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Screening and Characterization of Cellulase by *Bacillus megaterium* Isolated from Marine Sediments and its Antimicrobial Activity

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Abstract: Cellulase produced by *Bacillus megatarium* SSI, isolated from marine sediment was purified to homogeneity by ammonium sulphate precipitation, ion exchange chromatography on DEAE cellulose and gel filtration on Sephadex G-100. Purification of cellulase was achieved such as 1.36, 4.6 and 8.2 by ammonium sulphate precipitation, ion exchange chromatography and gel filtration respectively. The study revealed that the optimal conditions required for the maximum activity was cellulase were 9.0 pH and 50°C temperature. Significant enhanced activity of the enzyme was possible with carboxymethyl cellulose and other forms such as avicel. An additional input of metal ion like calcium supplemented to the medium influenced increased activity of the enzyme, however, EDTA did show a comparative effect. Apart from the enzyme activity, the designated purified protein *Bacillus megatarium* SSI inhibited the growth of *Fusarium oxysporum*, *F. odum*, *Botrys cinera*, *Alternaria alternate*, *Aspergillus niger* and *Penicillium sp.* Therefore, it was possible to establish *Bacillus megatarium* as a source of cellulase and antifungal agent.

Key words: CMC · Sephadex G-100 · DEAE · *Bacillus megaterium*

INTRODUCTION

The enzyme cellulase provides a key opportunity for achieving tremendous benefits of biomass utilization [1]. Cellulase is an extracellular enzyme, which is a polymer of β-1, 4-linked glucose units, a major polysaccharide constituent of plant cell walls. Therefore, it has become a considerable economic interest to develop processes for the effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials [2]. The breakdown of lignocellulose polysaccharides requires a combination of enzymes, which split off glucosidic lingkages between β-D-xylopyranosyl and glucopyranosil residues. A complete cellulase systems consists of three classes of enzymes: exo-1, 4-β-glucan cellobiohydrolase (EC 3.2.1.91), which cleave cellobiosyl units from the ends of cellulose chains; endo-1, 4-β-glucanases (EC 3.2.1.4), which cleave internal glucosidic bonds; β-D-glucosidase (EC 3.2.1.21), which cleaves glucose units from cellular oligosaccharides [3]. For cellulase system, a number of components and functional characteristics lead to efficient hydrolysis of cellulose [4].

The marine biosphere is one of the richest habitats of microorganisms. The oceans cover around 70% of the earth's surface and present themselves as an unexplored area of opportunity [5]. Microorganisms are increasingly becoming an important source in the production of medical and industrially important enzymes. Considering the fact that marine environment is saline in nature, which provides rare and unique microbial products particularly enzymes that could be safely used for human therapeutic purpose [6].

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Among the microorganisms, marine bacterial strains have been a great source of new compounds and their isolation all around the globe, from shallow coastal sediments to the deepest sediments [7].

From this point of view, the utilization of cellulosic materials for antimicrobial activity has received more attention. Most of these works are also confined to only terrestrial microbes' but not their marine counterparts. Hence the present study is aimed to trace a bacterium, which can produce more efficient cellulase enzymes in submerged fermentation.

MATERIALS AND METHODS

Isolation and Screening of Cellulolytic Bacterial Strain: The cellulase enzyme producing bacterial strain was isolated from marine sediments collected from the coastal regions of Kanyakumari district and southwest coast of India. The isolated organism was tested for cellulase production on CMC agar plates. The isolated bacterial isolates were streaked on CMC agar medium (g/l) (peptic digest of animal tissue, 5.0; beef extract, 1.5; yeast extract, 1.5; sodium chloride, 5.0, agar, 15, CMC cellulose, 10) and incubated at 37°C for 48 h. The isolated colonies on these plates were maintained on CMC agar slants at 4°C for further analysis. The purified colonies were further screened for their cellulase activity; pure cultures of bacterial colonies were transformed individually on CMC agar plates. After 72 hours incubation, the plates were flooded with 1% congo red and the plates were allowed to stand for 20 minutes at room temperature. Then the plates were thoroughly washed with 1M NaCl solution. A clear zone formed around the growing colonies of cellulase positive against the dark red background was taken as the indication of cellulase activity. The isolated bacterial colonies were further characterized for their morphological and biochemical characters by following standard keys of Bergey's Manual of Determinative Bacteriology [8].

