dutt, & Gulati, 2008) after flooding the carboxymethylcellulose (1.5%) agar plates with Lugol's iodine solution.

2.3. Enzyme production media and growth condition

The inoculums of bacterial strains with higher activity of cellulase on plate assay were prepared by overnight incubation of the bacteria in Zobell marine broth at 30 ± 2 °C and 150 rpm. These inoculums was transferred to the production medium containing salts (0.5% Yeast extract, 3.5% Artificial sea water medium) supplemented with 1.5% CMC-Na as a sole source of carbon. The pH was adjusted to 7.5–8.0 before sterilization at 121 °C for 15 min. The culture was incubated at 30 ± 2 °C on rotary shaker at 150 rpm. After 72 h of incubation, the production media was centrifuged at $12{,}000 \times g$ for 30 min at 4 °C and the collected supernatant was treated as crude enzyme.

2.4. Biochemical characterization and identification of the microorganisms

Biochemical and morphological analysis were done according to the Bergey's Manual of Systematic Bacteriology. For bacterial surface structure, scanning electron microscopy (SEM) was done using LEO 1430VP (UK) system.

2.5. 16S rDNA sequencing for strain identification

Genomic DNA for molecular identification of bacterial strain was extracted using a Wizard Genomic DNA Preparation Kit (Promega Co., Madison, USA). The PCR amplification of 16S *rRNA* was carried out in 50 μl reaction mixture containing 10× PCR buffer having 200 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 1.25U *Taq Polymerase*, 0.5 μM of each universal forward primer fD1(5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer rD1(3′-AAGGAGGTGATCCAGCC-5′) and 50–100 ng of template DNA. The PCR reaction was run for 35 cycles in a DNA Thermal Cycler (Model No. 580 BR 1693, Bio.Rad Co., USA). PCR amplified product then purify, were sequenced (Macrogen, South Korea) and compared with sequence in nucleotide database (NCBI) using the BLAST algorithm. Multiple sequence alignment was carried out with CLUSTAL W (Thompson, Higgins, & Gibson, 1994). The neighbour-joining phylogenetic analysis was carried out with MEGA programme (Tamura, Dudley, Nei, & Kumar, 2007).

2.6. Growth curve

The bacterial cultures were drawn from growth medium at an interval of 10 h and absorbance was taken at 600 nm. The growth curve was plotted with absorbance vs time and at the same instants enzyme activity was also calculated. The halotolerance of the strain was estimated with their growth at different concentration of NaCl ranging from 3.5 to 14%.

2.7. Enzyme assay

The cellulase activity was quantified by spectrometric determination of reducing sugars by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The enzyme: substrate 1% CMC (1:2) was prepared in 50 mM glycine–NaOH buffer (pH 10). The reaction mixture was incubated at 45 °C for 50 min. After incubation equal volume of DNS reagent was added and the mixture was heated to 99 °C for 15 min in a boiling water bath. The release of reducing sugar was measured by measuring absorbance at 546 nm. One unit of enzymatic activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute. Protein concentration of the enzyme was measured by Bradford method and standard curve was prepared taking bovine serum albumin (BSA) as standard (Bradford, 1976).

2.8. Purification of cellulase

Bacterial cells were removed from culture broth by centrifugation at $12{,}000 \times g$ for 20 min at 4 °C. Supernatant was overnight precipitated to 80% saturation with $(NH_4)_2SO_4$ at 4 °C and pellet was recovered by centrifugation at the same. The precipitate was dissolved in 50 mM glycine–NaOH (pH 10) buffer. For ion exchange chromatography, the sample was applied to DEAE Sepharose column equilibrated with same buffer. Fractions were collected with a linear gradient of 0.1–1 M NaCl in the same buffer. The fraction with highest enzyme activity were pooled and further purified by gel filtration on DEAE Sephadex A-50 column with same buffer. The fractions were eluted at a flow rate of 0.8 ml/min. Total of 30 fractions were collected and assayed for cellulase activity. Fractions showing maximum activity were analyzed for purity and other characterization studies.

