Isolation, characterization and identification of cellulase (Endo-β-1,4-glucanase) producing bacteria from diverse locations

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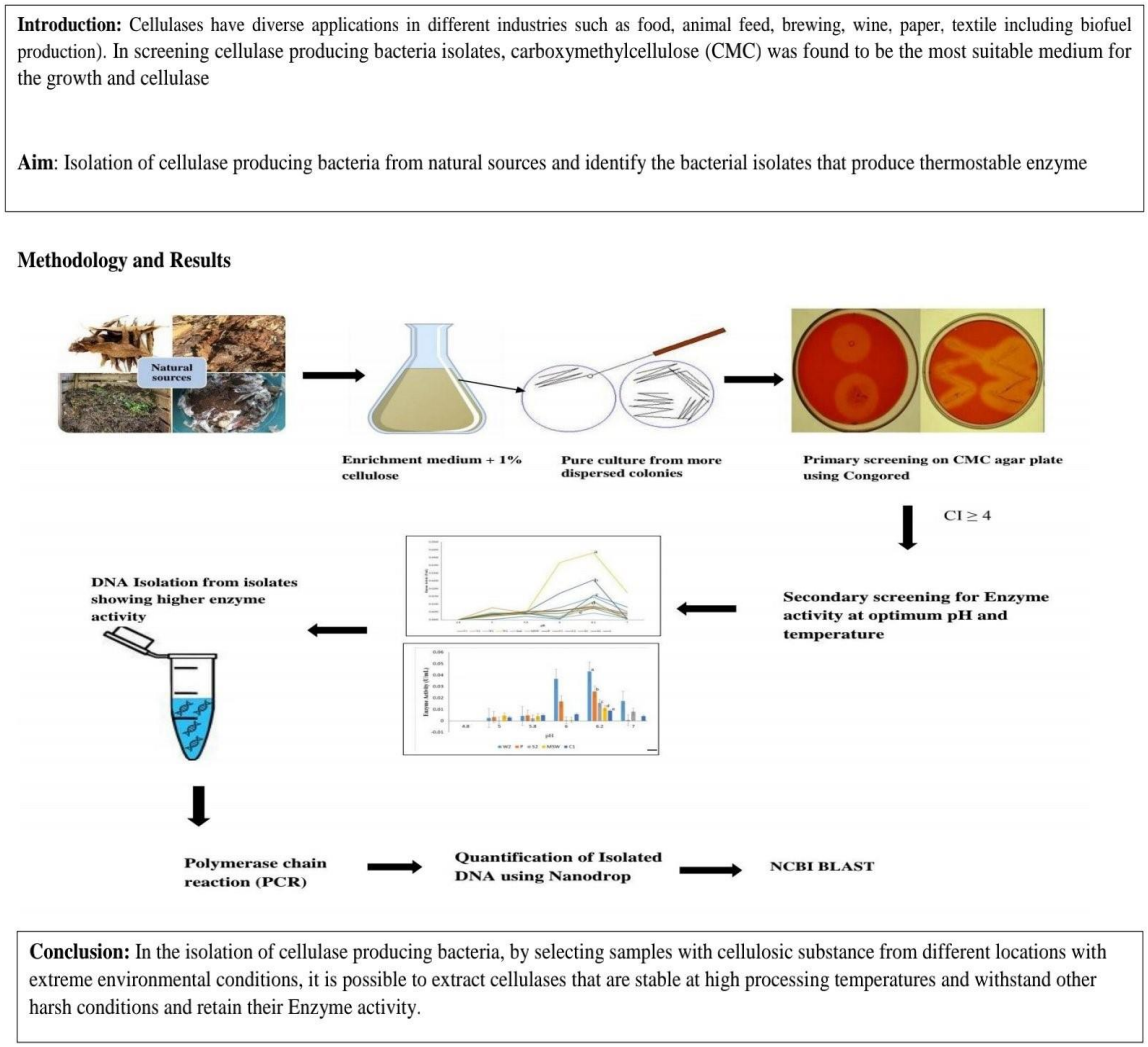
Graphical abstract



