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Bioprospecting of Cellulase Producing Extremophilic Bacterial Isolates from India

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SV and SRP designed the experiment. Author MS carried out sample collection and screening of cellulase producing bacteria. Author SV carried out DNA Isolation, phylogenetic analysis, and wrote the manuscript. Author VG helped in Interpretation of results. Author SRP corrected manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: To isolate and identify the potential extremophilic cellulase producing strain viz., psychrophiles, halophiles, thermophiles and to compare the Cellulase activity from samples collected from different geographical regions of India.
Place and Duration of Study: Bharathiar University, Department of Biotechnology, Molecular Microbiology Lab, Coimbatore, Tamilnadu, India, between January to April 2011.
Methodology: Cellulase-producing extremophilic bacteria viz., psychrophiles, halophiles, and thermophiles have been isolated from soil samples. According to morphology and pigmentation, 138 distinct bacteria were isolated and screened for cellulase activity by Gram's iodine–carboxymethylcellulose plate (CMC) assay. On the basis of the cellulase activity, six potent cellulase-producing isolates from each cluster viz., P14, P36, H6, H13, T2 and T3 were selected for 16S rRNA gene based identification. The strains were optimized for maximum cellulase activity at various temperature and pH range.
Results: The phylogenetic relationship revealed that P14 and P36 psychrophilic isolates possessed maximum identity with *Bacillus simplex* (100%) and *Arthrobactercitreus* (99%), with a cellulase activity of 14.10± 1.73 and 18.27± 0.71 UmL^{-1} respectively. Likewise,

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among halophiles, H6 and H13 were identified as *Bacillus subtilis* and *Bacillus endophyticus* (99%), with a cellulase activity of 14.87 ± 0.55 and 16.83 ± 0.44 U mL^{-1}, correspondingly. In thermophiles, T2 and T3 showed close proximity with *Bacillus amyloliquefaciens* and *Bacillus megaterium* (99%), with a cellulase activity of 21.53 ± 1.30 and 19.93 ± 0.38 U mL^{-1} respectively.
Conclusion: In the present study, the thermophilic isolates showed promising Cellulase activity compared to psychrophiles and halophiles.

Keywords: Cellulase; CMC plate assay; DNS assay; extremophiles; 16S rRNA gene.

1. INTRODUCTION

In recent times, extremophiles have received much attention by microbiologist to understand the metabolic properties of the cell and to study evolutionary relationships. Their products like extremophilic enzymes, proteins, antibiotics, and compatible solutes have vast industrial applications. Most of the organisms are isolated from sea water, river streams, glaciers, snowfields, volcanoes, and deep hydrothermal vents.

The extremophiles are well-adapted organisms, which grow optimally at deep-sea extreme conditions like high pressure, high salt, high or low temperature, pH etc. The enzymes thus obtained from extremophiles will have extreme stability and activity, which can be used as a biocatalyst for special applications. Furthermore, extremophiles are the potential source for novel enzymes with unique activity ranges [1]. Some of them have vital application in the bioconversion of renewable cellulosic biomass to sugars, especially for the production of ethanol by a fermentation process in large-scale industries [2]. Cellulases obtained from extremophiles have a broad range of industrial applications. Psychrophilic cellulases in particular, are very active at low temperature and are stable under alkaline conditions, which can be used as laundry detergents in textile industries, and animal feed [3,4]. Limited numbers of reports are available with respect to halophilic cellulases, which will have high commercial industrial importance [5,6]. In the case of thermophilic cellulases, they have high thermal stability with good activity at higher temperature, which finds application in biopolishing of cotton, color extraction of juices, color brightening, and saccharification of agricultural and industrial wastes [7]. Due to the above economic significance of overall extremophilic cellulases, the present study was designed to isolate and identify the potential extremophilic strain viz., psychrophiles, halophiles, thermophiles and to compare the enzymatic activity from the samples collected from different geographical regions of India.