2.9. SDS-PAGE and Zymogram analysis

To check the purity of enzyme, sodium deodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 8% gel according to Laemmli (1970). Gels were visualized by silver staining protocol of Hames and Rickwood (1996). The zymogram analysis was performed on the Native PAGE gel at 4 °C. Gel was soaked in glycine–NaOH buffer for 15 min and overlaid on the agarose gel (0.8%) containing 0.1% CMC. Following the incubation period of 50 min at 45 °C, the gel was stained with 0.1% Lugol's' iodine for 10 min and washed with NaCl (1%) until zone of clearance was visualized.

2.10. Influence of pH, temperature on cellulase activity and stability

The residual enzyme activity was measured at different pH as 8 (50 mM Phosphate buffer) to 9, 10, 11 and 12 (50 mM glycine–NaOH buffer) and temperature at 15–75 °C. Different concentration of NaCl ranging from 3 to 24%, were analyzed for enzyme activity. The thermal (15, 20, 25, 30 and 35 °C) and pH (8–12) stability of the pure enzyme was estimated with the pre-incubation for 30 min under the conditions optimized for maximum activity.

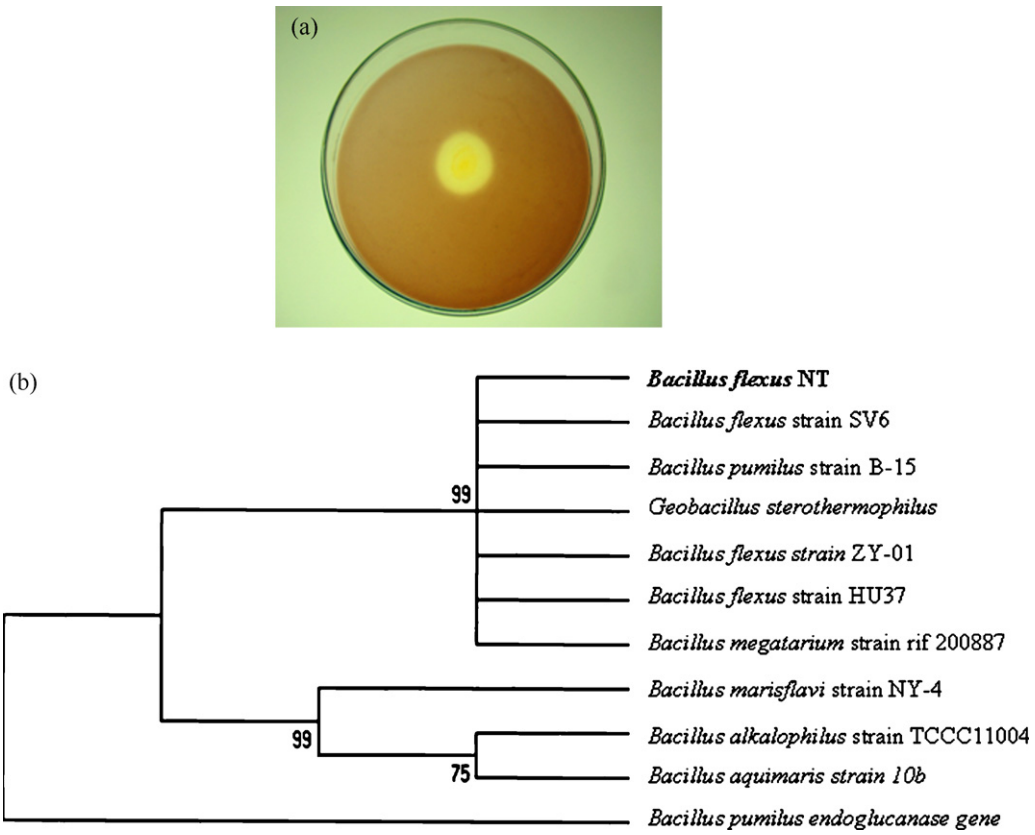

Fig. 1. Screening for cellulolytic activity of *Bacillus flexus*: (a) zone of clearance after flooding with Lugol's iodine on CMC agar plate; (b) phylogenetic tree of *Bacillus flexus* NT associated with other members of the genus *Bacillus using* 16S rDNA sequence. Number at each clade refers to bootstrap values and Bayesian posterior probability. Clades with bootstrap values less than 50% were omitted from the figure.