Abstract

The objective of the study was to isolate cellulase producing bacteria from natural sources and identify the bacterial isolates that produce thermostable enzyme. Bacteria were isolated from samples collected from different locations like mango leaf litter, termite gut, composting yard (municipal solid waste, compost, and soil), soil from decaying paper waste, decaying wood, and saline coastal belt soil by enrichment in broth containing 1% cellulose and subsequent culturing on plates and the purified cultures stored in slants of the solidified agar medium of the same composition as enrichment medium. Primary screening of the isolates for cellulase (Endo-β-1,4-glucanase) production was done by growing them on Carboxy Methyl Cellulose (CMC) agar medium and screening using indicator dye Congo red. Secondary screening of the positive isolates showing cellulolytic Index > 4 was done by crude cellulase enzyme extraction and assay using 1% CMC in 1N citrate buffer (pH 5.0) at different pH values (4.8-7.0) and temperatures (35- 70℃). Cellulase activity (EA) in U/mL was calculated based on the absorbance change per min. which depends on the amount of reducing sugar produced by the enzyme catalysed conversion of CMC to reducing sugar. Isolates were characterized morphologically and biochemically and six isolates (Gram -ve: T1, MSW, C1, S2 and Gram +ve: W2, P) having significantly higher EA were selected for DNA extraction. DNA was quantified and the DNA of four isolates (MSW, S2, W2, P) were selected for sequence analysis. The 16S rDNA sequencing was carried out through PCR amplification and Blast using NCBI blast similarity search tool resulted in the identification of MSW, S2, W2 as *E.coli, Sulfitobacter pontiacus, Bacillus subtilis* respectively and P as *Bacillus cereus* and *Rhodococcus erythropolis* strain e1. Isolates showed significantly higher cellulase activity (EA) at pH 6.2 and at varying temperatures. Bacterial isolates P and C2 showed significantly higher activity at 40℃, MSW at 60℃ and others at 50℃ (EA ranging from 0.0054 – 0.0604 U/mL). Isolates W2, P, S2 had EA ranging from 0.006 - 0.03 U/mL at 70℃. This study showed that the cellulase producers could be grown at different temperatures (40 - 70℃) on cellulose based substrate. In this study the substrate specificity indicates that the crude enzyme is an endo- β-1,4-glucanase that plays a prominent role in initiating and sustaining the hydrolytic process and randomly cleaves cellulose into glucose and olygomeric polysaccharides. Hence by selecting samples with cellulosic substance from different locations with extreme environmental conditions like high temperature, high salinity it is possible to isolate cellulolytic bacteria producing cellulase enzymes that are stable at higher processing temperatures and other harsh conditions which is of importance in industrial applications.

Keywords*: Endo-β-1,4-glucanase, Cellulolytic index, Cellulase activity, Thermostable*

1. Introduction

Cellulose containing wastes are cheap carbon sources and cellulose is a homopolymer of D-glucose units linked by β-1,4-bonds. Cellulase enzyme can effectively hydrolyse it into glucose units by the synergistic action of the enzymes endo-β-1,4-glucanase, cellobiohydrolase and β-D-glucosidase (Perez et al.,2002). Cellulases have diverse applications in different industries such as food, animal feed, brewing, wine, paper, textile including biofuel production. Cellulase enzyme that is available commercially is very expensive. The cost of cellulase enzyme affects the production cost and the economic viability of cellulose-based industries (Maravi et al.,2020). Cellulases are mostly secreted by fungi and comparatively limited studies have been carried out on cellulase producing bacteria. However production of cellulase by bacteria has been gaining attention because of their lesser generation time, adaptation to extreme environment, easy to modify their genes using genetic engineering, and faster secretion of extracellular enzymes in large quantities (Maki et al., 2009 Lynd et al., 2002). This could be considered as a merit point when it comes to cellulase. Few bacterial isolates *Pseudomonas fluorescens, Bacillus subtilis, E.coli* and *Serratia marscens* can produce large amount of enzyme (0.2 – 1 U/mL) that are capable of complete hydrolysis of cellulose into glucose for their normal growth and development (Sethi et al., 2013). In screening cellulase producing bacteria isolates, carboxymethylcellulose (CMC) was found to be the most suitable medium for the growth and cellulase production (Farjana et al.,2018). Among the three soluble extracellular enzymes (endo-β-1,4-glucanase, cellobiohydrolase and β-D-glucosidase) in the cellulase system, endoglucanase plays a prominent role in the process of cellulose hydrolysis because it hydrolyzes the glycosidic bonds randomly and shorten the cellulose chains in the initial stage of cellulose breakdown and cleaves cellulose into glucose and olygomeric polysaccharides (Yennamalli et al., 2011). The objective of the study was to isolate cellulase producing bacteria from diverse natural habitats such as mango leaf litter, termite gut, composting yard (municipal solid waste, compost, and soil), soil from decaying paper waste, decaying wood, and saline coastal belt soil and to characterize the bacteria and the purified cellulase enzyme.

2. Materials and methods

2.1 Collection of samples

Samples were collected from multiple sources to represent a range of environmental conditions. Specifically, mango leaf litter, termite gut, composting yard (municipal solid waste, compost, and soil), soil from decaying paper waste, decaying wood, and saline coastal belt soil were collected. After collection samples were placed on polythene bags and kept in a refrigerator at 4°C.

