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Research Article

CELLULASE PRODUCING THERMOPHILIC BACTERIA FROM HOT SPRING OF ASSAM

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Abstract-Thirty strains of bacteria were isolated from Garampani, under Nambor reserve forest in Golaghat district of Assam. Qualitative analysis was carried for the thirty isolates, though only eight isolates were found to have cellulose-degrading capacity. Analysis of the 16S rDNA sequences revealed degree of sequence similarity with *Bacillus cereus, Bacillus thuringiensis, Stenotrophomonas maltophilia, Bacillus pumilus* and *Bacillus* sp [3]. Growth curve analysis were done at 35°C, 45°C and 55°C. Thermal stability was evaluated at different temperatures ranging from 40°C-100°C as well as the stability of the enzymes were also assessed at different pH range of 2-10. *Stenotrophomonas maltophilia* (0.43 IU/ml) showed the highest cellulose activity followed by *Bacillus cereus* (0.39 IU/ml) and *Bacillus thuringiensis* (0.3 IU/ml) whereas *Bacillus cereus* (13.77 µg/ml) produced the highest amount of enzyme followed by and *Bacillus* sp (13.44µg/ml).

Highlights

- Eight isolates were found to have cellulose-degrading capacity
- Bacterial isolates showed high degree of sequence similarity with *Bacillus cereus, Bacillus thuringiensis, Stenotrophomonas maltophilia, Bacillus pumilus* and *Bacillus* sp.
- *Stenotrophomonas maltophilia* (0.43 IU/ml) showed the highest cellulase activity
- *Bacillus cereus* (13.77 µg/ml) produced highest amount of enzyme

Keywords- Thermophilic, cellulase, bacteria, cellulose, enzyme, endoglucanase

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Introduction

Thermophilic bacteria are important sources of heat stable enzyme [16], and geothermal environment such as hot springs are usually their favourable habitats. Potential enzymes such as amylase, protease, DNA polymerase, xylanases and chitinases have been identified and evaluated from thermophilic microorganisms [8].

Cellulases have attracted much interest due to their diverse application. Cellulases are used in food, brewery and wine, animal feed, textile, paper and pulp industries, along with agriculture and other research purposes. The major industrial application of cellulases are in the textile industry to produce the stone washed look of denims, in addition to household laundry detergents for refining fabric softness and brightness [7]. There are various benefits of using thermostable enzymes in industrial applications as compared to thermolabile enzymes [12]. The thermostable enzymes due to their ability to tolerate higher temperatures have a longer half-life. Moreover, the processes carried at higher temperature are advantageous as it prevents microbial growth and thereby reduces microbial contamination

The demand for thermostable enzymes has grown more rapidly than ever before, and has as such demand has become the driving force for intensive research on cellulases and related enzymes. Thermostable cellulolytic enzymes hydrolyse cellulose and ferment glucose to ethanol at high temperatures, which in turn can lower the production cost of ethanol significantly. Hence, it is important to find a novel source of thermostable cellulolytic enzymes to meet the growing demand for cellulases to understand their full prospective, continued multidisciplinary research on basic and applied aspects is vital. Therefore, based on the above background knowledge an attempt of screening and isolating cellulose producing bacteria from hot spring was undertaken. Characterization of cellulases based on pH, temperature, enzymatic activity was carried out and followed by molecular characterization of the isolates producing the highest amount of cellulase using 16s rRNA was done.

Materials and Methods

Source of Microorganisms

Water samples were collected from hot spring of Assam (Garampani, Dist. Golaghat). Cellulase-producing bacteria were screened out using CMC plates. Amongst the thirty isolates, ten isolates were found to be cellulose degrading. Further studies were carried out on the eight isolates.

Isolation and Culture of Thermophiles

Tenfold serial dilutions of each water sample were prepared in sterilized water and 0.1 ml of that diluted sample was spread on CMC medium [g/l):CMC, 10; Tryptone, 2; KH_2PO_4, 4; Na_2HPO_4, 4; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.001; $FeSO_4.7H_2O$, 0.004; Agar, 15 and pH adjusted to 7] [2]. The plates were incubated at 35^{0}C for 2-3 days and were flooded with iodine solution to check the cellulolytic activity of isolated strains by producing a zone of hydrolysis, which indicated the cellulose degradation.