2. MATERIALS AND METHODS

2.1 Sampling Site and Method

Soil and water samples were collected from different geographical locations of India for isolating broad range of extremophiles viz., Kashmir (33º 35' N, 76º 08' E), Assam (26º 19' N, 91º 00' E), Rajasthan (12º 47' N, 80º 16' E), and Chennai (12º 47' N, 80º 16' E) (Table 1). The mean air temperature at the time of sample collection were 6°C, 13°C, 48°C,36°C in those respective places. Plant debris if any, were removed from the top surface of the soil and employing sterile spatula, the samples were collected in sterile 50 mL tubes. Subsequently, the collected samples were placed in cooler and immediately transferred to

Bharathiar University, Coimbatore, Tamil Nadu, India. After reaching the laboratory, the samples were processed immediately to isolate distinct bacterial isolates.

2.2 Isolation and Cultivation of Extremophiles

One gram of the slurry was suspended in 9 mL of 0.9% (w/v) NaCl solution and shaken for 2 hour at 15ºC. Subsequently, 100 µL water sample was plated on nutrient agar medium supplemented with tryptone (1.0% w/v), yeast extract (0.5% w/v), NaCl (1.0% w/v), and agar (2.0% w/v) (HiMedia, India) and then incubated at 4ºC for 15 days [8] to isolate psychrophiles. The cultivation medium was additionally supplemented with sodium chloride (13%, w/v) and incubated at 37ºC for 4 days to enrich halophiles. Thermophilic bacteria were isolated by incubating plates at 60ºC for 4 days. Based on colony morphology and pigmentation, distinct bacterial strains were selected and subsequently streaked for 2–3 times in the medium to obtain pure cultures [6].

2.3 Screening of Cellulase Producers

The isolated strains were plated on carboxymethyl cellulose(CMC) plates (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% CMC sodium salt, 0.02% peptone, and 1.7% agar) and incubated with respect to extremophilic culture conditions. After incubation, when the plates were flooded with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 mL distilled water), the zone of clearance around the bacterial colonies represented the positive cellulase producers [3].

2.4 Preparation of Crude Cellulase

Fifty milliliters of grown culture (0.5 OD_{600}) was centrifuged at 10000 ×g for 10 min at 4ºC. In order to obtain a cell-free supernatant, the step was repeated twice, and the supernatant was subjected to further studies. 10 µL of supernatant was added in the well-coated CMC plates and incubated according to their culture condition viz., psychrophiles, 4ºC; thermophiles, 60ºC; and halophiles, 37ºC. The zone of clearance was calculated for all the isolates by measuring the diameter of the zone [9]. Crude cellulase activity was measured by the DNS (3,5-dinitrosalicylic acid) assay [10,11], through the determination of the amount of reducing sugars liberated from CMC solubilized in the 50 mMTris-HCl buffer, pH 7.0 [12]. Optical density was measured at 540 nm in Spectrophotometer (PerkinElmer Lambda 35, USA).

2.5 Phenotypic Characterization and Optimization of pH and Temperature for Cellulase Producers

The strains showing maximum enzyme activity in each group viz., psychrophiles, thermophiles, halophiles were subjected to phenotypic characterization and were optimized for maximum production cellulase enzyme at various temperature (7ºC - 60ºC) and pH range (4.0-11.0) using suitable buffers, 50 mM sodium acetate (pH 4.0 - 6.0), 50 mM sodium phosphate (pH 7.0 and 8.0), 50 mM glycine-NaOH buffer (pH 9.0-11.0), respectively [10]. However, the enzyme activity was not performed at extremities were there was no considerable bacterial growth at these temperatures.