Strain Identification

Genomic DNA Extraction: The isolated bacterial strain was grown in 2ml Zobell Marine Broth overnight at 35°C. The culture was spin at 7, 000 rpm for 3 min. The pellet was resuspended in 400 µl of sucrose TE (Tris EDTA). Lysozyme was added to a final concentration of 8 mg/ml and incubated for 1h at 35°C. To this tube, 100 µl of 0.5M EDTA (Ethylene Diamine Tetra Acetic acid) (pH 8.0), 60 µl of 10% SDS (Sodium Dodesyl Sulphate) and 3 µl of proteinase K from 20 mg/ml were added and incubated at 55°C overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamylalcohol (24:1) and ethanol precipitated. The DNA pellet was resuspended in sterile buffer [8].

Amplification of 16S rRNA Gene Sequence: Bacterial 16S rDNA was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA primers: forward primer 5' AGAGTTTGATCCTGGCTCAG 3' and reverse primer 5' ACGGCTACCT TGTTACGACTT 3'. Polymerase chain reaction was performed in a 50 µl reaction mixture containing 2 µl (10 ng) of DNA as the template, each primer at a concentration of 0.5µM, 1.5 mM $MgCl_2$ and each deoxynucleoside triphosphate at a concentration of 50 µM, as well as 1 U of *Taq* polymerase and buffer as recommended by the manufacturer (MBI Fermentas). After the initial denaturation for 3 min at 95°C, there were 40 cycles consisting of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min and then a final extension step consisting of 5 min at 72°C; Mastercycler Personal (eppendorf, Germany) was used. The amplification of 16S rDNA was confirmed by running the amplification product in 1% agarose gel in 1X TAE.

Cloning and Sequencing of 16S rRNA Gene Sequence: The amplified product (1500 bp) was purified using GFX ™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to manufacturer's instruction. The 16S rDNA amplicon was cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone™ PCR Product Cloning Kit # K1214, MBI Fermentas). Full length sequencing of the rRNA gene (about 1, 500 bp) for the isolated bacteria was carried out in Macrogen (Seoul, Korea).

Nucleotide Sequence Analyses: The full-length sequences obtained were matched with previously published sequences available in NCBI using BLAST. Multiple sequence analysis was carried out using CLUSTALX and further NJ plot and PhyloDRAW employed for constructing phylogenetic tree [8].

Enzyme Activity Assays

Endo-B-1, 4-Glucanase: Endo-B-1, 4-glucanase (EG) activity was determined by incubation of 1.0 ml of 0.2% (w/v) CMC in 0.025 M phosphate buffer (pH 7.0) with 1.0 ml of appropriate concentration of enzyme at 35°C. To stop the reaction, 1 ml of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 5 min after 30 minutes of incubation. The resulting samples were then

cooled to room temperature and the absorbance measured at 540 nm (A540). One unit of EG activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 >g of glucose within 1 min at 37°C [9].

Avicelase: Avicelase activity was determined by incubation of 1.0 ml of 0.2% avicel (w/v) in 0.025 M phosphate buffer (pH 7.0) with 1.0 ml of appropriate concentration of enzyme at 35°C. The cellulase enzyme activity was measured after 30 minutes of incubation. One unit of avicelase activity was defined as the amount of enzyme that could hydrolyze avicel and release 1 >g of glucose within 1 min at 37°C [9].

Filter Paperase: In this assay Whatman No. 1 filter paper was used as the substrate. Fifty milligrams of filter paper was dissolved in 0.025 M phosphate buffer (pH 7.0) and 1.0 ml of appropriate concentration of enzyme. The reaction mixture was incubated at 35°C for 30 minutes and the reaction was terminated by adding 2 ml of DNS. One unit of filter paperase activity was defined as the amount of enzyme that could hydrolyze filter paper and release 1 >g of glucose within 1 min at 37°C [10].