2.2 Isolation of cellulase producing bacteria

Bacteria were isolated from the collected samples using an enrichment technique using a medium containing 1 % cellulose powder (Saini et al., 2017). Each substrate (1g) was inoculated into 50 mL of enrichment medium with pH 7 and incubated at room temperature at 120 rpm for 4 days. Tenfold dilution of these enriched samples were made with saline and used to inoculate solidified agar medium of the same composition as enrichment medium and incubated at 37℃ for 48 hours. Colonies obtained were grown individually in Carboxymethylcellulose (CMC) broth for 24 hours at room temperature at 100 rpm and these bacterial broths were used to streak the CMC agar plates which were later incubated at 37℃ for 48 hrs. Much dispersed colonies were the pure bacterial colonies which were stored in slants at 4 ℃ and used for further studies.

2.3 Primary screening of carboxy methyl cellulase producers

Primary or qualitative screening of different isolates was done using indicator dye Congored (Teather and Wood,1982) for the production of carboxymethylcellulase (CMCase) enzyme specifically endo-β-1,4-D-glucanase. The different bacterial isolates were inoculated on to the surface of CMC agar medium by streaking and the plates were incubated at 37 ℃ until substantial growth was observed. Plates were removed from incubator and the carboxymethylcellulolytic potential was tested by flooding the plates with 0.1 % Congo red dye solution for ½ - 1 hour followed by destaining with 1M NaCl solution 2-3 times and plates were kept for 20-30 mins during which clear zones around colonies were visualized. Bacterial isolates which formed clear zones (positive isolates) were chosen to determine the cellulose hydrolysis capacity or cellulolytic index (CI) (Saptarini et al., 2014).

$$\mathrm{CI} = \frac{\text{Diameter of clear zone}}{\text{Diameter of colony}} \qquad \text{---------- (1)}$$

2.4 Activation and secondary screening of CM Case producers

All positive isolates that expressed CI value greater than 4 were screened quantitatively using secondary screening medium (pH=7.0) containing mineral salts with urea and peptone supplemented with CMC as ‘C’ source. Cultures in slants were activated by inoculating to nutrient broth and incubated overnight at 37℃ and 1% of the activated cultures were inoculated to the screening medium in flasks separately and incubated at room temperature (30℃) and 180 rpm for 3 days. Enzymes were extracted by centrifuging the flasks at 8,000 rpm at room temperature for 10-15 mins. The clear supernatants were used as the source of crude enzyme and stored under refrigerated condition at 5℃ for further study.

2.5 Optimization of culture conditions for endo glucanase activity

Optimum pH and temperature were determined by measuring the endo glucanase activity in the enzymatic reaction using carboxymethylcellulose as substrate. This was done by incubating the enzyme-substrate mixture for 30 mins at different pH (4.0 -7.0) and temperature (35℃ - 70℃) by changing one variable at a time. The Endo-$\beta-1,4-$glucanase activity was measured by colorimetric technique (DiNitro Salicylic acid (DNS) method - Miller, 1959).

The method of determining endo glucanase activity is based on the amount of reducing sugar released by the action of endo glucanase (500 µL) on a specific substrate (500 µL). After incubation for 30 min. 1 mL DNS was added to the solution to stop the reaction and the treated samples were boiled for 10 min. and cooled to room temperature and the absorbance was measured at 540 nm using a UV-visible spectrophotometer (Shimadzu: UV mini 1240) and absorbance values of were used to calculate the enzyme unit activity.

$$\text{Enzyme Unit Activity} = \frac{\text{Con. of Std.glucose} \times \text{Absorban}ce(test-blank) \times \text{Dilution factor}}{\text{Absorbance of Std.} \times \text{Vol.of enzyme} \times \text{Incubation time}} \quad \text{----------(2)}$$

One unit of endo-β-1,4-glucanase activity was defined as the amount enzyme that could hydrolyse CMC and release 1 mg of glucose within 1 min. of reaction (Shamugapriya et al., 2012).

2.6 Identification of isolates

Based on the endoglucanase activity values, six bacterial isolates that showed significantly higher activity were subjected to morphological and biochemical characterization.

Genomic DNA from selected bacteria was extracted using the kit method (Promega DNA purification kit – A1120). Pellets of bacterial cells were obtained by centrifuging the overnight culture in a sterile eppendorf tube. at 8,000 rpm. Pellets obtained after discarding the supernatant were used for DNA isolation. Gram (+) ve pellets for DNA isolation were subjected to an additional step where pellets from overnight culture was suspended in 50 mM EDTA (p H 8.0) and lytic enzyme lysozyme(250 µl) was added and incubated at 37 ℃ for one hour. Then the tubes were centrifuged at 8,000 rpm for 2 min. and the supernatants were removed and the pellets were recovered. Isolated DNA were quantified using 'Nanodrop' (Nano drop 2000 spectrophotometer Thermo scientific). DNA samples of the bacterial isolates that had concentrations above 100 ng/µL and the absorbance ratio 260/280 between 1.8 and 2.2 were subjected for PCR.

