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Optimization, Purification, and Characterization of Extracellular Mesophilic Alkaline Cellulase from Sponge-Associated *Marinobacter* sp. MSI032

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Abstract *Marinobacter* sp. (MSI032) isolated from the marine sponge *Dendrilla nigra* was optimized for the production of extracellular cellulolytic enzyme (CMCase) by submerged fermentation. Initial experiments showed that the culture medium containing 1% maltose as carbon source and 1% peptone and casein as nitrogen source supported maximal enzyme production at 27 °C and at a pH of 9.0. Further optimization carried out showed the maximal enzyme production was supported by the presence of 2% NaCl and 10 mM Zn^{2+} ions in the production media. The production of enzyme cellulase occurred at 48 h of incubation which proved the importance of this strain for cellulase production in large scale. Further, the enzyme was purified to 12.5-fold with a 37% yield and a specific activity of 2,548.75 U/mg. The purified enzyme displayed maximum activity at mesophilic temperature (27–35 °C) and at a broad pH range with optimal activity at pH 9.0. The purified enzyme was stable even at a higher alkaline pH of 12.0 which is greater than the pH stability that has not been reported in any of the cellulolytic isolates studied so far. Thus, from the present study, it is crucial that, instead of exploring the thermophilic resource that is limited in natural environments, the mesophilic bacteria that occurs commonly in nature can be added up to the database of cellulolytic bacteria. Thus, it is possible that a wide diversity of mesophilic bacteria associated with marine sponges opens up a new doorstep for the degradation of cellulosic waste material for the production of liquid fuels. This is the first report elucidating the prospects of sponge-associated marine bacterium for the production of extracellular alkaline cellulase.

Keywords Extracellular cellulase · Sponge bacteria · *Marinobacter* · Marine cellulase · Alkaline cellulase

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Introduction

Cellulose is the major polysaccharide compound in plants and is the most abundant organic compound on earth. Accordingly, its turnover in the carbon cycle is of prime importance for all living organisms [1–3]. Degradation of the cellulosic materials can be achieved chemically, enzymatically, or by the combination of both chemical and enzymatic methods [4–7]. The chemical hydrolysis of cellulose is achieved by treatment with acid. The acid hydrolysis of cellulose can be envisioned as a sequential reaction where cellulose gives glucose and further glucose gives undesirable side products. The formation of side products from glucose occurred at about the same rate as formation of glucose from cellulose. Therefore, the maximum glucose level in the hydrolysate was only 33%. Extensive improvements have been carried out, but the best yield obtained to date using this conventional technology is less than 50% glucose in the hydrolysate. Moreover, the economic and efficient hydrolysis of cellulase needs extensive effort [8]. Thus, the enzymatic hydrolysis of cellulose by the enzyme cellulase is preferred over chemical means. Cellulases (endo-1, 4-β-glucanase) are a group of hydrolytic enzymes which hydrolyze the glucosidic bonds of cellulose and related cello-oligosaccharide derivatives [9].

With the advent of new frontiers in the field of biotechnology, the spectrum of cellulase has expanded into various industries, including food, textiles, laundry, pulp, paper, agriculture as well as in research and development [10–15]. In combination with other enzymes, viz., protease, amylase, lipase, and lactase, modern-type heavy-duty detergents contain cellulase to increase their effectiveness as detergents. Cellulases (including carboxymethyl cellulases) added to laundry detergents improve the softness, color brightness, and dirt removal (soil) from cotton blended garments [10, 12, 14]. Cellulases are produced by a wide variety of microorganisms, but aerobic fungi *Trichoderma* sp. [5], anaerobic fungi *Neocallimastix* sp. [16], aerobic bacteria *Thermomonospora* sp. [17], and anaerobic bacteria *Clostridium* sp. [18] provide a promising source for the production of cellulase. Thus, the expanding application of the enzyme calls for an urgent need for the exploration of microorganisms from pristine environments as valuable source of this commercial enzyme. In the present study, we report the marine sponge-associated bacterium *Marinobacter* sp. MSI032 as a potential cellulase producer for industrial applications.

Materials and Methods

Sampling and Isolation of Bacteria

The marine sponge *Dendrilla nigra* was collected from the peninsular coast of India (Vizhinjam) by snorkeling. For the isolation of associated bacteria, 1 cm^3 of sponge tissue was excised from the middle of the whole sponge using a pair of sterile scissors. The excised portion was thoroughly washed three times with sterile seawater to remove any bacteria within current canals and then the tissue was homogenized with phosphate-buffered saline (PBS; 8 g NaCl, 0.20 g KCl, 1.44 g $Na_2HPO_4{\cdot}12H_2O$, and 0.24 g KH_2PO_4 in 1 l of distilled water, pH adjusted to 7.4 with HCl) using a sterile mortar and pestle. The resultant homogenate was serially diluted with sterile aged sea water (previously stored natural sea water autoclaved at 121 °C for 15 min at 15-psi pressure) and preincubated at

40 °C for 1 h for the activation of dormant cells. The aliquot was plated on various isolation media including marine sponge agar [19], marine agar, nutrient agar supplemented with 2% NaCl, thiosulfate citrate bile salts sucrose (TCBS) agar, modified nutrient agar, actinomycetes isolation agar, *Pseudomonas* agar, Emerson agar, halophilic agar, and anaerobic agar (HiMedia). Except for the anaerobic agar, all other inoculated plates were incubated at 30±2 °C in dark aerobic conditions until visible colonies appeared.