2.6 16S rRNA Gene Amplification, Sequencing, Phylogenetic Analysis

The potent cellulase producers from each group were identified through 16S rRNA gene sequencing. DNA was extracted [13]; 16S rRNA gene was amplified using universal primers fD (5'-GAG TTT GAT CCT GGC TCA G-3') and rD (5'-ACG GCT ACC TTG TTA CGA CTT-3') [14]. The reaction mixture (20 µL) consisted of 0.5U of Taq polymerase (Chromous Biotech, India), 2 µL of 10x buffer, 1.5 mM of $MgCl_2$, 0.4mM of dNTP mix, 10 µM of each primer, and 2 µL of template DNA (~20ng). The polymerase chain reaction (PCR) program was as follows: initial incubation at 94ºC for 5 min; 30 cycles followed by 94ºC for 1 min, 48ºC for 1 min, and 72ºC for 2 min, and then the final extension at 72ºC for 10 min. PCR amplicons were purified by agarose gel extraction kit (Qiagen). Then the amplicons were sequenced by the same set of universal primers [14]. The obtained sequences were subjected to BLAST analysis to identify the closely related species [15]. Those related 16S rRNA gene sequences were aligned by using CLUSTAL program [16] and phylogenetic tree was constructed by PHYLIP (Phylogeny Inference Package) version 3.69 [17] by using tree making algorithm, neighbor-joining (NJ) method. All 16S rRNA partial gene sequences were submitted to Genbank with accession no JX277544, JX277545, JX277546, JX277547, JX393831, and JX393832.

3. RESULTS AND DISCUSSION

3.1 Bacterial Isolation and their Abundance

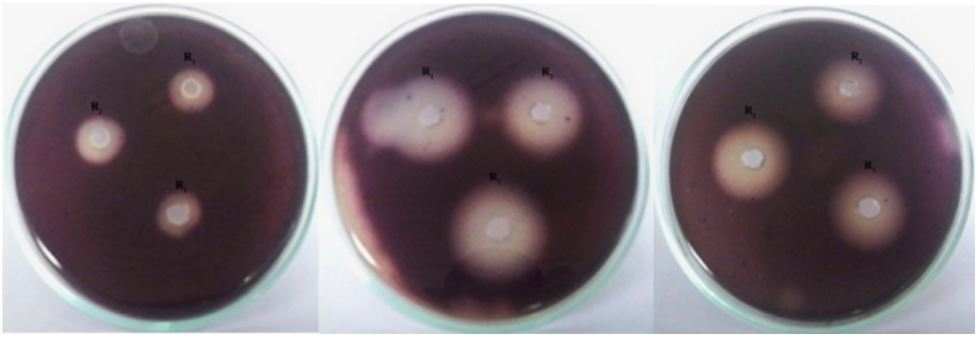
Initial screening of soil samples facilitated the isolation of 138 morphologically divergent bacterial colonies, which were used for further investigation (Table 1). Among them Gram positive bacteria was found to be predominant (68%). The total bacterial count in the soil samples varied from 9.1 x 10^4 to 14.2 x 10^4 cfu g^{-1} of soil. The nature of the soil despite being hard, soft, or sandy and pebble richness did not influence bacterial abundance. Among the total 138 strains, 60 were psychrophiles of which 23 were pigmented. The pigmentation of bacteria plays a significant role in survival of those bacteria in cold condition [18]. About 55 isolates belonged to thermophiles, which were isolated at 60ºC from Rajasthan. The majority of the isolates were spore-forming bacteria (data not shown), which are adapted to resist in high or extreme temperature [19]. The total count of thermophilic bacteria isolated from soil samples of Bikaner and Phalodi regions of Rajasthan were 11.7 × 10^4 and 9.8 × 10^4cfu g^{-1}, respectively. Compared to psychrophiles and thermophiles, halophiles showed a low bacterial count (Table 1). The 23 strains, which were categorized as halophiles, could grow when supplemented with 13% (w/v) NaCl. The total bacterial count of the halophiles was 9.1 × 10^4cfu g^{-1}.