DNA samples of four isolates were amplified through PCR with targeting fragment size 1400 bp using 16S rDNA primers (27F,5-AGAGTTTGATCATGGCTCAG-3 and 1492R, 5-

TACGGTTACCTTGTTACGACTT-3) in an OptiMax 96 thermal cycler under following conditions: 94 °C for 5 min, 3 cycles at 94 °C for 45 s, 57 °C for 30 s, 72 °C for 120 s; 3 cycles at 94 °C for 45 s,56 °C for 30 s, 72 °C for 120 s; 3 cycles at 94 °C for 45 s, 55 °C for 30 s, 72 °C for 120 s; 26 cycles at 94 °C for 45 s, 53 °C for 30 s, 72 °C for 120 s; and a final step at 72 °C for 5 min.1.5% of Agarose gel was used to visualize amplified amplicon of 16S rDNA fragments. Sanger sequencing of the PCR fragment was done at Molecular Biology Facility (MBSU, Department of Biological Sciences, University of Alberta, AB, Canada. Sequence contique was prepared aligning forward and reverse fragments and contique sequence was compared with other sequences available on NCBI Gene bank database using BLAST (Al-Dhabaan., 2019).

2.7 Statistical analysis

All the experiments were conducted in triplicates and the mean values were used to plot the graphs. Statistical analyses were carried out using Minitab 17.0 version. The data were analyzed using one-way ANOVA, where Tukey's multiple comparison tests were used to determine significant difference at $p < 0.05$.

3. Results and Discussion

Among varying number of bacterial isolates obtained from different samples, only a few isolates were recorded to produce the utilization zone on solidified agar plates with isolation medium supplemented with 1% cellulose. In the primary screening of bacterial isolates, the regions of the CMC agar plates where hydrolysis of CMC had occurred were clear and seen as yellow zones of clearance beneath and around the colonies against a red background. The appearance of a zone of hydrolysis around the colonies indicated the synthesis of extracellular cellulase by the bacteria and this enzyme had utilized the CMC (soluble cellulose) in the medium. Isolates showing clear zone indicated the presence of cellulolytic activity (positive isolates).

Even though, the isolates showed cellulase activity at a wide pH range (4.8 -7.0), the highest enzyme activity of almost all the isolates was found at the pH of 6.2 and significantly higher CMCase activity was obtained for the isolate W2 (0.0431 U/mL) (Figure 2 and 3).

Isolates showed enzyme activity over a wide range of temperatures (35℃- 70℃). Among the isolates having temperature optimum of 50℃ for CMCase enzyme activity, isolate W2 from decaying wood (0.0604 U/mL) had significantly higher enzyme activity and the isolate MSW from municipal solid waste was the second largest CMCase producer showed the highest EA at 60℃ (0.0423 U/mL) as in Figure 4 and 5.

Cellulose hydrolysis capacity (Table 1) and the enzyme activity of the four selected isolates (S2, MSW, P, W2) were found in the range of 7.75 to 11 and 0.0166 U/mL to 0.0604 U/mL respectively and the values were comparable with the cellulolytic index values in the range of 4 to 9 for different bacterial isolates in the qualitative screening (Gupta et al.,2012) and the CMCase activity value for the standard isolate *Cellulomonas fimi* (0.185 U/mL) and closer to the CMCase activity values in the range of 0.039±0.002 to 0.233±0.005 U/mL for cellulolytic bacteria isolated from oil palm meal samples (Khianngam et al., 2014).

Isolates W2, S2, P and C1 also show endo glucanase activity even at 70℃ with significantly higher enzyme activity value for W2 (0.0303 U/mL) and S2 (0.0074 U/mL), P (0.0065 U/mL) and C1 (0.0011 U/mL)- (Figure 4). In isolates W2 and MSW, CMCase activity was optimal at pH 6.2 and at 50℃ for W2 and at 60℃ for MSW with good stability at a wide temperature (35℃-70℃) and pH (4.8 – 7.0) range demonstrating their suitability in various industrial processes. As most of the industrial enzymatic reactions are carried out at high temperatures, cellulases isolated from thermophilic bacterial strains are important for industrial applications (Shanmugarajah and Kapilan, 2021; Zamost et al., 1991).

According to substrate specificity that the cellulase had on CMC, the extracted enzyme was an endo-β-1,4-glucanase.

Four gram negative isolates: T_1, MSW, C_1, S_2 and two gram positive isolates: W_2, P had significantly higher CMCase enzyme activity.