Preliminary Screening for Cellulase Producers

Plate assay was performed as per Teather and Wood [20] to screen the bacterial isolates capable of producing the enzyme cellulase. The isolates were streaked on nutrient agar plates (supplemented with 2% NaCl) and incubated at 37 °C for 18 h. Cellulolytic organisms were identified by overlaying the plates with soft agar (0.8%) containing 0.5% carboxymethylcellulose (CMC). The overlaid plates were incubated at 37 °C for 4 h. Then, the plates were flooded with Congo red (1 mg/ml), washed with 1 M NaCl after an incubation period of 15 min, and the organisms that produced yellow zones around their colonies were scored as cellulase producers.

Identification of the Potent Cellulase Producer

Biochemical, morphological, and physiological characteristics of the potential producer (MSI032) was determined by adopting standard methods [21]. The genomic DNA was isolated by CTAB/NaCl method. Universal 16S rRNA eubacterial primer (5′-GAGTTTG ATCCTGGCTCAG-3′; 5′-AGAAAGGAGGTGATCCAGCC-3′) was used for the amplification of DNA. Polymerase chain reaction (PCR) was carried out on a thermal cycler in a 50-μl reaction mix. PCR-amplified 16S rRNA gene product was cloned by the TA cloning method using a TOPO TA cloning® kit according to the manufacturer's instructions (Invitrogen) for sequencing. The 16S rRNA gene sequence obtained from the isolate MSI032 was compared with other bacterial sequences by using NCBI BLASTn [22, 23] for their pairwise identities. Multiple alignment of the sequence was carried out by ClustalW 1.83 version of EBI (www.ebi.ac.uk/cgi-bin/clustalw/) with 0.5 transition weight. Phylogenetic trees were constructed in MEGA 3.1 version (www.megasoftware.net) using neighbor-joining (NJ), minimum evolution (ME), and unweighted pair group method with arithmetic mean (UPGMA) algorithms. The conserved regions were analyzed by using BioEdit 7.0.4.1 version [24] with minimum length of 125 residues for all sequences and maximum entropy of 0.2 per position. Nucleotide composition of each aligned sequence was predicted by BioEdit software package. The sequence used in the analysis was deposited in GenBank, EMBL in Europe, and the DNA Data Bank of Japan with an accession number EF428029.

Quantitative Assay of Cellulase Activity

The enzyme assay was carried out according to the procedure as described earlier by Rani and Nand [25]. Diluted enzyme solution (0.1 ml) was mixed with 0.5 ml of substrate (1% CMC in 0.05 M glycine/NaOH buffer, pH 9.0) and incubated at 37 °C for 30 min. The reaction was terminated by addition of 2 ml of 3,5-dinitrosalicylic acid reagent [26] and the amount of reducing sugar (glucose) released was measured at 540 nm in a UV/Vis double beam scanning spectrophotometer (Thermospectronic) [27]. Thus, one unit of cellulase

activity was defined as 1 μM of reducing sugar as glucose released per minute per milliliter of enzyme source, under assay conditions and expressed as U/mg protein of the culture broth. All the experiments were performed in triplicates. The activity was expressed as mean±SD. The protein content of the broth was estimated [28] using bovine serum albumin as the standard.

Screening for Cellular Localization of Cellulase

The cellular localization of cellulase produced by *Marinobacter* MSI032 was determined. The culture was inoculated in a 250-ml Erlenmeyer flask containing 150 ml of production media (peptone 2 g, yeast extract 0.1 g, magnesium chloride 4.0 g, sodium sulfate 1.5 g, calcium chloride 0.75 g, potassium chloride 0.5 g, sodium carbonate 0.08 g, disodium phosphate 0.008 g, and sodium chloride 20 g in 1 l seawater). After 48 h of incubation at 37 °C at 250 rpm, the cell-free supernatant (CFS) was obtained by cold centrifugation (Eppendorf) at 8,000×*g* for 15 min. The pellet obtained was washed thrice with PBS and sonicated twice for 1 min. The cell lysate was centrifuged at 8,000×*g* for 10 min. A portion of the supernatant was stored at 4 °C as intracellular enzyme preparation. To another portion of the supernatant, triton X-100 (5:1 *v*/*v*) was added and incubated over ice for 10 min. The membrane proteins were precipitated by centrifugation at 8,000×*g* for 15 min. The pellet formed was dissolved in Tris–HCl buffer at pH 7.0. The extracellular, intracellular, and the membrane-bound protein preparations isolated were used as enzyme source and activity determined as described earlier.

Time Course of Cellulase Production

For the production of cellulase, the culture was inoculated in 500-ml Erlenmeyer flasks containing 300 ml production media and incubated at 37 °C at 250 rpm on a rotary shaker. The growth of the bacterial culture was measured at wavelength of 620 nm and expressed in terms of optical density. After incubation, the production media were centrifuged at 8,000×*g* for 20 min, and the resultant supernatant was filtered through a 0.22-μm membrane filter to collect the CFS. The CFS obtained was used as the enzyme source. The growth and cellulase activity was measured from the sample collected at every 12-h interval.