3.2 Cellulase Activity among Bacterial Isolates

Among 138 extremophiles, 22 strains have shown cellulolytic activity distributed viz., 9, 6, and 7, respectively, under psychrophiles, thermophiles, and halophiles. A clear distinct hydrolyzed zone was observed in the CMC plates of psychrophilic strains namely P13, P14, P24, P28, P31, P33, P36, P42, and P54. Among thermophiles, six isolates showed cellulase activity viz., T2, T3, T4, T5, T6, and T12. Similarly, cellulolytic property was observed in halophilic strains namely H1, H4, H5, H6, H7, H10, and H13. Among the isolated strains, while 30.43% of the halophilic strains showed maximum zone of clearance, psychrophiles and thermophiles showed only 30% and 22.2% respectively (Table. 1).All the 22 strains showing cellulase activity have been taken for further investigation.

Among the psychrophilic strains, P14 and P36 showed the maximum zone diameter of 12.57± 0.52 and 14.10± 1.73 mm, respectively. Further, the crude cellulase activity was found to be 14.10± 1.73 and 18.27± 0.71 U mL^{-1} in the cell-free culture supernatant. Thermophilic isolates grew vigorously and developed clear zone around the isolates on CMC agar plates within 20 hours.

The maximum cellulolytic activity of the thermophilic strains T2 and T3 on CMC plates was found to be 18.27± 0.71 and 18.83± 0.49 mm correspondingly. Similarly, cellulase activity viz., 21.53 ± 1.30 and 19.93 ± 0.38 U mL^{-1} was exhibited by the crude enzyme preparations from T2 and T3, respectively. In halophilic category, the strains H6 and H13 showed the maximum zone of clearance on CMC plate's viz., 15.20 ± 0.17 and 14.87 ± 0.55 mm, respectively. Crude enzyme preparations of both the strains showed cellulolytic activity as 14.87 ± 0.55 and 16.83 ± 0.44 U mL^{-1} in DNS assay (Figs. 1 and 2). Based on zone of clearance and the crude enzyme activity, P14 and P36 among psychrophiles, T2 and T3among thermophiles, and H6 and H13 among halophiles were identified as potent cellulase producers.



(a) (b) (c)

Fig. 1. Screening of cellulase producing extremophilic bacteria; (a) Psychrophilic strain;(b) Thermophilic strain; (c) Halophilic strain; R_1,R_2,R_3: in replicates

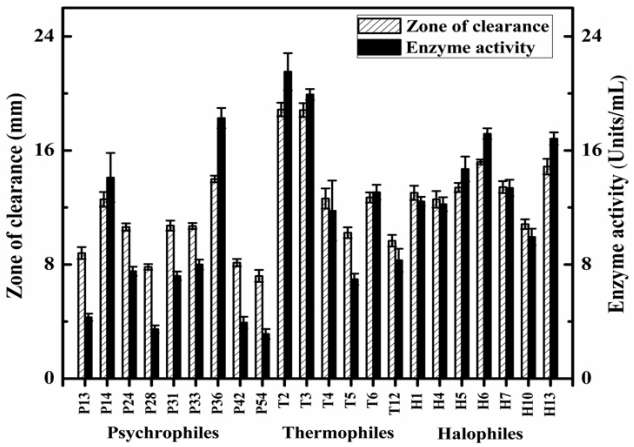


Fig. 2. Cellulase activity as assayed by plate assay and DNS method; the values are mean ± SD of three replicates

3.3 Molecular Identification of Cellulase Producers

The nearest phylogenetic neighbor of all six potent cellulase-producing extremophilic strains were identified following BLAST analysis of respective 16S rRNA gene sequences. According to the phylogenetic analysis, isolates P14, P36, T2, T3, H6, and H13 were identified as *Bacillus simplex* NBRC 15720^T (AB363738), *Arthrobactercitreus* DSM 20133^T (AM237348), *Bacillus amyloliquefaciens subsp. amyloliquefaciens* DSM 7^T (FN597644), *Bacillus megaterium* IAM 13418^T (D16273), *Bacillus subtilis subsp. Subtilis* NCIB 3610^T (ABQL01000001), and *Bacillus endophyticus* 2DTT (AF295302), respectively (Fig.3).