Enzyme Purification: The complete purification process was carried out at 4°C to maintain the enzyme activity. Enzymes (cell free supernatant of fermented broth obtained after centrifugation at 10000 rpm for 10 min) were precipitated by adding two volumes of acetone and kept for 24 h at 0 - 4°C to allow complete precipitation. The resulting precipitate was collected by centrifugation (10000 rpm for 30 min) and the pellet was air dried and resuspended in a minimal volume of 10 ml of buffer A (0.025 M sodium phosphate buffer, pH 7.0 containing 0.001 M ethylene diamine tetraacetic acid (EDTA) and 0.001 M 2- mercaptoethanol). The sample was dialyzed using dialysis membrane (150 µ) against same buffer. Then, ion exchange chromatography was performed on a column of DEAE-cellulose (2.5 -/30 cm) and the flow rate of the column was set at 1ml/mins. After loading the sample, the column was washed with the same buffer until the optical density of the elution at 280 nm was zero. The bound proteins were then eluted with a linear gradient of sodium chloride in the range of 0.1-1M in the equilibrating buffer. Fractions (2.0 ml each) were collected at a flow rate of 0.5ml/mins and the enzyme activity and protein content of the each fraction were determined. Active fractions showing enzyme activity was pooled and concentrated from the ion exchange chromatography. Then the pooled active fraction was centrifuged at 10000 rpm for 10mins. The resulted fraction was dialyzed against the same buffer without NaCl. The residue was dissolved in buffer a applied to gel filtration chromatography (Sephadex G-100). The column was washed with above mentioned buffer and eluted with the same buffer. A fraction of 2ml in sample was collected at a flow rate of 0.5ml/mins. The elute was observed for protein content and enzyme activity in each fractions. Fractions with high enzyme activity was pooled and subjected to centrifugation at 10000 rpm for 10mins and redisolved in a minimal volume of same buffer A. Then the purified fraction was obtained and stored in -20ºC.

Characterization of the Purified Enzyme

Molecular Weight Determination by SDS-PAGE: SDS-PAGE (12%) was performed according to the method of Laemmli under reducing conditions. The molecular weight was determined by interpolation from a linear semi-logarithmic plot of relative molecular weight versus the Rf value (relative mobility) using a standard molecular weight marker (97.4, 66.2, 39.2, 26.6, 21.5 and 14.4 kDa) [11].

Effect of pH on Purified Enzyme Activity: Stability of the purified cellulase was measured at different pH (4 to 9). The reaction mixture consisted 0.25 ml of cellulase and 0.5 ml substrate. The pH of the reaction mixture was adjusted using buffer A. To ensure the pH stability, the enzyme reaction mixture was incubated at 40°C for 1 h and the relative activity was measured at standard conditions [12].

Effect of Temperature on Enzyme Activity: The enzyme stability of the purified enzyme was studied by incubating the reaction mixture (0.2 ml protease + 0.5 ml substrate) at different temperatures ranging from 20° to 60°C for 1 h at 10°C interval and then relative activity of the samples were assayed at standard assay conditions [12].

Effect of EDTA Concentration on Cellulase Activity: The effect of EDTA on cellulase activity was tested by the supplementation of various concentrations on EDTA. For this, the cellulase enzyme (0.2 ml) and substrate (0.5 ml) were mixed with EDTA of different concentrations such as 0.02%, 0.04%, 0.06%, 0.08% and 0.1%. Then the mixture was incubated at 35°C for 1h and then relative activity of the enzyme was assayed at standard assay conditions [12].

Effect of NaCl Concentration on Cellulase Activity: The effect of NaCl on cellulase activity was studied by supplying various concentrations. The experiments were carried out individually at 1%, 2%, 3%, 4%, 5%, 6%, 7% and 8% NaCl with cellulase enzyme. Then the mixture was incubated at 35°C for 1h and then relative activity of the enzyme was assayed at standard assay conditions [12].

Effect of Metal Ions on Cellulase Activity: The impact of metal ions on cellulase activity was examined with metal ions such as calcium chloride, ferric chloride, zinc chloride, EDTA and magnesium chloride at 0.5mM concentrations. The reaction mixture (0.25 ml protease + 0.5 ml substrate) and individual metal ion solution were incubated at 35°C for 1h. Then the activity and stability of cellulase was assessed using standard method [12].

Antagonistic Activity: Antagonistic activity of marine bacterial isolates was tested as described by [13]. Initial screening for *in vitro* antagonistic activity was tested against fungal strains on PDA agar plates.