Optimizing the Production of Cellulase

Factors like different carbon, nitrogen sources, pH, temperature, salt concentration, and metal ions affecting the secretion of cellulase enzyme were optimized by adopting search technique varying parameters one at a time. The culture was inoculated in a 100-ml Erlenmeyer flask containing 25 ml of the production medium supplemented with different 1% carbon (fructose, glycerol, glucose, maltose, mannose, and sucrose) and 1% nitrogen (beef extract, yeast extract, tryptone, casein, peptone, ammonium sulfate, and sodium nitrate) sources, different NaCl concentrations (0.5–3.0% with increments of 0.5%) and 10 mM metal ions (calcium carbonate, ferrous sulfate, magnesium chloride, mercuric chloride, zinc sulfate, and manganese sulfate), and incubated under different temperature (20–50 °C, with increments of 10 °C) and pH (5–9 with increments of one unit) conditions. After incubation at appropriate conditions, the cells were removed by cold centrifugation at 8,000×*g* for 10 min. The resultant CFS was analyzed for cellulase activity.

Purification of Cellulase

Marinobacter MSI032 was grown in the production medium for 48 h at 37°C under submerged conditions. After incubation, CFS was obtained as described earlier. The enzyme from CFS was precipitated by ammonium sulfate at 60–80% saturation at 4°C. The precipitate was collected by centrifugation at 8,000×*g* for 15 min at 4°C and dissolved in 1 mM Tris buffer (pH 7.0). This aliquot was dialyzed against the same buffer overnight under refrigerated conditions. The partially purified enzyme was applied to a Sephadex G-200 column (1.5×40 cm) equilibrated with 1 mM Tris buffer (pH 7.0). Equilibration and elution were performed with 0.05 M sodium phosphate buffer to remove unbound proteins and then with a linear salt gradient from 0 to 3 M NaCl. The active fractions from the Sephadex G-200 column were pooled, concentrated, and applied to a DEAE Sepharose column (1.5×40 cm) which was previously equilibrated with 1 M Tris buffer (pH 7.0) containing 0.1 M NaCl. Elution was carried out by using the same buffer at a flow rate of 0.5 ml/min. All purification procedures were carried out at 4°C. Finally, the resulting enzyme preparation was desalted, concentrated by dialysis, and lyophilized. The quantitative activity of the purified enzyme was determined as previously described.

Characterization of the Purified Enzyme

Determination of Molecular Weight of the Purified Enzyme and Zymogram

The molecular weight of the partially purified enzyme was estimated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) with 4% stacking gel and 12% resolving gel [29]. Two PAGE gels were electrophoresed (BioRad) simultaneously and one gel was stained with silver nitrate. Molecular weight was determined by comparing the relative mobility of standard protein molecular weight markers of 14.9 and 94.7 kDa. The other reference unstained gel was incubated with 1% soluble CMC at 37°C for 30 min and then stained with Congo red.

Effect of pH and Temperature on Stability and Activity of the Purified Alkaline Cellulase

The purified enzyme was tested for the optimum pH for the enzyme activity. The enzyme activity was determined using buffers of same ionic strength and different pH (4–12). The enzyme activities obtained herewith were expressed as relative activity in reference to the enzyme activity at pH 9.0. The stability study was tested through 1 h preincubation of the purified enzyme in appropriate buffers including 0.05 M citrate buffer (pH 4–6), 0.05 M sodium phosphate buffer (pH 7–8), 0.05 M glycine buffer (pH 9–10), and 0.05 M potassium phosphate (pH 11–12) at 37°C. The remaining activities of cellulase were measured immediately after processing the aliquots as described previously. The activity of the enzyme has been reported as relative activity with reference to that at pH 9.0. The temperature stability of the purified enzyme was tested by preincubating the enzyme at different temperatures for 1 h. The residual activity was measured at different time intervals from 20 to 60 min with an increment of 20 min. The sample preincubated at 37°C was used as reference to calculate the residual activity.

Results and Discussion

Isolation of Sponge-Associated Microbes

In the present study, based on the colony morphology and stability in subculturing, 57 heterotrophic bacteria were isolated from the marine sponge *D. nigra*. Among these, 13 isolates were obtained on modified marine agar followed by seawater agar (eight), TCBS agar (eight), modified nutrient agar (seven), actinomycetes isolation agar (seven), *Pseudomonas* agar (six), Emerson agar (five), halophilic agar (two), and anaerobic agar (one) (HiMedia).

Screening for Cellulase Producers

Among the 57 isolates, 18 isolates were scored as cellulase producers by plate assay. Particularly one isolate, MSI032 exhibited large clear zone around the colonies on CMC agar plates. This strain was selected for further studies for the production, purification, and characterization of the enzyme in order to employ it for large-scale industrial processes.

Characteristics of Cellulase-Producing *Marinobacter* MSI032

Considering the biochemical and physiological tests performed, the strain MSI032 was identified as *Marinobacter* sp. The isolate MSI032 was a Gram-negative, non-motile rod. The strain was found to be catalase and methyl red negative. It utilized citrate and was found to be negative for Voges–Proskauer test and indole production. The strain grew well at various concentrations of NaCl ranging from 1% to 3% (*w*/*v*). An optimum growth occurred at 2.5% NaCl and no growth was observed in a medium without supplementation of NaCl. The strain grew well between 27 and 50 °C, with an optimum growth at 27 °C and at a wide pH range of 5.0 to 9.0 with an optimum growth at pH 9.0.