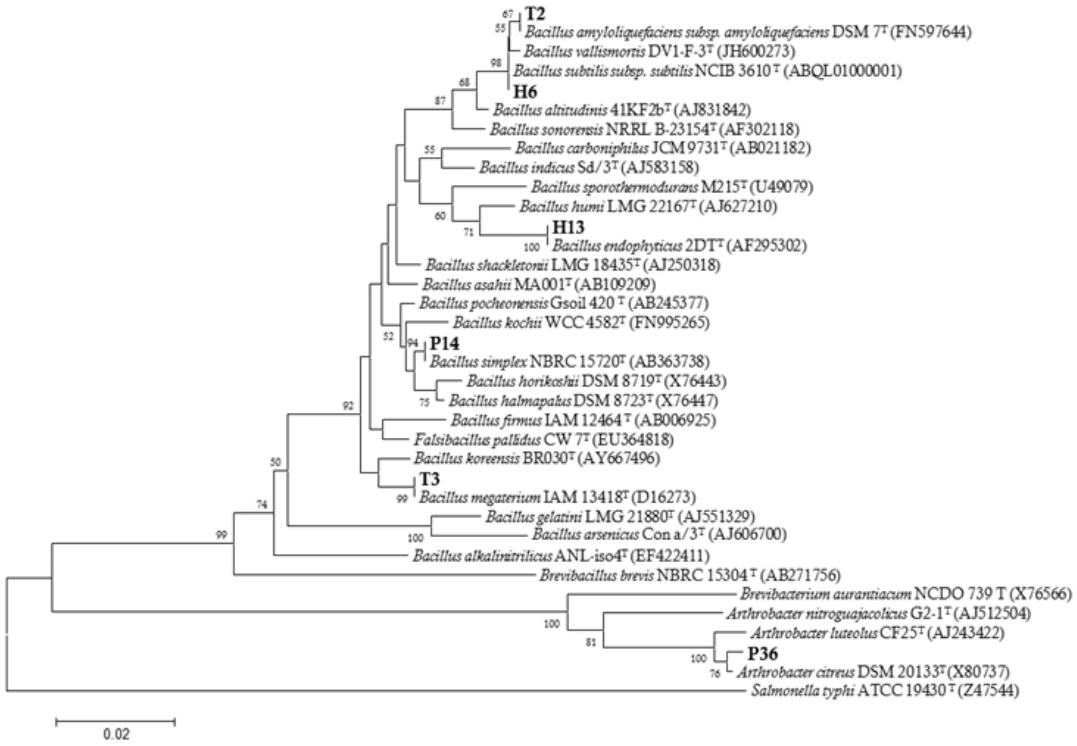


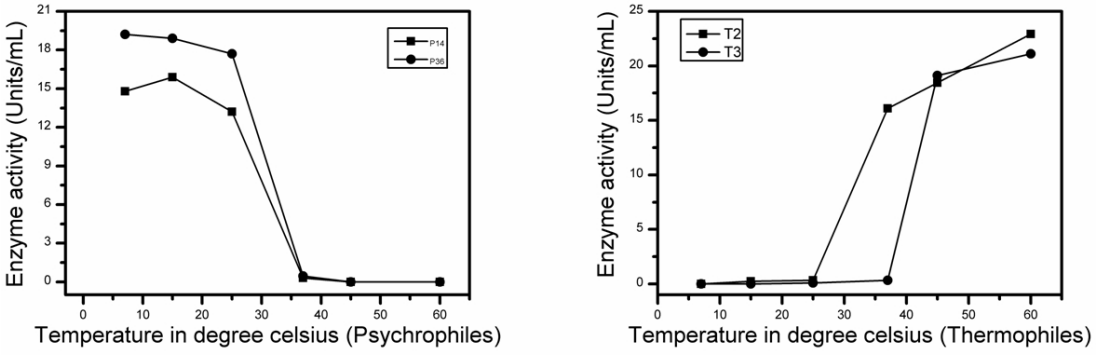
Fig. 3. Phylogenetic analysis of psychrophilic (P14, P36), thermophilic (T2, T3), halophilic isolates (H6, H13) based on 16S rRNA gene. Neighbour-joining method, *Salmonella typhi* was used as out group in the tree. GenBank accession numbers are given in parentheses. Numbers at the nodes were percentage bootstrap values based on 1000 resampled datasets, Only Bootstrap values >50% were shown

