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PRODUCTION OF CELLULASE FROM NATURAL BACTERIAL ISOLATES FROM SOIL SAMPLE OF DISTRICT JAUNPUR, U.P., INDIA

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ABSTRACT

The present study aims at isolating, screening, characterizing and optimizing cellulolytic bacteria isolated from soil samples. Different soil samples were collected from different locations of district Jaunpur, Uttar Pradesh, India. The morphological, biochemical and molecular characterization of the isolates were carried out for their identification. A total number of 23 isolates were screened at preliminary level for cellulolytic activity out of which three potent cellulase producers were selected for further study. On the basis of 16S rDNA sequencing these three isolates were identified as *Bacillus velezensis* PS-14, *Bacillus velezensis* PS-09 and *Bacillus paramycoides* PS-05. The optimal conditions for cellulase enzyme production was also determined and maximum activity was obtained at pH 7 and 40 ^{0}C temperature, while 2% inoculum level and 48 hr incubation period were found to be optimum for cellulase production by all the strains. Glucose and Carboxymethyl cellulose was proved to be the best carbon sources, while ammonium choride and peptone was observed to be the most favourable nitrogen source for all three bacterial isolates.

KEY WORDS: Cellulase, *Bacillus*, Molecular Characterization, Optimization

INTRODUCTION

These days, cellulase is a more affordable raw material, and its industrial uses are more sophisticated. These efforts have produced a huge platform for cellulose research focused on it's applications. Cellulases catalyze hydrolysis of cellulose, which is a linear polymer comprised of D-glucopyranose units connected by β 1-4 glycosidic linkages as studied by Danalache *et al.* (2018). Cellulase is a whole system of enzymes that breaks down β 1, 4 linkages in the cellulose polymer to release glucose units. This system is made up of endoglucanase, exoglucanases and β glucosidase according to Paudel *et al.* (2015).

Cellulases are used widely in a variety of industries, including chemicals, food & feed, pulp & paper, textiles, drinks, transportation, electronics and most significantly energy, De Souza and Kawaguti (2021), Cipolatti *et al.* (2019). According to recent data, there is a 29.71% market demand for cellulase in animal feed, a 26.37% market demand in food and drinks, and a 13.77% market demand in the textile industry, (Guerrand, 2018). Additionally, the use of cellulase is dramatically increasing each year and according to the Global Cellulase Market Growth 2021–2026 research (2021), they will reach 2300 million USD by the end of 2025, with a 5.5% yearly growth rate for the 2018–2025 period.

Many investigators have reported that anaerobic and aerobic bacteria, Kumar *et al.* (2019), Sampathkumar *et al.* (2019), Soni *et al.* (2018), Singhania *et al.* (2017) fungi and actinomycetes, Kumar *et al.* (2019), Ramesh *et al.* (2020), Shida (2016) are potent cellulase enzyme producers. These microorganisms have an effective enzyme breakdown system and secrete free or cell surface-bound cellulases. Bilal and Iqbal (2020) studied that

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due to bacteria's rapid growth and high cellulase synergistic activity, they are the most effective cellulose degraders among all other types of microorganism.

The present investigation is focused on the isolation, screening and identification of the potential cellulase producing bacteria from soil environment and their optimization for different nutritional and environmental parameters for maximum cellulase production

MATERIALS AND METHODS

Sample collection

The soil samples used in the study were collected from Jaunpur district (82^{0}44'E longitude and 25046'N latitude), Uttar Pradesh, India. Four separate locations were selected such as Purvanchal University Campus, an agricultural farm, an area where agricultural waste is dumped and a wholesale vegetable market for sampling. Using a sterile spatula, soil samples were collected from the surface, and a sterile poly bag was used to transport them to the laboratory.

Isolation and screening of cellulolytic bacteria

For the isolation of cellulase producing bacteria, serial dilution and spread plate method were employed using carboxymethyl cellulose (CMC) agar, pH adjusted to 7. The media was sterilized by autoclaving it at 121 ^{0}C and 15 lbs pressure. In a test tube, 10 ml of sterile distilled water were added to 1 g of soil. Tenfold serial dilution of the above sample was made and 0.1 ml of aliquot was spread on the media. The plates were incubated for 24 to 48 hr at 37^{0}C. Bacterial colonies were purified by streaking for repeated times. Pure culture was stored at 4^{0}C. For screening, pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48 hr, 1% congo red was used to flood the plates and then they were left to stand for 15 min and finally rinsed with 1 M NaCl for counter staining. A clear zone of hydrolysis around colonies indicate a positive result for cellulolytic activity as investigated by Shah *et al.* (2021). The diameter of zone of hydrolysis on the plates was indicator of best cellulase producer. Isolates having a large clear halo yellow zone were selected for their identification and production of enzyme.