2.11. Effects of additives on enzyme activity

The effect of various metal ions and reagents were examined on the activity of purified enzyme. The additives used in this study were the salts of Cd^{2+}, Co^{2+}, Pb^{2+}, Hg^{2+}, Cr^{2+}, Mg^{2+}, Mn^{2+}, Li^{2}, Ca^{2+}, Na^{+} and EDTA (5 mM each) and residual activity was calculated as relative (%) considering control as 100%.

Different solvents used in this study were toluene, Dimethylsulfoxide, heptane, ethanol, butenol, methanol, cyclohexane, chloroform, dichloromethane, benzene, isopropanol and acetone. The concentrations (5v/v) of each solvent were added to reaction mixtures and the residual enzymatic activity was measured.

2.12. Kinetic determination

Kinetic studies were performed with different CMC concentrations as 0.05–0.35% in 50 mM glycine–NaOH buffer (pH 10) at 45 °C for 50 min .The kinetic constant K_m and V_{max} were determined according to Lineweaver–Burk double reciprocal plot.

2.13. Enzymatic product analysis

The enzymatic degradation of polysaccharide (CMC) was examined with the change in molecular weight estimated through chromatogram generated after Gel permeation chromatography (GPC) (Water Allaince, model 2695) equipped with Refractive Index Detector (Waters 2414). Enzyme was incubated with 1% CMC for 3 days at 45 °C, degraded product was loaded to GPC column ultrahydrogel 120 & 500 and hydrolyzed monosaccharide fraction was collected from GPC at the same retention time as of glucose standard. Alditol acetate derivatives were prepared using a method of Siddhanta et al. (2001).

Analysis of alditol acetate reducing sugar derivatives was carried out in a GC-MS 2010 (Shimanzu, China) using SGE BP-225 capillary Column (25 m × 0.22 mm, 0.25 μm film thickness). The injection temperature was 230 °C and the column temperature was ramped from 160 to 230 °C at a rate of 10 °C/min.

3. Results and discussion

3.1. Isolation and taxonomic characterization of cellulolytic bacteria

Microorganisms associated with the degraded green seaweed *U. lactuca*, rich of cellulosic content as its cell wall component, were screened for extracellular cellulase enzymes. By enrichment culture technique, 6 (NT1–NT6) different cellulose degrading bacteria were isolated. Of which, bacteria NT1 showed maximum zone of clearance on CMC agar plates after flooding with Lugol's iodine and the same was selected for further studies (Fig. 1a). The strain was found to be gram positive, short rods, aerobic and motile in nature. The phylogenetic analysis of the strain with16S rDNA gene sequence exhibited its maximum homology (99%) with *Bacillus flexus* strain SV6 (Fig. 1b). Recently, a wide variety of genera have been reported for producing the enzyme cellulase include *Bacillus subtilis* (Kim et al., 2009) *Marinobacter* (Shanmughapriya et al., 2010), *Vibrio* (Gao, Ruan, Chen, Zhang, & Xu, 2010), *Penicillium* (Jeya et al., 2010), *Aspergillus* (Tao et al., 2010).

3.2. Growth curve

The selected strain NT1 exhibited the optimum growth at 3.5% salt (NaCl) concentration with an extended exponential phase of about 20 h. On the contrary, at higher salt concentration (7 and