Taxonomic affiliation of the 16S rRNA sequences of the isolate MSI032 was retrieved from classifier program of RDPII. The 16S rRNA sequence of the isolate was blasted using megablast tool of GenBank (http://www.ncbi.nlm.nih.gov/). This revealed that the isolate was a *Marinobacter* strain. Representatives of maximum homologous (98–99%) sequences of each isolate were obtained from seqmatch program of RDPII and were used for the construction of phylogenetic affiliation. The phylogenetic tree was constructed using only the culturable *Marinobacter* strains. The isolate showed a unique cluster with a *Marinobacter* strain HZBC2 (Fig. 1). Among the 25 closest neighboring strains used in the phyogenetic analysis, ten strains were *Marinobacter hydrocarbanoclasticus* including a type strain ATCC 27132T. Therefore, the strain MSI032 might have the characteristics of *M. hydrocarbanoclasticus*. NJ, ME, and UPGMA bootstrapping values of this clade was 95–99. Considering the significances in bootstrap values, interior branch lengths, and diversification rate as shown in Fig 1, "*Marinobacter* MSI032" can be claimed to be a new strain of *Marinobacter* taxa.

Screening for Cellular Localization of Cellulase

No cellulase activity was observed with the membrane-bound and intracellular protein preparations. The CFS used as an enzyme source displayed a cellulase activity of 180 U/mg. Thus, the localization of the cellulase enzyme was confined as extracellular rather than intracellular or membrane bound. The extensively studied cellulase system of

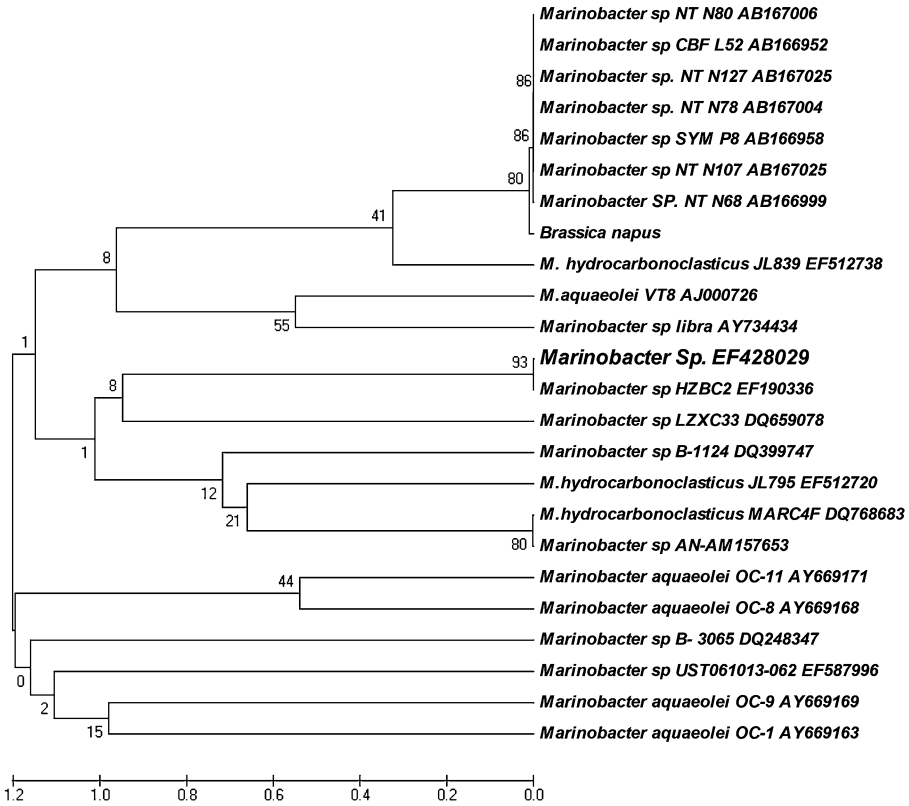

Fig. 1 UPGMA phylogenetic tree of isolate MSI032 and their closest NCBI (megaBLAST) relatives based on the 16S rRNA gene sequences. Bootstrap values calculated from 1,000 resamplings using UPGMA are shown at the respective nodes when the calculated values were 50% or greater

Clostridium thermocellum, isolated from fermenting manure, is a multi-component cellulolytic complex (cellulosome) present on the cell surface. This complex facilitates the attachment of cells to cellulose [30]. In contrast to this, *Marinobacter* MSI032, which produce an extracellular cellulase not associated with the cells and thus do not attach themselves to the cellulose, have been reported in the present study. Such an extracellular cellulase obtained goes well in accordance with the cellulase system of a mesophile *Clostridium* sp. strain C7, which produces an extracellular cellulase complex and facilitates hydrolysis of crystalline cellulose [31].

Time Course of Production of Cellulase

In general, cellulase production by bacteria occurs during the late growth phase of the organisms. Thus, maintenance of the cultures and culture conditions for longer periods pose economic drawbacks for the development of industrial processes. Thus, surprisingly, production of cellulase by *Marinobacter* MSI032 occurs at an earlier stage of fermentation suggesting the usefulness of the strain in industrial processes. Under optimal conditions, 203.9 U/mg cellulase production was reached in the batch culture of *Marinobacter* MSI032 at 48 h of the fermentation when the cell growth reached the early stationary phase (Fig. 2).