Determination of Growth pattern of the isolates

Nutrient Broth (50 ml) media were prepared and autoclaved in the 100 ml flask at 121^{0}C and 15 p.s.i. pressure for 15 minutes. The isolates were inoculated in the

NB media and incubated for 24 hrs. Volumes of broth cultures were adjusted with sterile water in the 2 ml eppendorf tube to obtain similar optical density (uniform cell population/ml) at 600 nm. A 0.1 ml volume from each of these bacterial cultures were inoculated separately in the flask containing 100 ml liquid NB media and incubated in an incubator shaker at 182 rpm and 35^{0}C as well as 55^{0}C separately. Three 100 ml capacity replicate flasks were maintained at each culture. The optical density of the cultures was recorded in a spectrum of 600 nm by aseptically drawing approximately 3 ml of the cultures at 4 hrs intervals [21].

DNA extraction, PCR amplification of the rDNA internal transcribed spacer (ITS) region, and Sequence analysis

Isolation of the genomic DNA [6] from the eight cellulase producing strains were carried out by the modified protocol of Cardinal et al., 1997. Amplification and sequencing of the isolates were performed using a pair of universal primers: US16S F (5'- AGA GTT TGA TCC TGG CTC AG ') and US16S R (5'- ACG GCT ACC TTG TTA CGA CTT -3'). The amplification was conducted in a 25 µL reaction mixture containing 100 ng genomic DNA, 10 mMTris-HCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 200 ng of each primer, and 1 unit Taq DNA polymerase (Promega). The reaction mixtures were denatured at 94^{0}C for 3 minutes and subjected to 35 cycles of 1 minutes at 94^{0}C, annealing at 55^{0}C for 1 minutes, extension at 72^{0}C for 1 minutes, and a final extension step of 7 minutes at 72^{0}C. The amplified PCR product was resolved on a 1.2% agarose gel to observe the integrity of the PCR amplified product.

The purification of the PCR amplified products was carried out using GenElute PCR Clean-up kit (Sigma, India).The purified products were sequenced by ABI 377 automated DNA Sequencer (Applied Biosystems, USA). The sequenced 16S rDNA gene from both the ends were further pre-processed and assembled to a consensus sequence using CodonCode Aligner version 4.1 (Codon Code Corporation, USA). Finally, the consensus sequence of each strain was compared with the 16S rDNA sequences of bacteria available in the public domain of NCBI using BLAST search. Sequences producing significant alignment with the query isolate were considered (cut off % identity >96%) for multiple sequence alignment in Clustal W. Phylogenetic analysis of 16S rDNA gene sequences for each strain along with their closest relatives was performed using the Kimura 2-parameters method incorporated in MEGA 6.0 to assign each taxa (isolates in the present study) in their respective taxonomic position [23]. Moreover, each 16S rDNA gene sequence of the isolate was further assessed using EZTaxon server version 2.1 to ensure the correctness in the identification of each strain. The Its rDNA gene sequences of the 8 strains were submitted to GenBank of NCBI through sequence submission tool BankIt and where they were assigned GenBank Accession numbers. In addition, the strains identified in the present study were taken together for the construction of unrooted phylogenetic tree in MEGA 6.0.

Isolation of extracellular cellulase enzyme

Bacterial isolates were grown in 100 ml of CMC broth in an incubator shaker at 170 rpm and 35^{0}C.CMC broth was centrifuged at 5000 rpm, for 10 minutes at 4^{0}C so that the cell debris was collected in the form of pellet and the supernatant was collected in a beaker which contains the crude enzyme [17].