RESULTS AND DISCUSSION

Isolation, Screening and Molecular Identification of Cellulolytic Bacteria from Marine Sediment: A total of nine bacterial strains were isolated from marine sediments, Kanyakumari District, Tamil Nadu, India. The isolated bacterial strains were screened for cellulase producing ability on CMC agar. Among the nine bacterial strains, *Bacillus megaterium* showed the maximum cellulolytic activity on CMC agar plate. The zone formation around the bacterial growth indicates the cellulase positive. Hence the strain was identified as a cellulase producer and it was taken for further experimental studies. Based on the morphological, physiological and biochemical characteristics, the isolated bacterial strain was identified as *Bacillus megaterium* by the following standard keys of Bergey's Manual of Determinative Bacteriology (Table 1). Phylogenetic studies revealed that the 16S rRNA gene sequence of *Bacillus megaterium* has 98% similarity with the nearest match in the Gene bank (Fig 1). This report is identical with the earlier report of Singh *et al*. [14] who reported the purification and characterization of cellulase from *Bacillus* sp. isolated from Kanyakumari paddy field.

Purification of Cellulase from *Bacillus megaterium*: Cellulase enzyme from *Bacillus megaterium* was successively purified by ammonium sulfate precipitation, ion exchange chromatography on DEAE-cellulose and gel filtration chromatography on Sephadex G-100. The results of the purification of cellulase from *Bacillus megaterium* are summarized in Table 2. Acetone precipitation gave purification fold of about 1.36. Dialyzed enzymes from the ammonium sulphate precipitation steps were subjected to ion exchange chromatography on DEAE Cellulose. Fig. 2 shows the anion exchange profile of *Bacillus megaterium*. In total, 50 column fractions were obtained and they were tested individually for cellulase activity. The elution profiles of cellulase on DEAE cellulase from the *Bacillus megaterium* gave a single peak of cellulase. The enzyme did not bind to the ion exchanger when NaCl ion gradient was applied. The percentage recovery of the enzyme was 49.3%, while the purification fold of about 4.69% obtained. The most active fractions of this peak was pooled and concentrated for further purification. Again, the pooled sample from active peaks of DEAE cellulose was applied on Sephadex G 100. Fig. 3 shows the gel filtration profile of *Bacillus megaterium*. The recovery and purification fold were 35.95 and 8.2, respectively. The purified enzyme seemed to be homogeneous, because it is shown as a single band in SDS–PAGE.

Table 1: Morphological, cultural and biochemical tests for identification of the candidate bacterial strain

Biochemical tests	Results
Colony Morphology	
Configuration	Circular
Margin	Entire
Elevation	Convex
Surface	Smooth and shiny
Opacity	Opaque
Gram's staining	Gram positive
Endospore staining	Sub-terminal spores
Motility	+
Shape	Elevated
Size (µm)	Length: 1.5-2.5µ, Width: ~ 1.5µ
Carbohydrate fermentation test	
a. D-glucose	+
b. Mannitol	+
c. Lactose	-
d. Sucrose	+
Indole production	-
Methyl red test	-
Voges-Proskauer test	+
Citrate utilization test	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Casein hydrolysis	+
Urease test	-
Catalase test	+
Oxidase	-
Nitrate utilization test	+