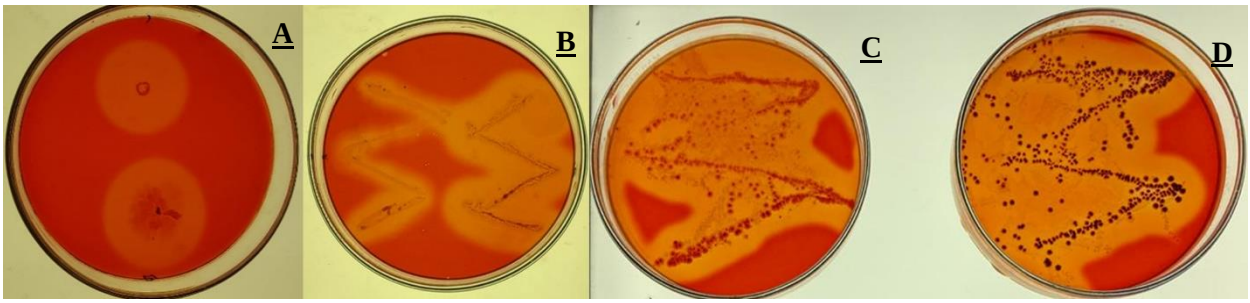


Figure 1: CMC agar plates (spot inoculated (A) and streaked (B, C & D) showing zones of clearance after the inoculation of the bacterial isolate

Table 1: Various bacterial isolates along with their size of zones of clearance and hydrolysis capacity

Source of the organism	Sample code (mm)capacity	Colony diameter(mm)	Hydrolytic zone diameter	CI/ cellulose hydrolysis capacity
Termite gut bacteria	T1	3	15.5	5.16
	T2	2.2	10	4.54
Decaying wood	W1	2	20	10
	W2	2	22	11
Composting yard soil	Soil	2	7.2	3.6
Municipal Solid Waste	'MSW	2	17.5	8.75
Compost	C1	3	25	8.33
	C2	2	12	6
Saline coastal belt soil	S1	2	9	4.5
	S2	2	15.5	7.75
Decaying paper	P	2	19	9.5
Leaf litter	L	1	3.5	3.5

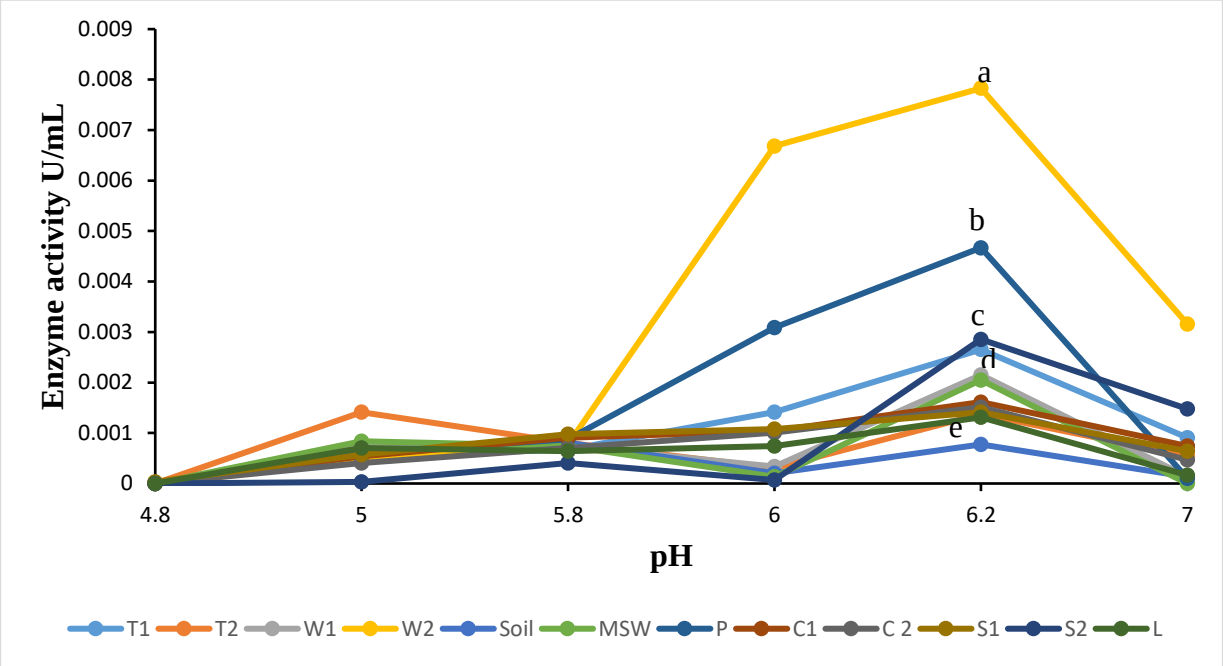


Figure 2: Effect of different pH values on the activity of endoglucanase produced by different bacterial isolates. Each value in the figure is represented as mean ± SD (n = 3). Values in the same column followed by a different letter (A-E) are significantly different ($p < 0.05$).