Cellulase concentration by Ammonium sulphate precipitation

Ammonium sulfate precipitation is a method used to purify proteins by altering their solubility. The volume of the protein solution was measured, poured into a beaker with a magnet bar and placed in an ice bath or at 4^{0}C on a stirrer. The required amount of ammonium sulphate was calculated (70% cut). Stirring the solution and adding salt to it in small portions, allowing the salt to dissolve before adding the next portion. When all the salt has been added, the beaker was taken off stirrer and was left it at 4^{0}C for some time, ideally overnight [3]. To start the process of dialysis, the first dialysis bag needs to be activated. Dialysis bag activation was done by boiling the bag in distilled water for 1 minutes twice and then transferred to 0.1% SDS solution and boiled twice for 1 minutes, then again, it was boiled in distilled water for 1 minutes, and then finally transferred to the distilled water. Once the bag is activated, the crude enzyme was transferred to the bag and was sealed. This bag was kept in 100 mMTris buffer for 90 minutes and was again changed and kept overnight in the fridge. After keeping it for overnight, the buffer was again changed and kept for 90 minutes.

Characterization of the enzyme

Iodine solution clearing zone assay is suitable for qualitative display of cellulase activity [24). The overnight incubated CMC plates with circular growth of the bacterial isolates were flooded with iodine solution and left for 15 minutes with intermittent shaking and finally it is washed with distilled water 2-3 times. The clearing zone of enzymatic activity will be visible around the batch of growth. The water elutes the dye in the clearing zone where the cellulose has been degraded into simple sugars by the enzymatic activity.

Estimation of the enzyme concentration by Bradford method

Protein concentration was determined according to the method described by Bradford (1976). One ml of Bradford reagent was added to 50µl of the sample and the extinction was measured after 5 minutes at 595 nm [5]. Different concentrations of bovine serum albumin (BSA) were used as a protein standard: 10, 20, 40, 60, 80, and 100 µg/ml distilled water. One ml of Bradford reagent was added to 50 µl BSA standard and the extinction was measured after 5 minutes at 595 nm.

Effect of Temperature and pH on the Activity of Cellulase

To determine the temperature optimum of crude enzyme, the endoglucanase activity was estimated at temperatures ranging from 40^{0}C- 100^{0}C under the standard assay conditions, using 1.0% CMC solution as substrate.

To determine the thermal stability of crude enzyme, 10µg/ml of crude enzyme were pre-incubated for 30 minutes at 40^{0}C, 50^{0}C, 60^{0}C, 70^{0}C, 80^{0}C, 90^{0}C and 100^{0}C for 30 minutes each and then 10µl of each crude enzyme were placed on CMC plates and incubated at 35^{0}C for 2 days. The plates were then flooded with iodine solution to check the zone of hydrolysis [3]

To determine the pH stability of crude enzyme, 10µg/ml of crude enzyme were placed on CMC plates of different pH ranging from 2- 10 and incubated for 2 days. The plates were then flooded with iodine solution to check the zone of hydrolysis [9].

Quantitative Assay of cellulase activity

The IUPAC recommended endoglucanase (CMCase) assay is a fixed conversion method, which requires 0.5 mg of absolute glucose to be released in the reaction condition. The reducing end concentration is measured by the DNS method [25]. Enzyme dilution series were prepared, of which at least two dilutions must be made of each enzyme sample, 0.5 ml of the diluted enzyme solution was added into test tubes with a volume of at least 25 ml. The enzyme solution and substrate solution was equilibrated at 50^{0}C.0.5 ml of the CMC solution was added to the test tubes and mixed well.Incubated at 50^{0}C for 30 minutes.3.0 ml of DNS solution were added and mixed well. Boiled for exactly 5 minutes in vigorously boiling water. The tubes were placed in an ice-cooled water bath to quench the reaction. 20 ml of distilled water was added and sealed with parafilm. Mixed by inverting the tubes several times. The absorbance was read at 575 nm based on the substrate blank. The substrate blank (0.5 ml of CMC solution + 0.5 ml of citrate buffer) and the enzyme blanks (0.5 ml of CMC solution + 0.5 ml of dilute enzyme solutions) were prepared. Substrate and enzyme blanks were identically treated as the experimental tubes. The glucose standards were prepared. The glucose released by the enzyme solutions with deduction of the enzyme blank absorbance based on the glucose standard curve was calculated.