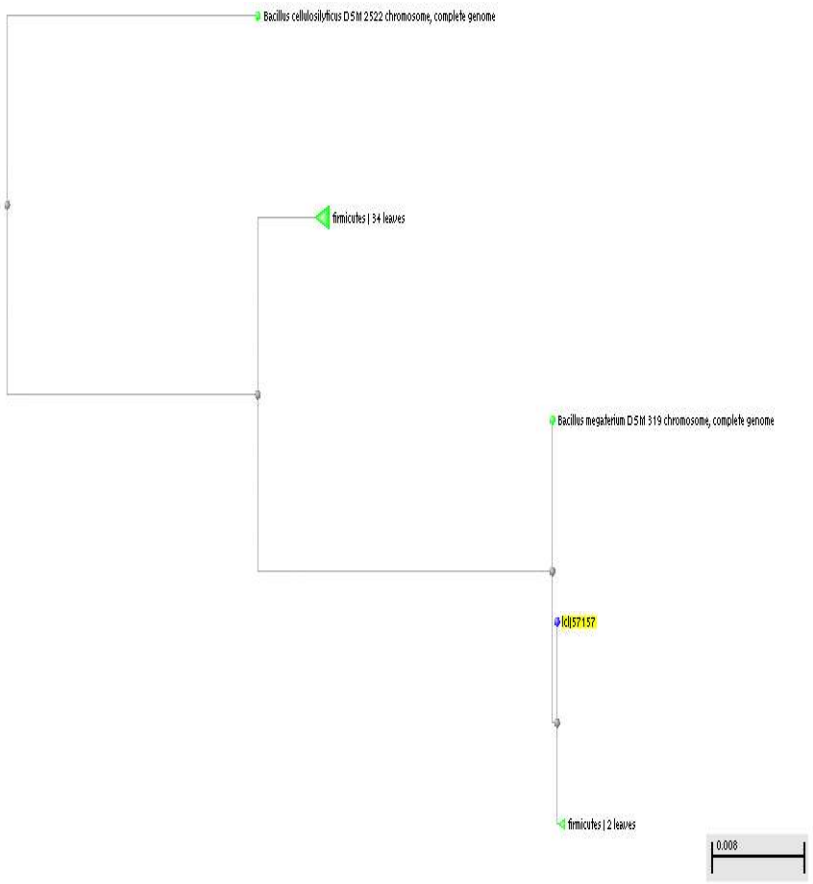


Fig. 1: Rectangular view of phylogenetic tree

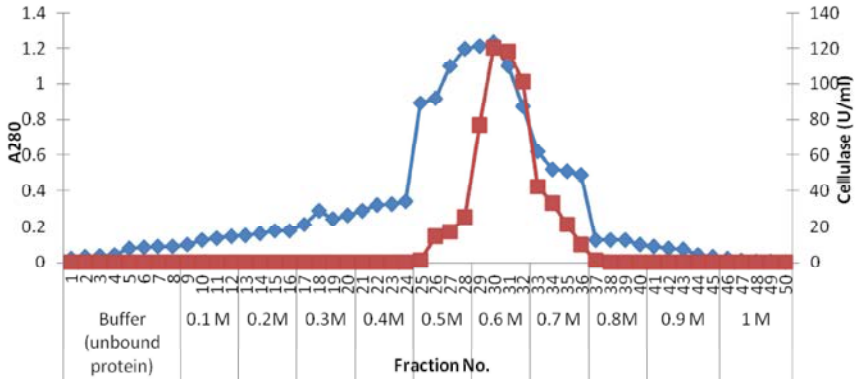


Fig. 2: Elution profile of *Bacillus megaterium* purified on DEAE- cellulose

Table 2: Summary of the results of the purification of cellulase from *Bacillus megaterium*

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture supernatant	501	162	3.09	1	100.00
Acetone precipitation	459	109	4.21	1.36	91.6
DEAE- cellulose	247	17	14.52	4.69	49.3
Sephedox – G100	180	7.01	25.6	8.2	35.9

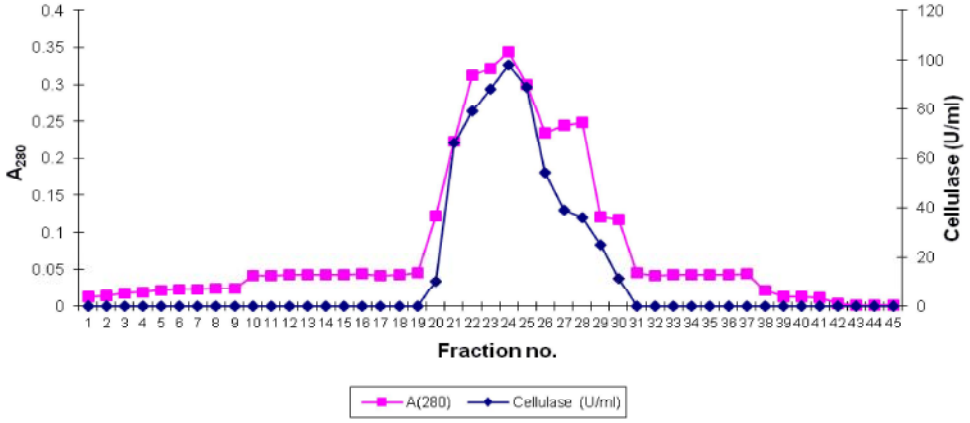
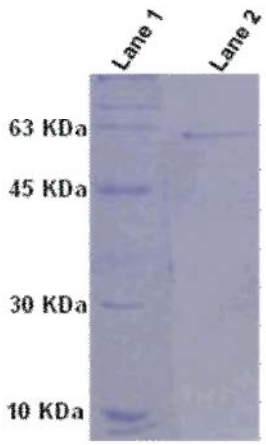