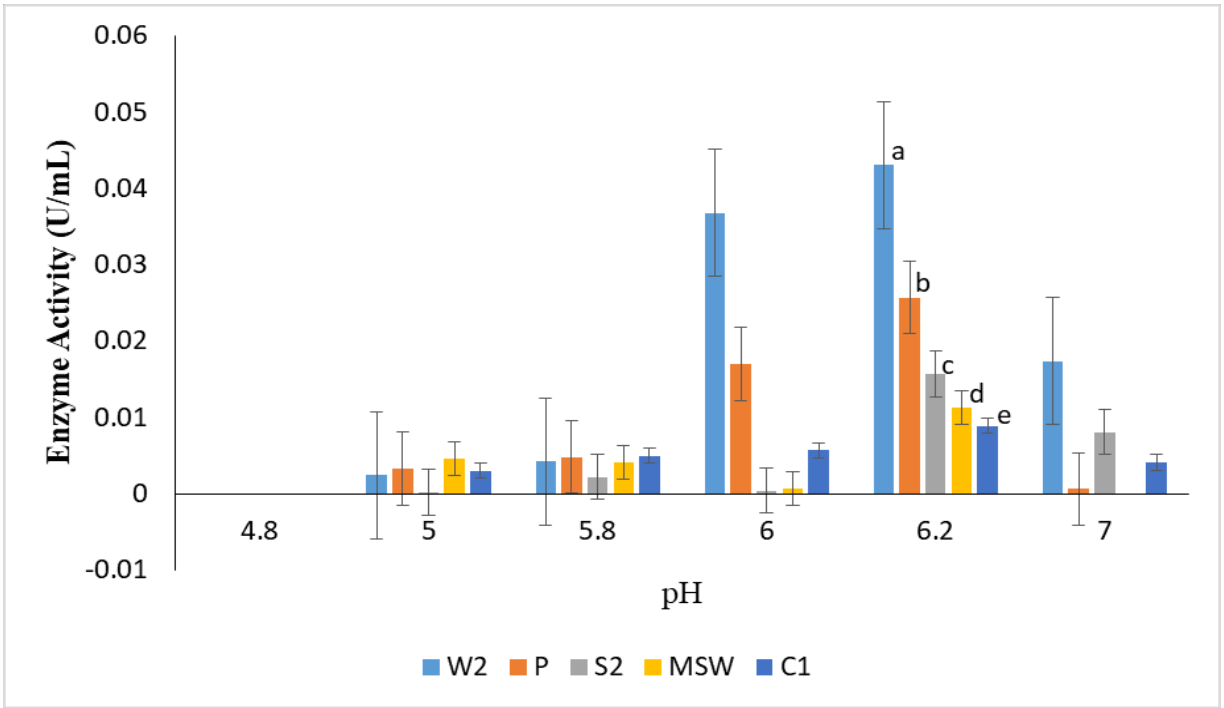


Figure 3: Effect of different pH values on the activity of endoglucanase produced by different bacterial isolates. Each value in the figure is represented as mean ± SD (n = 3). Values in the same column followed by a different letter (A-E) are significantly different ($p < 0.05$). Remove all the bars at 4.8