Results and Discussion

Soil and water samples were collected from hot spring of Assam (Garampani, Dist: Golaghat). A total of thirty organisms were isolated which were able to grow on the CMC agar plates but only 8 of them were able to produce clearing zone and proved positive to cellulolytic activity by Iodine solution staining. Further studies were carried out on these eight isolates (G1, G2, G3, G4, G5, G6, G7 and G8) which were found to be cellulose degrading [Fig-1]. Ponnambalam *et al*. (2011) reported that compared to fungi, bacteria are not effective producers of the

cellulase enzyme and therefore, it is difficult to get visible clearing zone, because of this the isolated microorganisms have to be inoculated in concentric circular patches in the plates which helps in concentrated growth of the colonies and accumulation of enzyme produced in that concentric region will show clearing zone indicating cellulolytic activity [15]. The positive isolates were taken into consideration for further study. A similar study was done by Maki *et al*. (2011) for screening and identification, whereby the iodine solution was found to be quick and efficient for identifying several good cellulase producing bacteria from a wide variety of samples. Iodine assay is an effective method of isolation as well as semi quantification of cellulolytic activity [14]. Visual scoring of cellulase activity was done in CMC plates after staining with Iodine and the isolates showed the development of cellulose clearance zone with iodine solution. Kasana *et al*. (2008) did screening and qualitative estimation of cellulase production by microorganisms on CMC agar plates using iodine solution [10].

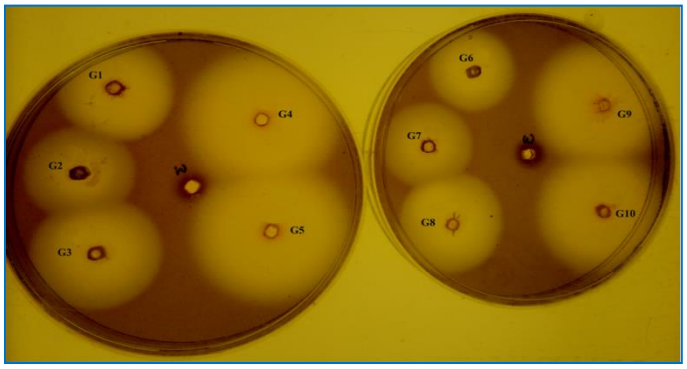


Fig-1 Zone of hydrolysis shown in the plates

When the isolates were grown at 35°C, all the isolates showed sigmoidal growth, which is characteristics of bacterial growth [Fig-2]. The growth curve analysis of all isolates at 45°C revealed that the isolate G2 has the shortest lag phase [Fig-3]. The growth characteristics of all the isolates are found different at 55°C [Fig-4]. When the isolates were grown at 35°C, all the isolates showed sigmoidal growth, which is characteristics of bacterial growth. Among all the isolates, G6 and G8 are slow growing. The growth curve analysis of all isolates at 45°C revealed that isolate G2 has the shortest lag phase followed by G7 and G8. All isolates showed variations in their log phase. The growth characteristics of all the isolates are found different at 55°C. All the isolates showed almost same lag phase and slight variation in their log phase compared to their lag phase at 35°C and 45°C. Here all the isolates showed very slow growth but long log phase. So, it can be concluded that all the isolates showed varied growth characteristics depending upon the temperature provided during their growth. Sen et al. (2010) reported that the thermophilic bacteria survived at relatively high temperatures [19], between 45°C and 80°C and are classified as obligate and facultative. Obligate thermophiles require such high temperatures for growth, whereas facultative thermophiles can thrive at high temperatures, but also at lower temperatures (below 50°C). Shimura et al. (1999) also did growth curve study of a thermophilic *Bacillus* sp. JF8 and were found to tolerate up to a temperature of 75°C [21] and the optimum growth temperature was 60°C.