The strain P14 was identified as *Bacillus simplex* is a Gram positive bacterium, which showed cellulolytic activity of about 14.10± 1.73 U mL^{-1}. The strain was able to grow well between 4ºC to 25ºC, which has an optimum growth at 15ºC and it can tolerate salt concentration up to 5%, pH range of 7-12 (Table. 2) Further the strain was found to have maximum cellulase activity at 15º C with pH of 9.0 (Figs. 4a and 5a). To best of our knowledge, there were no previous reports regarding the cellulolytic activity of *B. simplex*. However, relative species of *Bacillus* namely *B. licheniformis, B. flexus, B. subtilis, B. megaterium* have shown pronounced cellulase and other hydrolytic enzyme activity [20-23].

The second isolate P36 showed close proximity with *A. citreus,* which are Gram positive, aerobic, and motile bacteria [24]. The present study showed cellulolytic activity of about 18.27± 0.71 U mL^{-1} for the isolate P36. The strain was having optimum activity at 4ºC and a pH range of 7. Further, it showed up to 7% salt tolerance level (Table 2) which might be one of the potent strains to be used in textile industries as detergents due to their activity at low temperatures and high salt tolerance [25]. A study was performed by Immobilized cells of *A. citreus* were shown to degrade corrosive organic solvents like phenol which designates the prominence of these bacteria [26].

Both of the isolates T2 and T3 showed maximum activity at the temperature of 60ºC with the pH range of 7-9 (Figs. 4b and 5b). The isolate T2 was found to have similarity with *B. amyloliquefaciens.* Earlier, the cellulolytic activity of the organism was studied from rice hull, which is one of major cellulosic waste materials found on earth. The enzymes from the strain showed greater thermo stability ranges from 40 to 80ºC [27]. The other isolate T3 was found to have activity of 19.93 ± 0.38 U mL^{-1}, which was identified as *Bacillus megaterium* through 16S rRNA gene sequencing. The isolate is a rod-shaped bacterium often found in chains, which was found to survive in extreme conditions such as desert environments [28]. From the Earlier reports, it was found that *B. Megaterium* isolated from the soil sample was found to have Chitosanase activities which is belonged to β-1,4- linked D-glucosamine residues [29] .

Isolate H6 showed close proximity with *Bacillus subtilis,* which is a Gram positive; obligate aerobe and spore-forming bacteria. The cellulolytic activity of this bacterium was studied earlier by utilizing the coir waste along with saw dust as a substrate, which resulted in an enormous amount of cellulase via solid-state fermentation [30]. The isolate H13 showed close proximity with *Bacillus endophyticus,* was a newly identified novel species isolated from the inner tissue of cotton plants *Gossypium* sp. [31]. Both of the isolate have optimum cellulase activity at the temperature and pH range of 25ºC - 37ºC and 7-9 respectively (Figs. 4c and 5c). To the best of our knowledge, there is no previous report regarding the cellulase activity of *Bacillus endophyticus.* Hence the present organism could be the first strain of *Bacillus endophyticus* to exhibit cellulase activity.



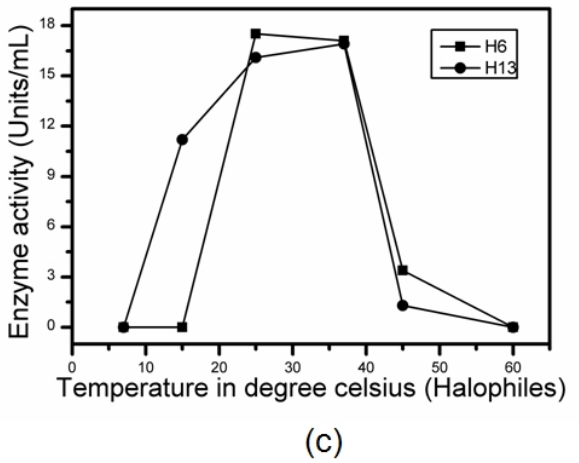


Fig. 4. Optimization at various temperature conditions (7ºC to 60ºC) for the maximum activity of cellulase from potent isolates