D

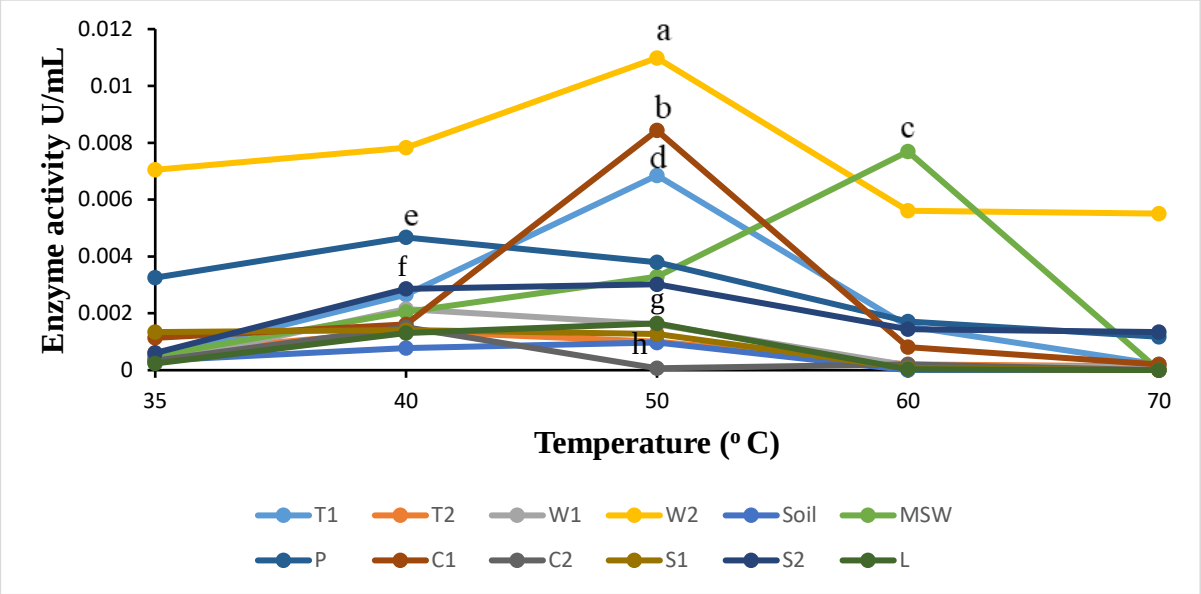


Figure 4: Effect of different temperatures on the activity of endoglucanase produced by different bacterial isolates. Each value in the figure is represented as mean ± SD (n = 3). Values in the same column followed by a different letter (a-h) are significantly different ($p < 0.05$).

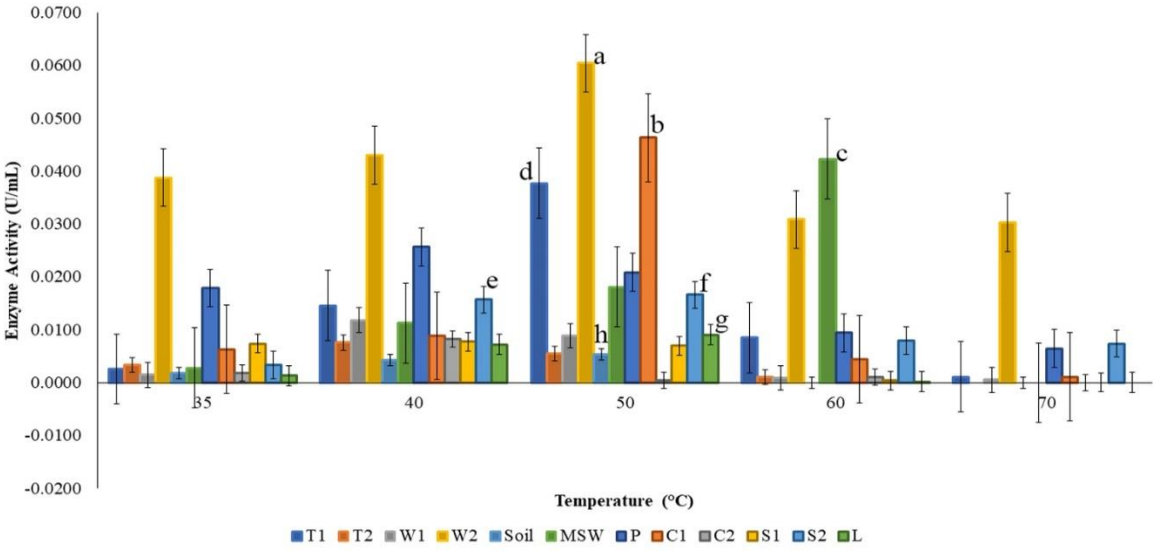


Figure 5: Effect of different temperatures on the activity of endoglucanase enzyme produced by different bacterial isolates. Each value in the figure is represented as mean ± SD (n = 3). Values in the same column followed by a different letter (a-h) are significantly different ($p < 0.05$).

Table 2: Morphological and biochemical characteristics of selected bacterial isolates

Isolates / Test	T1	MSW	C1	S2	W2	P
ram staining	–	–	–	–	+	+
ıtalase	+	+	+	+	+	+
trate utilization	–	–	+	–	+	+
ethyl red	+	+	+	+	–	+
ges Proskauer	–	–	–	–	–	–
S production	+	–	–	–	–	+
dole test	–	–	–	–	–	+
ease test	+	+	+	+	+	+
arch hydrolysis	–	+	+	+	+	–
ucose fermentation	–	–	–	–	–	+

All the four selected isolates (MSW, S2, W2, P) were catalase and urease positive and have the ability to catalyse the breakdown of H_2O_2 and hydrolyse urea and produce NH_3. Among the four selected isolates, isolate P gave positive results for indole, H_2S production and glucose fermentation tests and has the ability to utilize tryptophan, produce H_2S through the reduction of sulphur containing amino acids and metabolize glucose respectively. Gram positive bacterial isolates W2 and P were found to be citrate (+) ve and have the ability to utilize citrate as carbon source . Gram negative isolates MSW and S2 were citrate (-) ve. All four isolates gave negative results for Voges Proskauer test and were unable to produce acetone in broth culture. Bacterial colonies of isolates MSW, S2 and W2 gave positive result for starch hydrolysis and MSW, S2 and P were (+) ve for Methyl red test. Based on the morphological and biochemical test results, the isolates MSW and W2 were identified to belong to the genus *E. coli* and *Bacillus* sp. respectively.