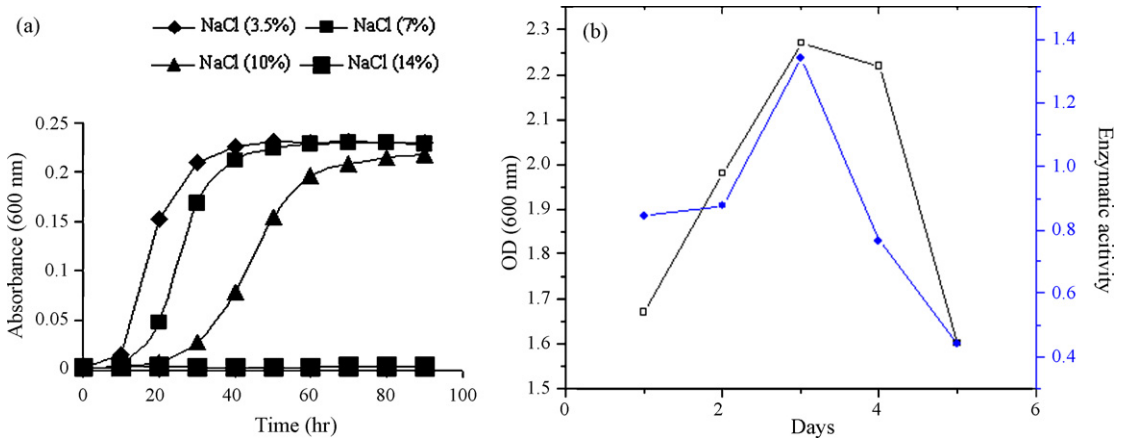

Fig. 2. (a) Bacterial growth curve with different NaCl concentrations and (b) time study of bacterial growth along with enzymatic activity. Samples were withdrawn after every day, growth and enzymatic activity was measured.

10%) the exponential phases get shorten but with longer stationary phase (Fig. 2a). The optimum growth at 3.5% salt concentration and tolerance limit up to 10% salt confirmed the origin of this strain from marine source and also signify its moderate halophilic character (Shivanand & Jayaraman, 2009). The salt concentration above 14% did not support the bacterial growth. Further, the simultaneous estimation of growth and enzymatic activity reveled the incubation period of 3 days as optimum for the production of extracellular cellulase (Fig. 2b).

3.3. Purification and molecular mass characterization of cellulase

The three-step purification of the enzyme from the cell free supernatant of production media includes ammonium sulphate precipitation, ion exchange and size exclusion chromatography (Table 1). The purified enzyme showed 22.31-fold increases in the activity with a final yield of 25.03%. The purity was confirmed by SDS-PAGE showing a single protein band of molecular weight about 97 kDa (Fig. 3). The *in situ* estimation of enzyme activity also showed the distinct single band. The purified enzyme showed maximum activity and specific activity as 0.338 U/ml and 92.38 U/mg respectively which is similar to all *Bacillus* sp. reported earlier for cellulase production (Bischoff, Rooney, Li, Liu, & Hughes, 2006; Kumar et al., 2009; Singh, Batra, & Sobti, 2004).

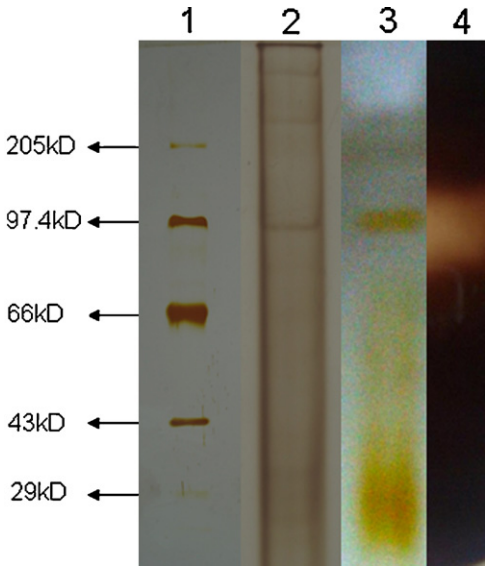

Fig. 3. SDS-PAGE analysis of cellulase produced by *Bacillus flexus* NT. *Lane 1* molecular mass markers, *Lane 2* active fractions of DEAE sepharose chromatography, *Lane 3* active fractions of DEAE sephadex chromatography and *Lane 4* zymogram.