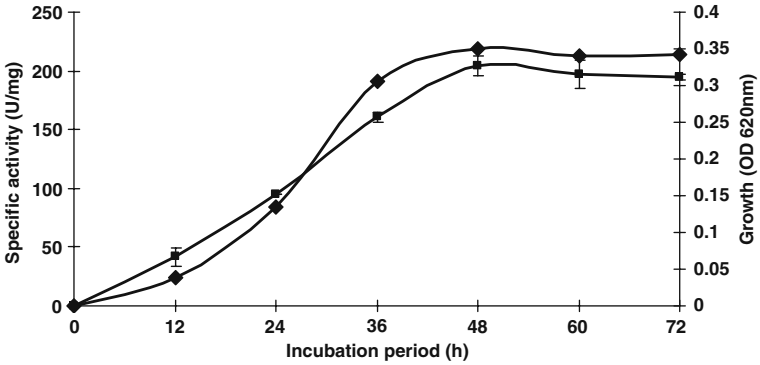

Fig. 2 Effect of growth kinetics on cellulase (*filled squares*) and biomass production (*filled diamonds*). Results represent the means of triplicates and *bars* indicate standard deviation. Absence of *bars* indicates that errors were smaller than symbols

The trend was similar to the incubation period required for the cellulase production by *Clostridium papyrosolvens* [32]. But the production of CMCase and filter paperase by *Streptomyces albaduncus* was obtained after 96–120 h of fermentation [33].

Effect of Nitrogen Source on the Production of Cellulase

The effect of nitrogen source on the growth and production of enzyme was studied (Fig. 3). The presence of different organic nitrogen sources (tryptone, casein, peptone, yeast extract, and beef extract) significantly influenced the production of cellulase. The supplementation of peptone and casein needs special mention as there was a stimulation of enzyme production by 32% and 34%, respectively, compared to that of the control (without nitrogen sources). Ito et al. [34] reported that higher production of cellulase enzyme required the presence of complex nitrogen sources. The results obtained in the present study were in accordance with CMCase production by *Bacillus* sp., where the

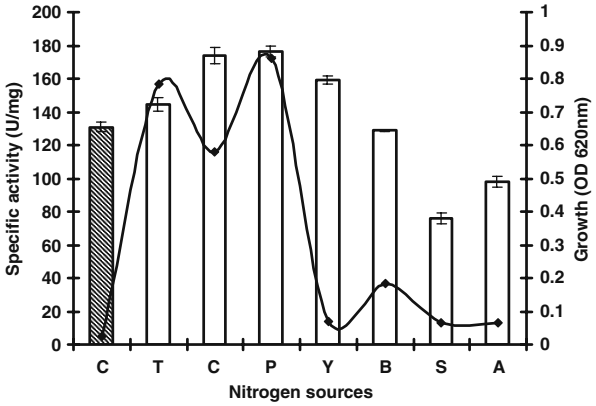

Fig. 3 Effect of different nitrogen sources on cellulase (*bars*) and biomass production (*filled diamonds*). The cells were cultivated in the production medium. All the data are given as mean±SD, $n=3$. *C* control, *T* tryptone, *C* casein, *P* peptone, *Y* yeast extract, *B* beef extract, *S* sodium nitrate, *A* ammonium sulfate

presence of tryptone as nitrogen source stimulated the production [35]. Highest yield of cellulase by *Clostridium* was obtained in a medium supplemented with 0.3% yeast extract [32]. The lower cellulase production observed in the presence of inorganic nitrogen sources apparently suggested reduced utilization of inorganic nitrogen by *Marinobacter* MSI032.

Effect of Carbon Sources on the Production of Cellulase

The presence of carbon sources influenced the production of cellulase. The media supplemented with CMC served as a reference. CMC was replaced by readily available carbon sources (fructose, maltose, sucrose, glycerol, glucose, and mannose). The presence of carbon sources other than CMC increased cellulase production. The presence of maltose in the production media enhanced cellulase production by 195% which was higher than that achieved on reference media (Fig. 4). This showed that the cellulase produced by *Marinobacter* MSI032 was not induced by the presence of the substrate and thus the enzyme can be constitutive and not controlled by a mechanism involving catabolite repression. But this proposition needs to be studied through extensive molecular mechanism that underlie behind the control of synthesis of cellulase. Similar to the results obtained, catabolite repression has not been observed for the production of cellulase by *Bacillus* sp. No. 1139, *Bacillus* sp. KSM-19, KAM-19, KAM-64, and KSM-520 [36, 37], and *Bacillus* sp. VG1 [38]. But there are reports where the supplementation of cellulose showed an induction of cellulase production by *C. papyrosolvens* [32]. There are also some cases where the addition of CMC in the cultivation medium showed no effect on the enzyme yield [39, 40].

Effect of Temperature on the Production of Cellulase

Maximum production of cellulase by *C. papyrosolvens* and *Clostridium celerecrescens* was attained at 35°C [32, 41]. However, *C. thermocellum* showed maximum production at 70°C [35]. Optimum production of alkaline CMCase by *Bacillus* sp. VG1 was attained at 45°C [38]. Thus, it can be concluded that the effect of temperature on any enzyme production