Fig. 3: Gel filtration Chromatographic elution profile of *B. megaterium* cellulase



Lane 1: Molecular Marker
Lane 2: *Bacillus megaterium*

Lane 1: Molecular Marker
Lane 2: *Bacillus megaterium*

Fig. 4: SDS – PAGE profile of purified cellulase from *Bacillus megaterium*

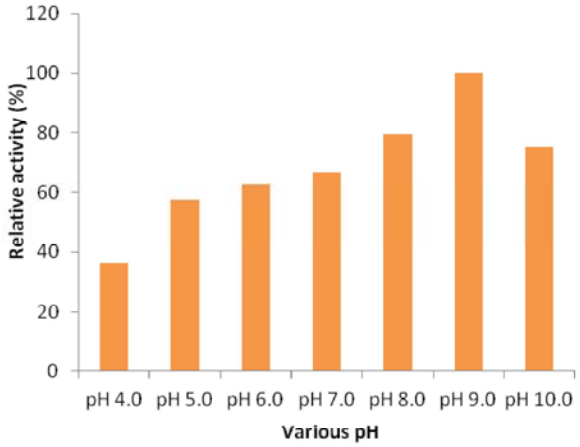


Fig. 5: Effect of pH on cellulase activity

Molecular mass of *Bacillus megaterium*: SDS-PAGE analysis of the purified enzyme revealed a single band with a molecular mass of 56 kDa (Fig. 4). The molecular weight of endoglucanase varies with different bacteria. In *Pseudomonas fluorescens* it is 36 kDa [15] while in *Bacillus* strains it is 54 kDa [16, 17]. But in *Thermomonospora* and *Cellulomonas* sp. YJ5 it is 38 kDa and 43.7 kDa respectively [10, 18].

Effect of pH on Enzyme Activity and Stability: The production of cellulase from *Bacillus megaterium* was found to be most active at pH 9. Cellulase activity improved progressively with increase in pH up to pH 9.0 and a further decrease in cellulase activity (Fig. 5). At pH 4, only 36% of the maximum enzyme activity was obtained, increased to 81% at pH 8. This report is harmonizing with the previous report of Kim *et al* [19], who reported that the purified cellulase from *Bacillus* sp HSH was optimally active at pH 9 – 10. Also reported that the cellulase from *Bacillus sphaericus* was active alkaline range [14].

Effect of Temperature on Cellulase Activity and Stability: The effect of cellulase activity was studied by incubating the reaction mixture at various temperatures (20–70°C). Enzyme activity was found to be high at 50°C (Fig 6). The relative enzyme activity was 36.4%, 48.2%, 71.0%, 46.8% and 32.1% at 20, 30, 40, 60 and 70°C respectively. This kind of stability of enzyme might be suitable for industrial process requirements as it possessed prolonged stability under high temperature. The present study is similar to previous study of Mawadza *et al.* [9]. They have reported that the effect of temperature on cellulase activity from *Bacillus* sp was optimum at 50°C [16] also showed the maximum enzyme activity of *Bacillus amyoliquefaciens* recorded in 50°C.