3.4. Influence of pH, temperature on cellulase activity and stability

The activity profile of purified enzyme showed the optimum residual activity at pH 10 and temperature 45 °C (Fig. 4a and b). The enzyme retained its activity (100%) even after a pre-incubation of 30 min at pH 10 (Fig. 4c). The activity was found to be stabilized in the pH range of 8–12 and thus evident for alkali-tolerance nature of enzyme. The present study correlate well with earlier reports for alkaline extracellular cellulase enzymes reported from *Marinobacter* sp. MS1032 (Shanmughapriya et al., 2010), *Bacillus* sp. HSH-810 (Kim et al., 2005), *Vibrio sp.* G21 (Gao et al., 2010), *Stachybotrys atra* BP-A (Picart, Diaz, & Pastor, 2008) that have shown their characteristic pH tolerance in the range of 6–12.

Cellulases that are active and stable at high temperatures and alkaline pH ranges are of special interest in the detergent industry, where enzymes and detergent additives must be active in harsh washing conditions (Simonaka et al., 2006; Wang et al., 2005). Moreover, cellulases can be applied to facilitate de-inking in the flotation cells in paper recycling industry, which usually operate under alkaline and high temperature conditions (Bajpai & Bajpai, 1998). One of the main industrial applications of cellulases is biostoning or biopolishing of denims. The cellulase with high activity at alkaline pH investigated in the present study promises its potentiality for the use in detergent industries. An alkaline cellulase from *Thermomonospora* sp. has been reported for diminishing the back staining compared to commercial cellulases of fungal origin (Anish, Rahman, & Rao, 2007).

Table 1
Summary of purification of cellulase from the *Bacillus flexus*.

Purification steps	Protein (mg)	Total activity (U/ml)	Specific activity(U/mg)	Purification fold	Yield
Crude enzyme	326.41	1.35	4.14	1	100
$(NH_4)_2SO_4$ precipitation	29.78	0.662	22.82	5.51	49.07
DEAE Sepharose	14.6	0.583	41.64	10.05	43.18
DEAE Sephadex A-50	4.27	0.338	92.38	22.31	25.03