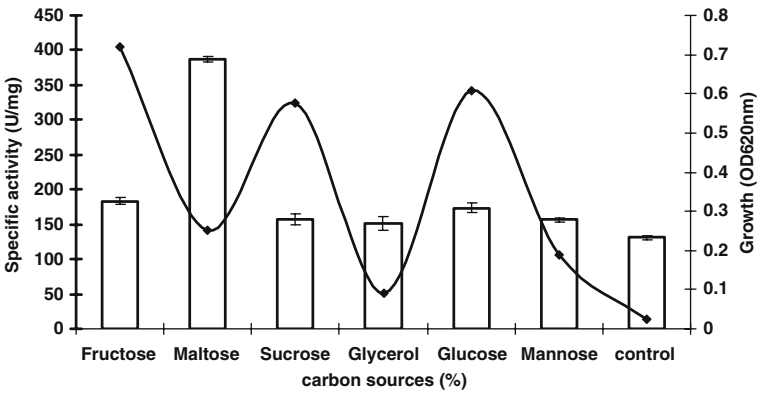

Fig. 4 Effect of different carbon sources on cellulase (*bars*) and biomass production (*filled diamonds*). The cells were cultivated in the production medium. All the data are given as mean±SD, $n=3$

depends on the growth of the organisms and stability of the enzyme produced. Hence, optimum temperature for enzyme production depends upon whether an organism is mesophilic or thermophilic. The effect of temperature on bacterial growth and cellulase production was studied. Maximum cellulase production occurred at 27°C, whereas the production decreased drastically at higher temperatures (Fig. 5). Thus, 27°C was favorable for leaching of cellulase enzyme from *Marinobacter* MSI032. The decreased production at higher temperatures can be due to the denaturation of enzymes at increased temperatures or a relation can be drawn between the growth of the organism and the production of the enzyme. Thus, the study envisaged that the optimal production of the enzyme occurred at mesophilic temperature required for the growth of *Marinobacter* MSI032.

The fermentation of cellulose is of considerable interest as potential source of liquid fuels. A huge number of studies deal with the cellulolytic thermophilic bacteria, since it has been reported that rate of cellulose breakdown occurs 50% more rapidly in thermophilic conditions than mesophilic temperatures. But controversially, Pfeffer [42] found two temperature optima for the digestion of cellulose at 42 and 60°C. Thus, it is crucial that, instead of exploring the thermophilic resource that is limited in natural environments, the mesophilic bacteria that occur commonly in nature can be added up to the database of cellulolytic bacteria. Thus, it is possible that a wide diversity of mesophilic bacteria associated with marine sponges opens up a new doorstep for the degradation of cellulosic waste material for the production of liquid fuels.

Effect of pH on the Production of Cellulase

pH is one of the most important factors that determine the growth and morphology of microorganisms. Microorganisms are more sensitive to the concentration of hydrogen ions present in the medium. The results suggested that the enzyme synthesis was stimulated at pH 6.0 and maximum production was obtained at pH 9.0 (Fig 6). Since the organism was alkalophilic, no growth was observed at pH 5.0 and subsequently no cellulase activity. Song et al. [38] observed optimal CMCase production at pH 9.0. In contrast, the production of cellulase has been reported at acidic [43] and neutral pH [44, 45].

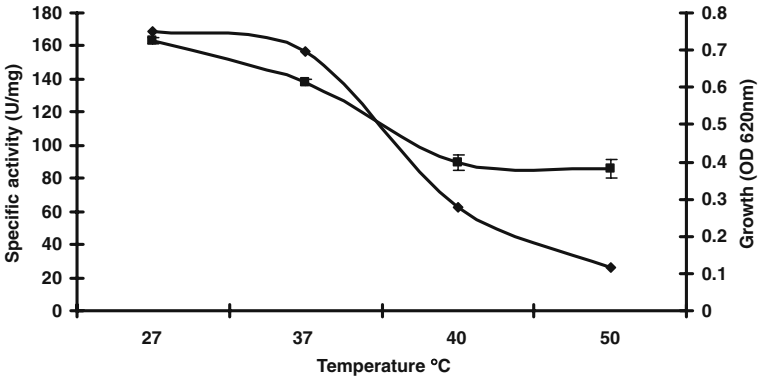

Fig. 5 Effect of temperature on cellulase (*filled squares*) and biomass production (*filled diamonds*). The values are mean±SD, $n=3$. Absence of *bars* indicates that errors were smaller than symbols

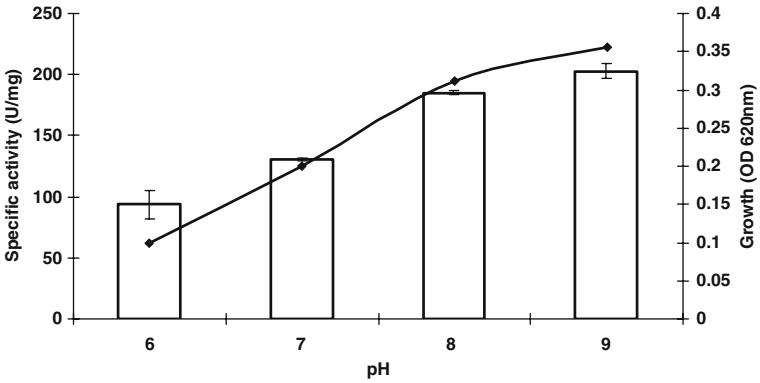

Fig. 6 Effect of pH on cellulase (*bars*) and biomass production (*filled diamonds*). The values are mean±SD, $n=3$. Absence of *bars* indicates that errors were smaller than symbols

Effect of Metal Ions and Salt Concentration on the Production of Cellulase

Metal ions influence enzyme production by microorganisms in submerged fermentation [25]. Maximum cellulase production (approximately 344%) was obtained in a medium supplemented with zinc ions. Calcium, iron, and manganese strongly inhibited cellulase production by 73%, 65%, and 88%, respectively, in comparison with the control (Fig. 7). Thus, the increased production of cellulase in the presence of zinc ions can be due to increased stability of the leached-out enzyme in the presence of zinc ions.