PCR amplification of the genomic DNA of the isolates showed an amplification product of ~1500bp using 16S rDNA universal primers (forward US16F and reverse US16R primers) [Fig-8]. The genomic DNA of the 10 isolates was extracted using phenol: chloroform method. DNA was quantified using Nano Drop spectrophotometer and the concentration 549.59 ng/µl-3123.12 ng/µl was recorded. The DNA samples were also found pure in terms of RNA and protein contamination. In the present investigation, the absorbance ratios (260/280) for the DNA extracts from all samples were found high (2.0 is indicative of pure DNA [18]. The blast results showed that the bacterial isolates have a high degree of sequence similarity with *Bacillus cereus, Bacillus pumilus, Bacillus* sp. and *Bacillus thuringiensis.* The phylogenetic analysis of the sequences revealed that the isolate G1 showed highest homology (>97%) with *Bacillus cereus* (KF278103), G2 with *Stenotrophomonas maltophilia* (KF278104), G3 and G8 with *Bacillus thuringiensis* (KF278105), G4 with *Bacillus pumilus* (KF278106) and G5, G6, G7 with *Bacillus* sp (KF278107). However, further such studies involving more number of isolates (taxa) can better depict their phylogenetic position at community level. [Fig-9]. Khalil *et al*. (2011)reported *Bacillus* sp. from hot springs of Saudi Arabia after 16S rRNA phylogenetic analysis [11]. Similar study was carried out by Shankar *et al*. (2011) where cellulase producing *Bacillus pumilus* EWBCM1 was identified using 16S rRNA sequence analysis [20]. Lin *et al*. (2012) carried out investigation on the catalytic performance of cellulases produced by Bt strains and explored cellulase-activities of *Bacillus thuringiensis* strains by detecting their ability to form halos in CMC, plates suggesting that Bt strains could produce cellulases with high activities, which might have the potential ability to liberate glucose from cellulose [13].

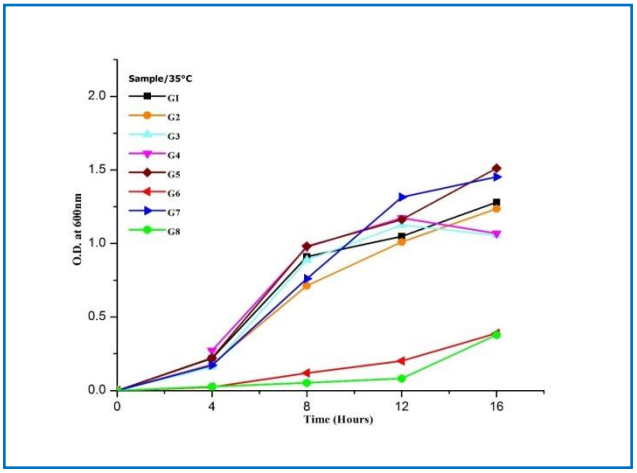


Fig-2 Bacterial growth curve (35°C)

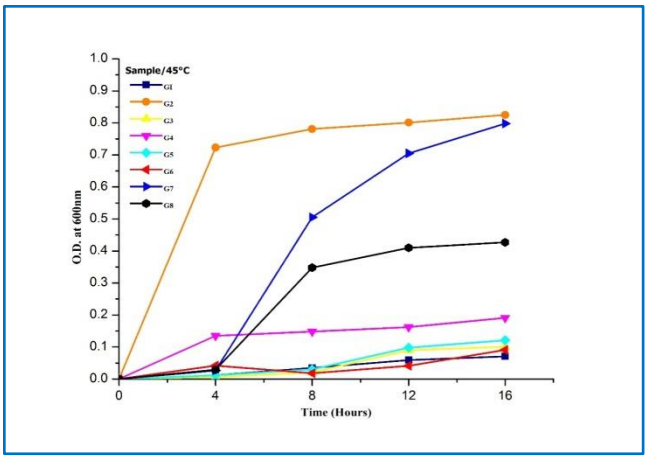


Fig-3 Bacterial growth curve (45°C)