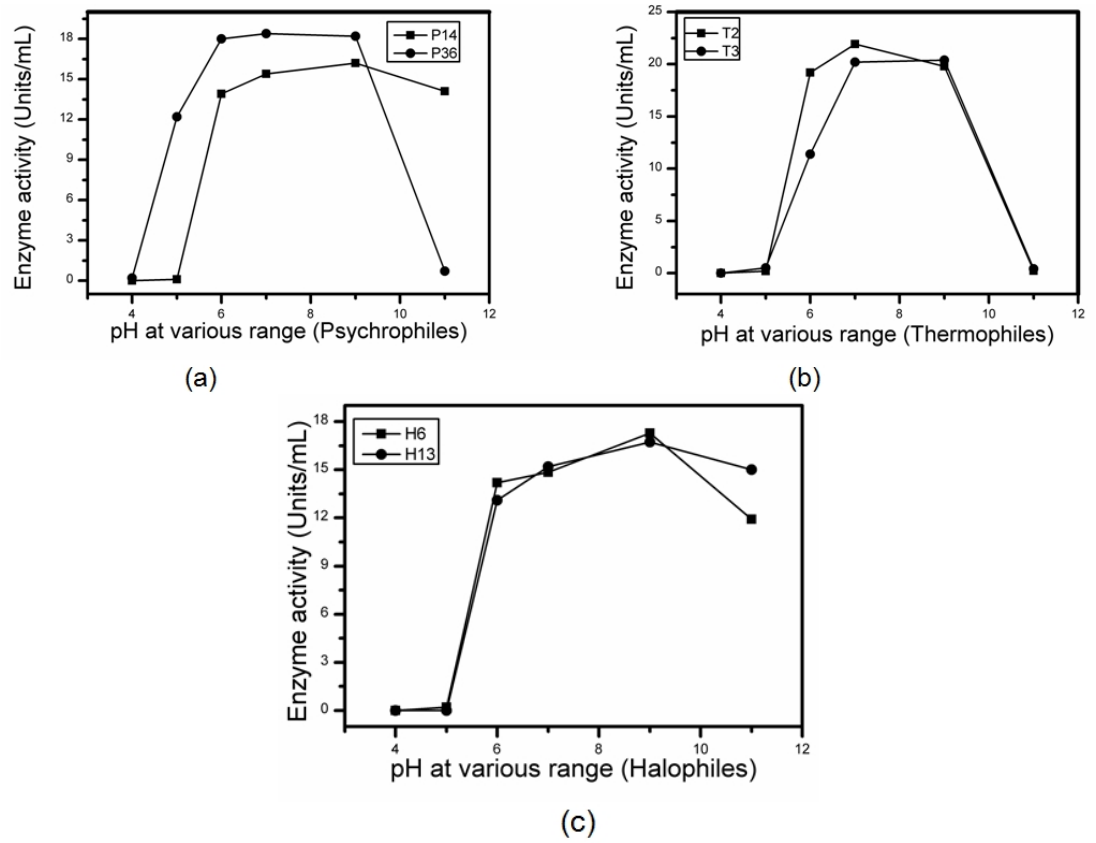


Fig. 5. Optimization at various pH (4.0 to 11) for the maximum activity of cellulase from potent isolates

Table 1. Bacterial abundance (cfu g^{-1}) and screening of cellulase producers from soil samples of different geographical locations in India