Since the organism was from a marine origin, the effect of NaCl concentration on the production of enzyme was studied. From the results, it was envisaged that supplementation of NaCl to the culture medium was a prime requirement as the enzyme production seems to reduce drastically in the absence of NaCl. This was because the presence of NaCl favored

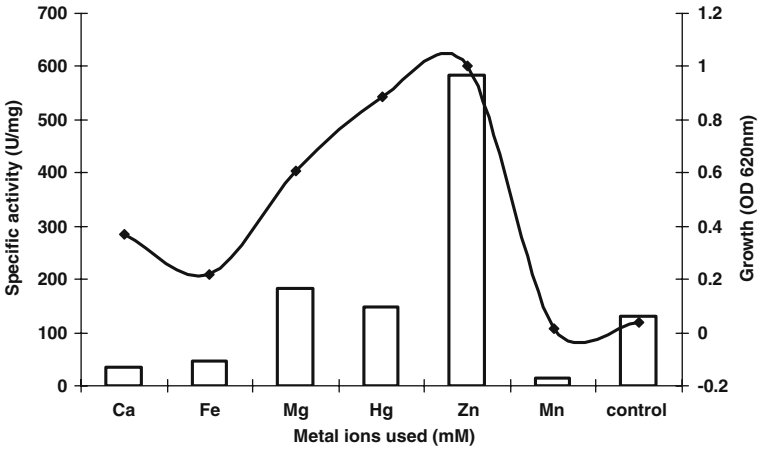

Fig. 7 Effect of metal ions on cellulase (*bars*) and biomass production (*filled diamonds*). The values are mean±SD, $n=3$. Absence of *bars* indicates that errors were smaller than symbols

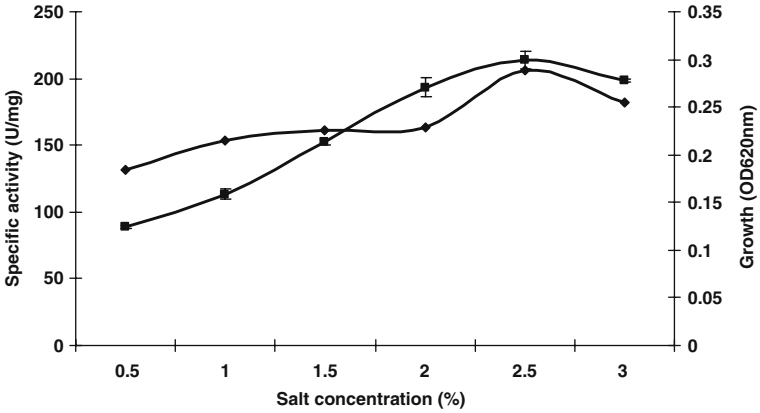

Fig. 8 Effect of NaCl concentrations on the production of cellulase (*filled squares*) and biomass production (*filled diamonds*).The values are mean±SD, $n=3$. Absence of *bars* indicates that errors were smaller than symbols

the growth of the isolate and thus production. Thus, optimum cellulase production was obtained in a medium supplemented with 2.5% NaCl (Fig. 8).

Purification of Alkaline Cellulase from *Marinobacter* sp. MSI032

The enzyme was purified by a three-step strategy including ammonium sulfate precipitation and dialysis, gel filtration, and ion-exchange chromatography. The recovery of dialyzed enzyme was 68% followed by 52% in gel filtration and 37% in ion-exchange chromatography (Table 1). The overall purification attained 12.5-fold purity with specific activity of 2,548.75 U/mg. In a previous report, the three-step purification of alkaline cellulase from *Bacillus* sp. HSH-810 gave an 8.7-fold pure alkaline cellulase with specific activity of 71 U/mg [46]. The molecular weight of the purified cellulase was about 68 kDa as per the SDS–PAGE profile and activity staining (Fig. 9). This is within the range of molecular masses (40–70 kDa) of other bacterial alkaline cellulases [18, 46, 47]. However, Kim et al. [48] reported an alkaline cellulase with a molecular mass of 80 kDa from a *Bacillus* sp.

Table 1 Purification of alkaline cellulase from *Marinobacter* MSI032.

Purification step	Total activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	77.07	0.378	203.9	100	1
Ammonium sulfate fractionation 60–80%	52.41	0.147	356.83	68	2
Sephadex G-200	40.08	0.018	2,202.12	52	10.8
DEAE Sepharose	29.52	0.012	2,548.75	37	12.5

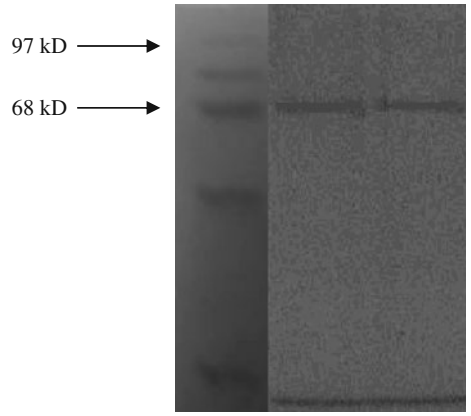

Fig. 9 SDS–PAGE profile depicting the molecular weight of purified cellulase enzyme

Effect of pH and Temperature on Activity and Stability of the Purified Alkaline Cellulase

The purified enzyme showed optimal activity at pH 9 (Fig. 10) and retained about 65% activity at pH 12. A similar trend of activity was reported for *Bacillus* sp. HSH-810 [48]. Other *Bacillus* sp. strains KSM-N252 [47] and KSM-635 [6] showed optimum pH of 9.0 but retained only 0–35% activities at pH 12. Thus, the alkaline cellulase purified in the present study displayed activity that was stable over a broad pH range which is much higher than those that reported till date.