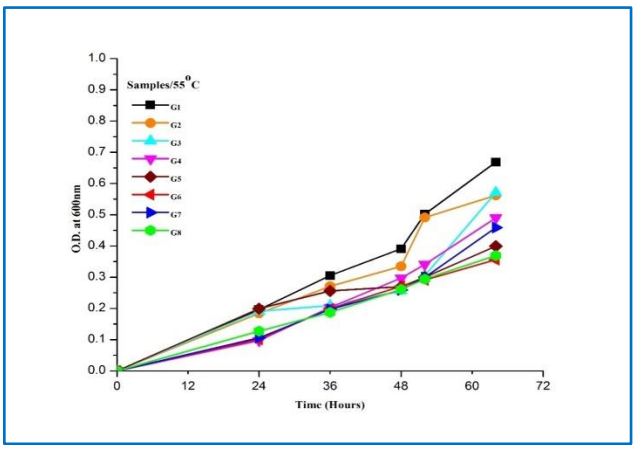


Fig-4 Bacterial growth curve (55°C)

The crude enzymes were incubated at different temperatures *viz.* 40^{0}C- 100^{0}C for 30 minutes along with commercial cellulase (Himedia, India) to determine the temperature sensitivity for cellulase activity [Fig-5]. The crude enzymes were stable even at temperature upto 100^{0}C whereas the commercial cellulase was stable only up to 60^{0}C only. The crude enzymes were incubated at 40^{0}C-100^{0}C along with commercial cellulase (HiMedia, India) to determine the temperature at which cellulase is optimally active. Crude enzymes were stable at temperature up to 100^{0}C but the commercial cellulase was stable only upto 60^{0}C. Earlier studies on the effect of temperature, the enzyme exhibited its optimal activity at 50^{0}C and pH 6.0 [4]. A similar study was carried out by Vijayaraghavan *et al*. (2011), where the enzyme was found stable in the pH range 5.0 to 7.0 and stability was maintained for 30 minutes at 50^{0}C [24]. The crude enzymes were incubated at different pH range (pH 2-10) along with a commercial cellulase enzyme to determine the optimum pH for their activity [Fig-6]. Isolated crude enzymes did not show any activity at acidic pH, but were active at alkaline pH (upto pH 10) whereas commercial cellulase enzyme showed activity for the whole pH range (pH 2-10). Crude were visually scored at pH 2-10 along with commercial cellulase (HiMedia, India) to determine the pH optimal for cellulase activity. The crude enzymes did not show any activity at acidic pH whereas the commercial cellulase was active in the pH range 2-10. Optimum pH for cellulases activity for the crude enzymes was found to be between pH 7-10. Similar study was carried out by Immanuel *et al*. 2006, where cellulolytic endoglucanase activity of *Cellulomonas* sp., *Bacillus* sp. and *Micrococcus* sp. was found to be optimal at pH 7 and temperature 40^{0}C.

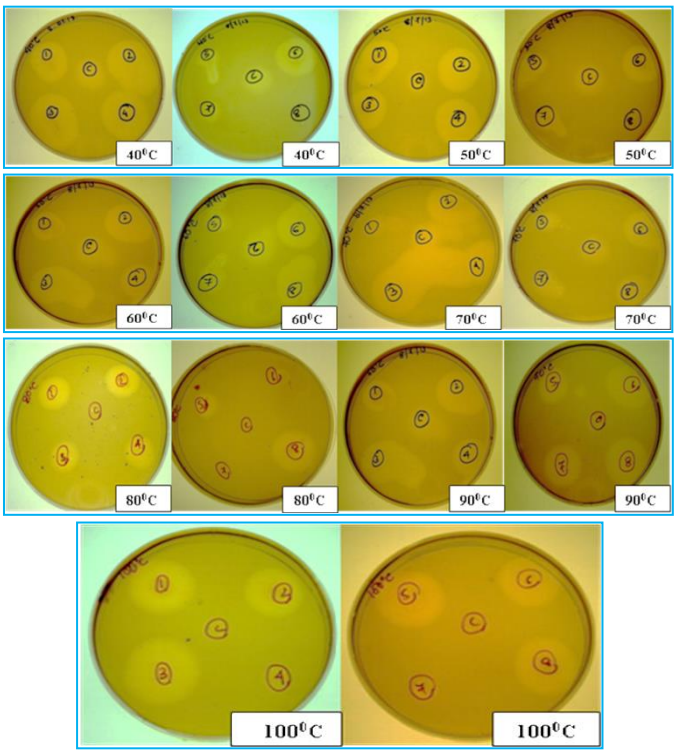
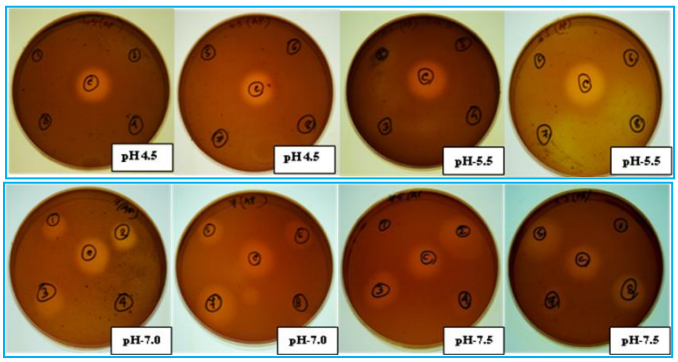