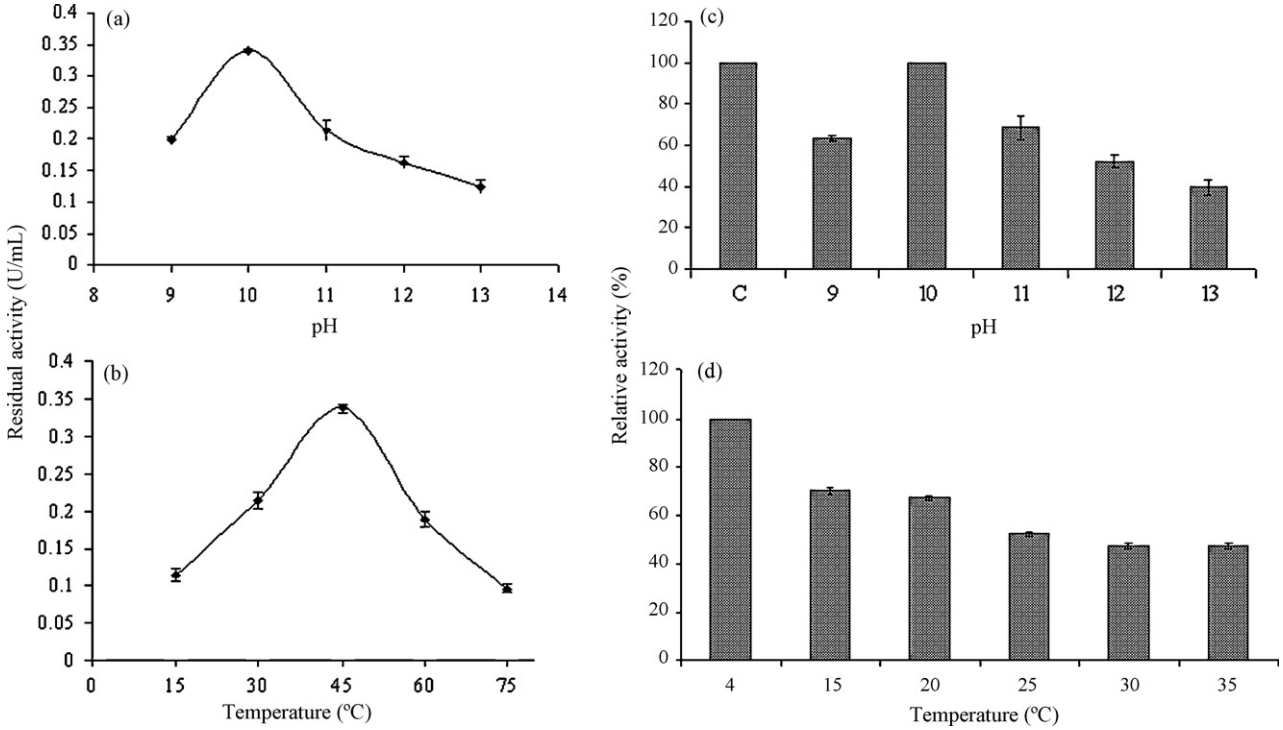

Fig. 4. Effects of temperature and pH on activity and stability of cellulase: (a) residual activity under different pH from 9.0 to 13.0 at 45 °C; (b) residual activity at different temperatures (15–75); (c) the pH stability; and (d) thermal stability of the enzyme.

The thermal stability analysis revealed for the decline in its activity with increase in temperature. The enzyme exhibited 100% activity at 4 °C (Fig. 4d) and declined to 47% at 35 °C. It could able to retain 28% activity at 75 °C with a pre-incubation of 30 min. The decline in the activity with temperature is due to fluidity of protein confirmation. The results of earlier studies evaluated the optimum temperature in the range of 40–60 °C for cellulase producing bacterial strains belonging to genus *Bacillus* (Endo et al., 2001; Hakamada et al., 2002). Recently, Rastogi et al. (2010) reported thermostable cellulases from *Bacillus* sp. with an optimum activity at 70 and 75 °C and also could retained 89 and 78% stability at 70 and 75 °C respectively after an incubation of 24 h.

3.5. Effect of salinity on cellulase activity

The enzyme could retain 70% of its activity at salt concentration (NaCl 15%) and it reduced to 18% at higher salt concentration of 21% (Fig. 5a). The activity of endo-β-1,4-glucanase Cel5A from *Vibrio Sp*.G21 and Egl-AG from alkaliphilic *Bacillus agaradhaerens* has been found to be induced in the presence of NaCl (Gao et al., 2010; Hirasawa et al., 2006). In contrast to alkaline cellulases, only a few salt-tolerant or halophilic cellulases have been reported (Hirasawa et al., 2006; Voget, Steele, & Streit, 2006). Johnson, Lanthier, and Gochnauer (1986) described a cellulase from halophilic actinomycete *Actinopolyspora halophila*, exhibited optimal cellulase activity at 15% (w/v) NaCl. Simankova, Chernych, Osipov, and Zavarzin (1993) characterized an anaerobic eubacterium, *Halocella cellulolytica* that could produces cellulase at 20% NaCl. These studies indicated that NaCl is important either for the stability or activity of the enzyme. The high-salt tolerance of the enzyme is further a prevail with its alkali-tolerance nature towards its potential applications in the industry related with biotechnology. In comparison to the earlier reports of cellulases from *Bacillus* sp. present study firstly demonstrated the potential physiological characteristic of *B. flexus* for excreting the halotolerance cellulase enzyme with alkali tolerance nature too.

3.6. Effect of additives and solvents on cellulase activity

Majority of *Bacillus spp*. producing alkaline cellulase showed different type of inhibition as well as activation with different additives depending on the type of cations (Christakopoulos et al., 1999). The influence of various metal ions and reagent on cellulase activity was studied at 5 mM concentration. Enzyme activity was found to be stimulated by Cd^{2+} and Li^{+} to 120 and 101% respectively. While the activity was found to be strongly inhibited by divalent ions of Ca^{2+}, Cr^{2+}, Co^{2+}, Zn^{2+} Mn^{2+} and metal chelator EDTA as summarized in Fig. 5b.