Table 3: Properties of DNA of the bacterial isolates

Bacterial Isolate	Amount of DNA(ng/µL)	Ratio of Abs. value 260 nm/280nm
T1	18.7	1.72
MSW	522.5	1.98
C1	18 .0	1.57
S2	66.8	1.84
W2	88.1	1.85
P	48.2	1.93

According to the sequence analysis, the following four isolates that showed significantly higher endo-β-1,4-glucanase activity than the other isolates tested were identified as,

W2 – *Bacillus subtilis* DmB55 (Accession No. HQ111354.1)

MSW – *E.coli* K12 (Accession No. U00096)

S2 – *Sulfitobacter pontiacus* DSM1004 (Accession No.GCA 900106935.1)

P – *Bacillus cereus* and *Rhodococcus erythropolis* e1 (Accession No.EU 729.738)

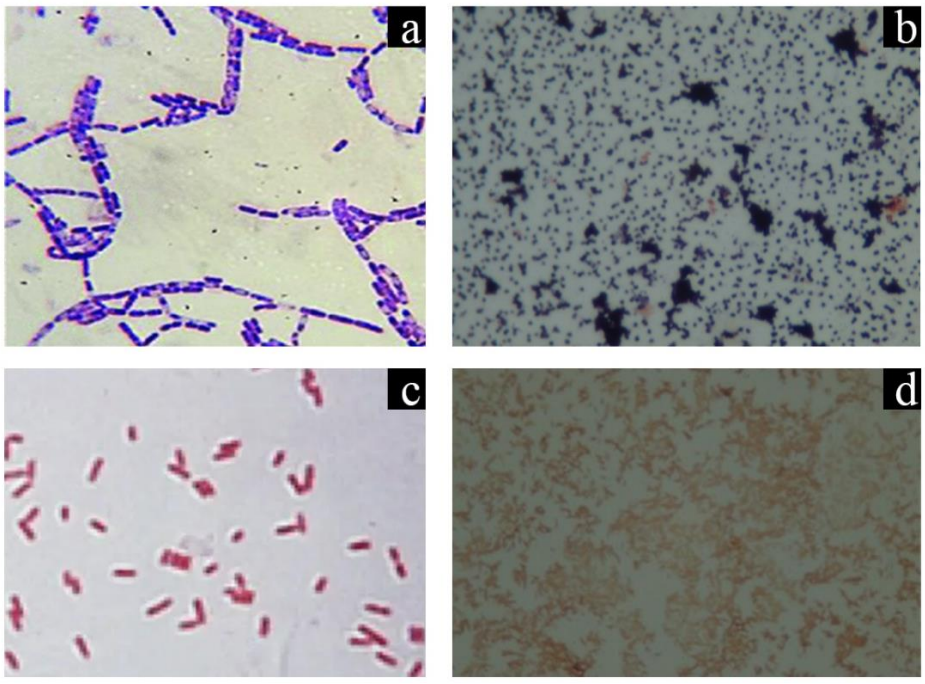


Figure 6: Stained microscopic slides of bacteria having significantly higher endo glucanase activity

[a-Bacillus subtilis (+ve rod), b-Mixed cells of Bacillus cereus (+ve rods) and Rhodococcus erythropolis (+ve cocci), c-E.coli (-ve rod), d-Sulfitobacter pontiacus (-ve short rod)]

4. Conclusion

The following four isolates that showed significantly higher endo-β-1,4-glucanase activity than the other isolates tested were identified as, W2 – *Bacillus subtilis*), MSW – *E.coli* K12 S2 – *Sulfitobacter pontiacus* and P – *Bacillus* and *Rhodococcus erythropolis* based on comparative BLAST results. Cellulase producing isolates can be grown at different temperatures ranging from 40℃ - 70 ℃ and the maximum activity is found at pH 6.2 on suitable cellulose substrate. In this study the substrate specificity indicates that the crude enzyme be an endo-β-1,4-glucanase which plays a prominent role in initiating and sustaining the hydrolytic process and randomly cleaves cellulose into glucose and olygomeric polysaccharides. In the isolation of cellulase producing bacteria, by selecting samples with cellulosic substance from different locations with extreme environmental conditions, it is possible to extract cellulases that are stable at high processing temperatures and withstand other harsh conditions and retain their EA.

Acknowledgement

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