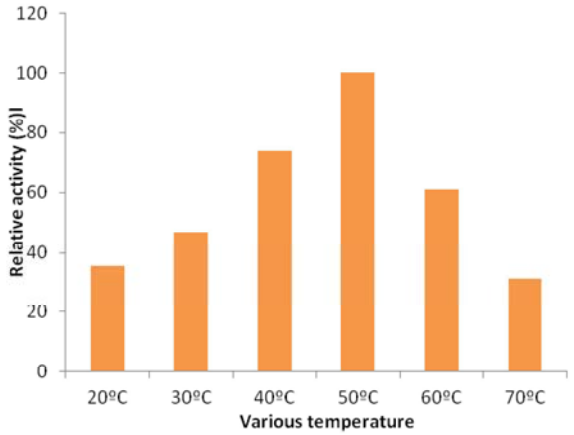


Fig. 6: Effect of temperature (°C) on cellulase activity

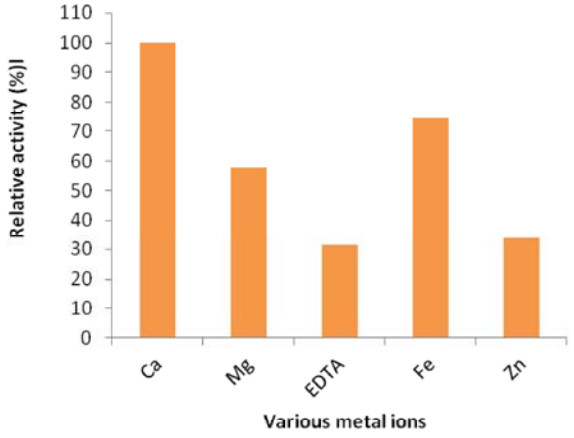


Fig. 7: Effect of metal ions on cellulase activity

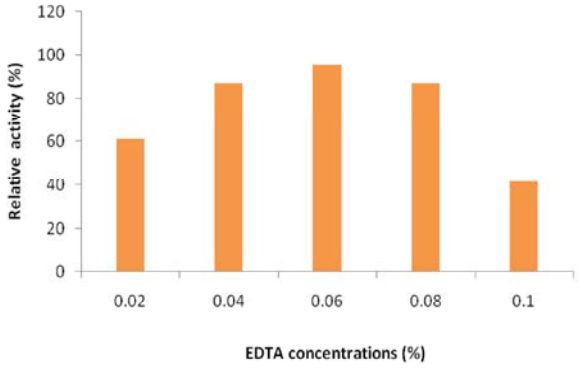


Fig. 8: Effect of EDTA concentrations on cellulase activity

Effect of Metal Ions on Cellulase Activity: The effect of divalent ions on enzyme activity was investigated by allowing the divalent ions (0.001 M) to react with the cellulase sample. Among divalents, Ca+ enhanced the relative enzyme activity when compared with the control (100%). Mg, EDTA, Fe and Zn inhibited the enzyme

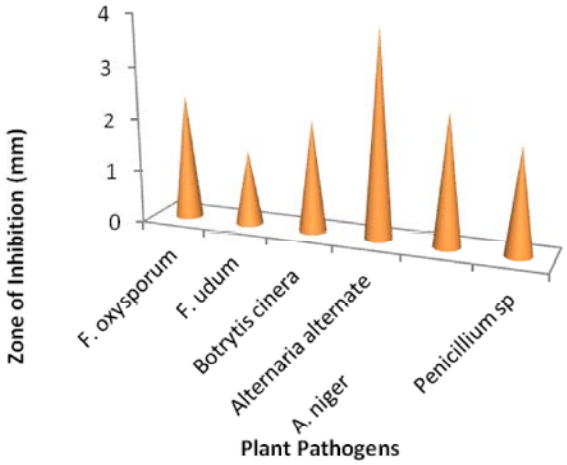


Fig. 9: Antimicrobial activity of purified cellulase from *Bacillus megaterium* against selected plant pathogens

activity and the relative activity was 58%, 33%, 74% and 37%, respectively (Fig 7). In contrary to this result, cellulase was highly active in *Bacillus subtilis* YJI, *Bacillus amyoliquefaciens* DL-3 in the presence of Mn [16].

Effect of EDTA on Enzyme Activity: Among the various concentrations of EDTA, the maximum activity was recorded in 0.6mM (Fig. 8). EDTA is a metal chelating agent and inhibition of the enzymes by EDTA suggests that the enzyme activities probably depend on chemical activities and that they may contain inorganic groups, which formed inactive complexes with EDTA [15].

Antifungal Activity of Purified Cellulase from Candidate Bacterium: SS1 protein was tested for the presence of antifungal activity against five strains including *Fusarium* oxysporum, *F. odum*, *Botrys cinera*, *Alternaria alternate*, *Aspergillus niger* and *Penicillium* sp. (Fig. 9). The diameter of the inhibition zones for these strains was 2.4, 1.4, 2.1, 3.9, 2.5 and 2 mm in the above order. Marine *Bacillus* species have been used for insect biocontrol, industrial enzyme production, bacteriocin production and antibiotic production. The present study is similar to the earlier study of [20], who has reported that the *Bacillus* has been used as biological control agents because of their advantages over Gram-negative bacteria and antifungal activity. The *Bacillus licheniformis* have been successfully used for biocontrol of plant pathogenic fungi, such as *Botrytis cinerea* and *Phytophthora capsici* [21].

CONCLUSIONS

This study gives us a hint as well as the microbial wealth of cellulase producing bacteria *Bacillus megaterium* which can be harnessed for biotechnological processes.

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