Recently, Ng et al. (2009) and Tao et al. (2010) reported that endoglucanase from *Geobacillus sp.*70PC53I and *Aspergillus glaucus* XC9 was stimulated by Ca^{2+}, Zn^{2+}, Co^{2+} whereas in present study activity was strongly inhibited by same divalent ions. The rate of inhibition of the purified enzyme activity was in the order of $Ca^{2+} > Co^{2+} > EDTA > Zn^{2+}$ and remained partially affected by $Mg^{2+} > Li^{+} > Na^{+} > Pb^{2+}$. These findings are similar to those obtained with *Bacillus sphericus* JS1 and *Salinivibrio sp. Strain* NTU-05 (Kumar et al., 2009; Wang et al., 2009). The inducing effect of heavy metal Cd^{2+} and tolerance towards high salt concentrations (NaCl 15%) revealed the unique characteristic of the cellulase from *B. flexus*.

The residual enzyme activity remained 100% in the presence of ethanol and isopropanol and partially inhibited with toluene (87%), DMSO (82%), methanol (85%). Whereas it was found to decrease up to 32, 59 and 58% respectively with acetone, cyclohexane and benzene (Fig. 5c). Also the activity of enzyme Cel5A obtained from uncultured bacteria was also reported to be unaffected in presence of ethanol and methanol (Voget et al., 2006).

3.7. Kinetic analysis

Substrate concentrations showed the complete enzymatic saturation at 0.2% (w/v) of CMC. The Michelis–Menton kinetic parameters (V_{max} and K_m) determined from Lineweaver–Burk double reciprocal plot was 370.17 U/ml/min and 6.18 mg/ml

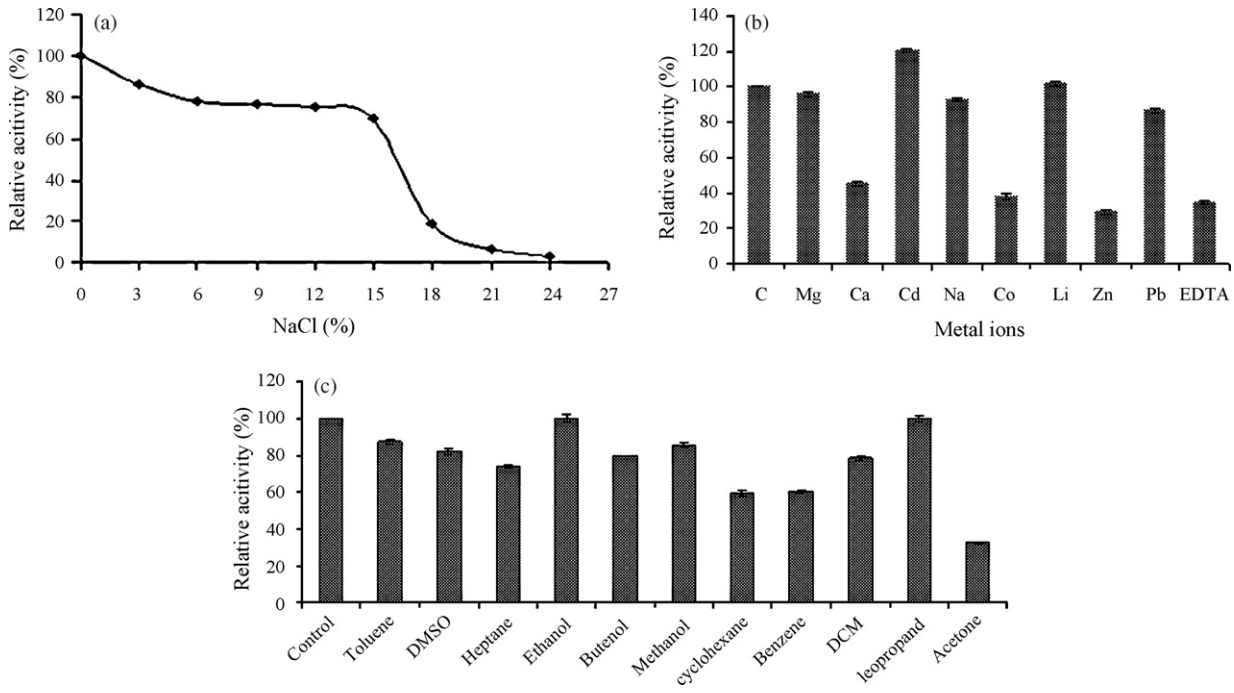

Fig. 5. Effect of different additives on cellulase activity: (a) different NaCl concentrations (3–27%), (b) different metal ions and EDTA (5 mM) each, (c) different solvents (5v/v). The values represent averages from triplicate experiments. Error bars represent the standard deviation.