Fig-5 Stability of crude enzymes at different temperatures



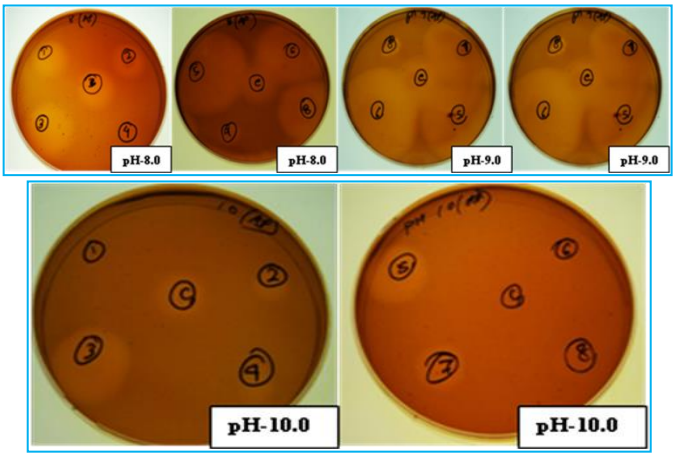


Fig-6 Stability of the crude enzymes at different pH

Stenotrophomonas maltophilia (0.43 IU/ml) showed the highest cellulase activity followed by *Bacillus cereus* (0.39 IU/ml) and *Bacillus thuringiensis* (0.3 IU/ml [Fig-7]. Enzyme concentration was determined by the method described by Bradford (1976). Cellulase activity was determined by DNS method [25] and *Bacillus cereus* (13.77 µg/ml) produced the highest amount of enzyme followed by and *Bacillus sp* (13.44 µg/ml). Shankar and his co-workers (2011) studied cellulase production by *Bacillus pumilus* EWBCM1 and found the maximum cellulase production was obtained at 37^{0}C temperature (0.2740±0.006 IU/ml), followed by this, at 40^{0}C temperature (0.2407 ± 0.006 IU/ml). On the other hand, the minimum amount of cellulase production was observed at temperature 60^{0}C (0.0296 ± 0.006 IU/ml). Similar works were also done by Vijayaraghavan and his co-workers (2012), they studied characterization of cellulase enzyme from *Bacillus sp*. where the enzyme activity was 12.5, 18, 16, 7 and 1.5 U/mg proteins at 30, 40, 60, 70 and 80^{0}C, respectively.