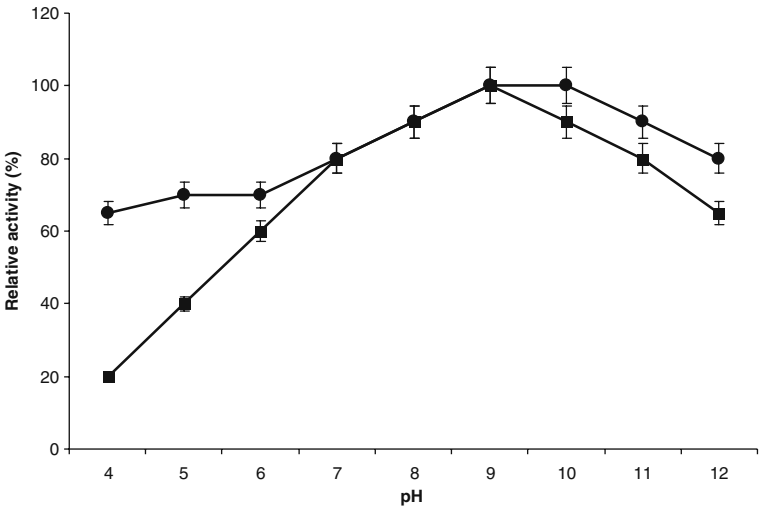

Fig. 10 Effect of pH on the activity (*filled squares*) and stability (*filled circles*) of the purified alkaline cellulase from *Marinobacter* sp. MSI032. The enzyme activity was assayed at 37°C for 10 min and the residual activity was measured after 1-h preincubation of the enzyme at 4°C in various buffers. The values are mean±SD, $n=3$. Absence of *bars* indicates that errors were smaller than symbols. The values are shown as percentages of the maximum activity under standard assay conditions

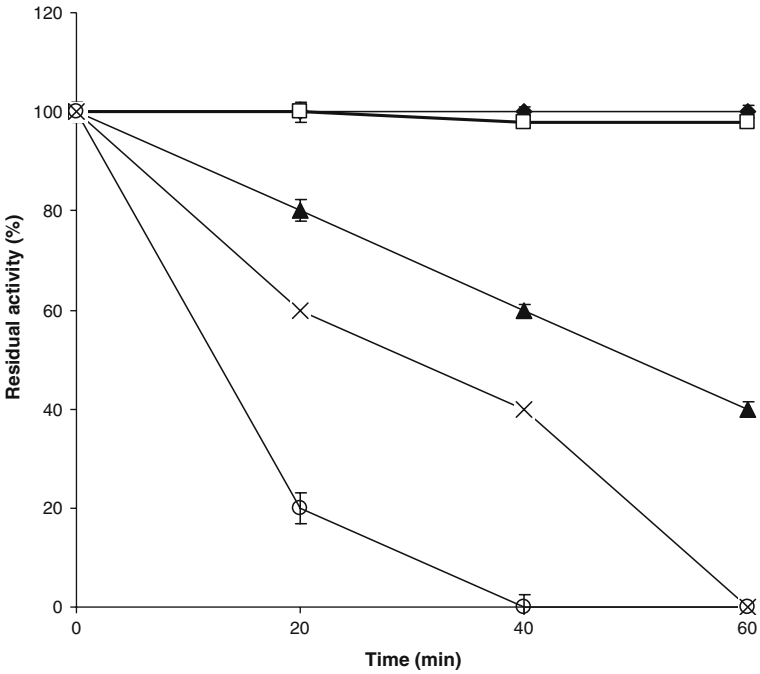

Fig. 11 Effect of different temperature on the activity of alkaline cellulase at 27°C (*filled diamonds*), 30°C (*open squares*), 37°C (*filled triangles*), 40°C (*multiplication symbols*), and 50°C (*open circles*). Temperature stability of the purified enzyme was tested by preincubating at different temperatures for 1 h. The residual activity was measured by standard cellulase assay conditions. The preincubated samples at 4°C were used as a reference to calculate the residual activity. Data are presented as mean±SD, $n=3$

The thermostability was tested by preincubating the enzyme for 1 h at various temperatures and the remaining activity was measured. The residual activity was still retained up to 100% and 98% of the control after incubation at 27 and 30°C, respectively (Fig. 11). The present findings revealed that the enzyme was stable up to 37°C. However, the enzyme was inactivated rapidly at temperatures higher than 40°C. In contrast, most alkaline cellulases from *Bacillus* sp. showed a thermal stability in the range of 40 to 60°C [17, 36, 46–49].

Conclusion

Recently, our research group initiated a holistic exploration of sponge-associated heterotrophic bacteria for the production of extracellular enzymes [50–52]. Literature also evidenced that sponge-associated bacteria has seldom been exploited for the production and optimization of industrial enzymes. Cellulase, one of the industrially important enzymes, has been exploited in the recent work. Though there are reports for the production of cellulase from marine sources [54], this paper would be the first describing the production and characterization of an extracellular cellulase from a sponge-associated *Marinobacter* sp. Apart from industrial implications, the enzyme seems to play a crucial role in endosymbiotic associations of marine nematodes and bacteria [53]. Therefore, the present study needs to be extended further to explain the functional role of cellulase produced by sponge-associated bacteria in the marine ecosystem.

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