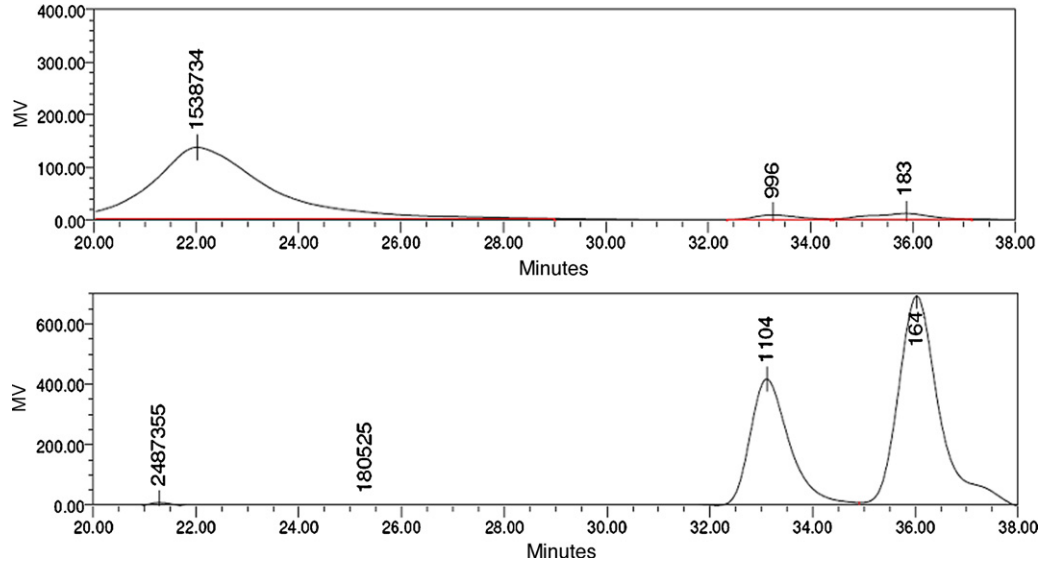

Fig. 6. Gel permeation chromatography of hydrolyzed product showing complete degradation of CMC.

respectively. Recently, Wang et al. (2009) demonstrated the kinetic properties of the halostable cellulase from *Salinivibrio* sp. strain NTU-05 with K_m value 3.03 mg/ml and V_{max} with 142.86 mol/min/mg.

3.8. Enzymatic product

GPC spectrum of enzymatic hydrolyzed CMC confirmed its degradation into oligosaccharides and monosaccharide (Fig. 6), thus presented the hydrolyzing potential of enzyme investigated. Further the liberated monosaccharide was confirmed as glucose after their derivation by alditol acetate method and subjected to GC–MS analysis. Thus the study confirmed the enzymatic hydrolysis of cellulose into its monomeric unit of glucose which is of high value platform molecule.

4. Conclusions

Bacillus flexus, capable of hydrolyzing CMC was isolated from degraded seaweed species *U. lactuca*. The strain *B. flexus* NT of marine origin is first time described as a source of extracellular alkali-halo tolerating cellulolytic enzyme in the present study. Moreover, the enzyme was found to tolerate the presence of various solvents with its alkaline and saline stability. Furthermore the heavy metal ion Cd^{2+} was the inducers of this enzyme. The *in vitro* functional activity was confirmed with zymogram and resulted molecular weight was about 97 kDa. The pH and temperature stability study showed that the enzyme was stable at temperature range from 15 to 35 °C. The stabilized activity of the enzyme from neutral to alkaline pH coupled with potential activity in 15% NaCl concentration with heavy metal ions represent as unique proper-

ties that forward its potential at industrial level mainly in detergent, paper-pulp, leather and textile that require such extreme conditions for functionality.

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