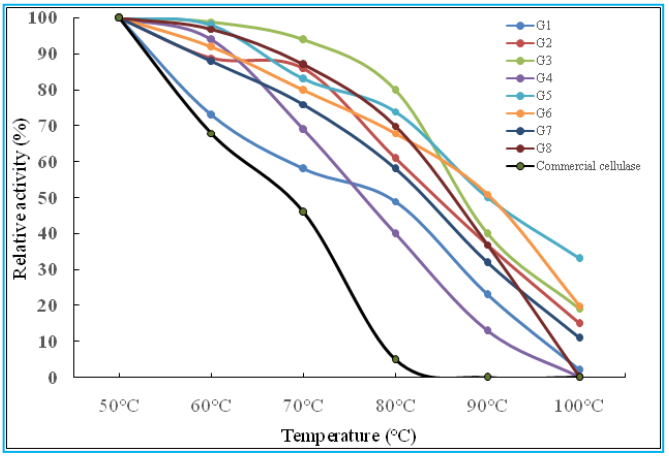


Fig-7 Relative activity (%) of enzymes at different temperatures

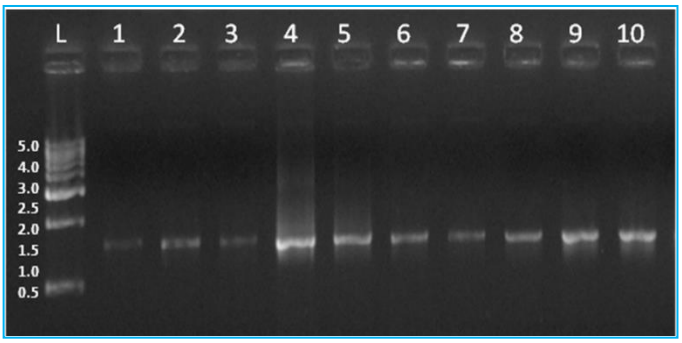


Fig-8 PCR Amplification of 16SrRNA
(L=Ladder-1 kb (GeNei), Lane no. 1=G1, Lane no. 2=G2, Lane no. 3=G3, Lane no. 4=G4, Lane no. 5=G5, Lane no. 6=G6, Lane no. 7=G7, Lane no. 8=G8, Lane no. 9= G9, Lane no. 10=G10)

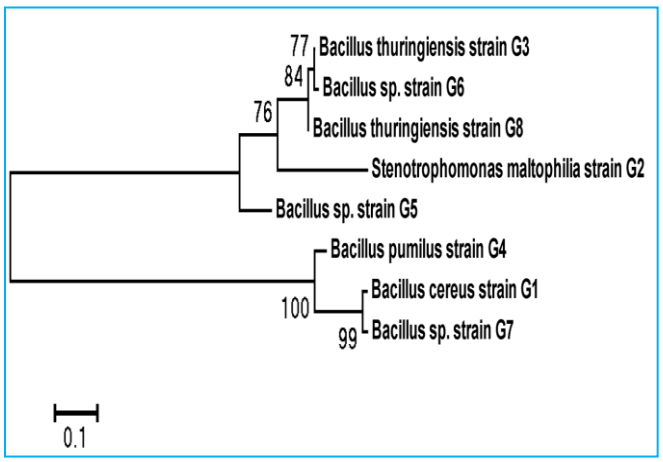


Fig-9 Neighbour-joining Tree of the identified strains

Conclusion

To summarize, we isolated eight nos. of isolates (G1, G2, G3, G4, G5, G6, G7 and G8) based on their ability to degrade cellulose. The isolates showed sigmoidal growth at 35^{0}C, which are characteristics of bacterial growth. The growth curve analysis of all isolates at 45^{0}C revealed that the isolate G2 has the shortest lag phase.).PCR amplification of the genomic DNA of the isolates showed an amplification product of ~1500bp fragment using 16S rDNA universal primers (forward US16F and reverse US16R primers). The blast results showed that the bacterial isolates have a high degree of sequence similarity with *Bacillus cereus, Bacillus pumilus, Bacillus sp.* and *Bacillus thuringiensis.* The crude enzymes were stable even at temperature upto 100^{0}C whereas the commercial cellulase was stable only upto 60^{0}C only. Isolated crude enzymes did not show any activity at acidic pH but were active at alkaline pH (up to pH 10) whereas commercial cellulase enzyme showed activity for the whole pH range (pH 2-10). *Stenotrophomonas maltophilia* (0.43 IU/ml) showed the highest cellulase activity followed by *Bacillus cereus* (0.39 IU/ml) and *Bacillus thuringiensis* (0.3 IU/ml) whereas *Bacillus cereus* (13.77 µg/ml) produced the highest amount of enzyme followed by and *Bacillus* sp (13.44µg/ml).

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