Extremophiles	Sampling site	GPS position	Sample nature	Incubation temperature	Cfu g^{-1}	Number of isolates	Strains	Cellulase producing strains	Percentage (%)
Psychrophiles	Bharanzar, Kashmir,	33º 35'N 76º 08'E	Brownish, Very soft, sparse vegetation	4º C	10.8 x 10^4	28	P1 to P28	P13, P14, P24, P28	14.28
	Monapur, Barpeta	26º 19'N 91º 00'E	Red soil, sticky, dry vegetation	4º C	14.2 x 10^4	32	P29 to P60	P31, P33, P36, P42, P54	15.62
Thermophiles	Bikaner, Rajasthan,	12º 47'N 80º 16'E	Sand Pebbles	60º C	11.7 x 10^4	27	T1 to T27	T2, T3, T4,T5,T6,T12	22.22
	Phalodi, Rajasthan,	26º 43'N 73º 56'E	Sand soil, granular	60º C	9.8 x 10^4	28	T28 to T55	Nil	0
Halophiles	Muttukaadu, Chennai,	12º 47'N 80º 16'E	Soil sediment from the coastal area, moisture, bluish brown colour.	37º C	9.1 x 10^4	23	H1 to H23	H1, H4, H5, H6, H7, H10, H13	30.43

Table 2. Phenotypic characteristics of the potent cellulase strain at different temperature, salt concentration and pH range

Strain name	Temperature							Salt concentration					pH range				
	4ºC	10ºC	15ºC	25ºC	37ºC	45ºC	60ºC	2%	5%	7%	12%	13%	3	5	7	10	12
P14	++	++	++	+	-	-	-	++	++	-	-	-	-	-	++	++	+
P36	++	++	++	++	-	-	-	++	++	+	-	-	-	+	++	++	-
T2	-	-	-	-	+	++	++	++	-	-	-	-	-	-	++	+	-
T3	-	-	-	-	-	++	++	++	+	-	-	-	-	-	++	++	-
H6	-	-	-	++	++	-	-	++	++	++	++	++	-	-	++	++	-
H13	-	-	-	+	++	-	-	++	++	++	++	++	-	-	++	++	+

++: Strongly positive; +: Weakly positive; -: Negative

Bacillus species are known to produce cellulases, some of which are exploited in the industries. Effectiveness of the cellulases produced by the *Bacillus* species varies from species to species. Cellulase enzymes were extracted from several halophilic microorganisms, which had many industrial applications [32]. Recently, 99 extremely halotolerant *Bacillus* strains, most of them able to grow at 20–25% (w/v) salt, were isolated from hypersaline environments. The enzymes produced from these isolates will have great applications in biotechnological industries [33]. Extensive research works were also carried out in mapping the genome of the species, which could lead to the identification of prominent genes responsible for cellulase production in the microorganisms. Similar studies were reported in various *Bacillus* species [34,35]. Thus results are in concurrence with the previous reports on cellulases produced from *Bacillus* genera.

Further all the isolates belonged to facultative alkaliphiles which were able to grow at a broad pH range from 7 to 12 (Table 2). Earlier reports also confirmed that the *Bacillus* species were generally able to withstand high pH conditions [36,37]. The cellulase produced from these bacteria is especially used as detergent additives for laundry and to dishwashing detergents [38]. Ito and coworkers [39, 40] then isolated the alkaliphilic *Bacillus* sp. from the soil and succeeded in producing an alkaline cellulase as a laundry detergent additive in an industrial scale.

4. CONCLUSION

In this study, we have isolated 22 cellulase-producing organisms that are capable of withstanding extreme conditions such as high salt, high and low temperatures. The cellulase activity was estimated through zone of clearance and DNS method which yielded six potential isolates. Among them, Isolates P14(*Bacillus simplex*) and H13 (*Bacillus endophyticus)* showed novel cellulase activity. Thus in the present study, we have found that the Thermophilic isolates showed promising activity compared to psychrophiles and halophiles respectively. Such results could cater the industrial needs for identifying promising cellulase producers. Eventually our strains can be further studied towards industrial applications pertaining to scale-up and enzyme characterization.

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COMPETING INTERESTS

Authors have declared that no competing interests exist

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