Identification of Bacteria

For the identification of isolates, various morphological and biochemical tests were performed Cappucino and Sherman (1992). The molecular identification of the cellulase producing bacterial isolates was carried out using 16S rDNA gene sequencing. The results were then compared with Bergeys Manual of Determinative Bacteriology, Buchana and Gibbons (1974).

Identification by 16S rDNA sequencing

Molecular characterization of the isolates was carried out at APICAL SCIENTIFIC Laboratory, Selangor, Malaysia. Isolated bacteria were subjected to DNA extraction using Bacterial Lysis Buffer of Bacterial DNA Barcoding Kit (1st BASE, KIT-1100-50). The bacterial 16S rDNA, full-length 1.5 kb, was amplified using universal primers 27F and 1492R. The total reaction volume of 25 µl contained gDNA purified using in-house optimized protocol, 0.3 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 400 µM each), 0.5 U of thermostable DNA polymerase, supplied PCR buffer and water. The PCR was performed as follow: 1 cycle (94 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 10 sec; 53 °C for 30 sec; 68°C for 1 min) for denaturation, annealing and extension. After PCR completion, aliquots of 1 µl PCR were run on 1% TAE agarose gel. The PCR products were purified by standard PCR clean-up method. The purified PCR products were subjected to bidirectional sequencing with universal primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Using the National Center for Biotechnology Information (NCBI) database's nearest culture sequence, the sequenced data were further examined using the Basic Local Alignment Search Tool (BLAST), Altschul *et al*. (1990).

Assay of Cellulase activity

Cellulase activity was estimated by using dinitrosalicyclic acid (DNS) reagent as given by Miller (1959) by estimating reducing sugar released from CMC. The production medium containing the culture was centrifuged at 10,000 rpm for 10 min to get the crude enzyme extract as supernatant. The reaction mixture contains 1 ml of 0.5% CMC in 0.05 M phosphate buffer (pH 7), to this add 0.1 ml of supernatant and afterwards 0.9 ml of buffer was added to make the final volume upto 2 ml. This enzyme assay mixture was incubated at 50 °C for 30 min in a water bath. The reaction was halted after 30 min by adding 3 ml DNS reagent. After 5 min in

a boiling water bath finally the tubes were allowed to cool to room temperature. At 575 nm the absorbance was recorded, Bhagat and Kokitkar (2021). Cellulase production was estimated by using glucose calibration curve. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1 µmol of glucose per min under the standard assay conditions.

Process Optimization

The parameters analyzed for the optimization were pH, temperature, carbon source, and inoculum size for the selected bacterial isolates. Once the fermentation process is completed for different parameters the crude enzyme sample was collected from each to check the enzyme activity. Cellulase activity was determined by using DNS method.

Effect of pH: pH values ranging from 6, 6.5, 7, 7.5, 8, 8.5 were selected to observe the effect of pH on cellulase production.

Effect of Temperature: For determining optimum temperature selected isolates were grown in the production medium and incubated at 30 ^{0}C , 35 ^{0}C, 40 ^{0}C, 45 ^{0}C, 50 ^{0}C & 55 ^{0}C.

Effect of Incubation Period: For determining optimum incubation period, desired isolates were incubated at different time intervals, i.e. 24, 48 , 72, 96 and 120 hrs.

Effect of Carbon source: The effect of carbon source was studied by replacing the carbon source with simple sugars (glucose, sucrose, fructose and maltose) and compound sugars (cellobiose, starch, CMC and cellulose).

Statistical analysis All the experiments conducted in present investigation were conducted in triplicates and data presented were in the form of mean ± SE from their independent experiments. Graphpad prism version 5.01 was used to plot the graphs.

Table 1. Results of Biochemical Tests

S. No.	Biochemical tests	Cultures name		
		PS-14	PS-09	PS-05
1.	Catalase test	-	-	-
2.	H_2S Production	-	-	-
3.	Amylase test	-	-	-
4.	Indole test	+	+	+
5.	Methyl red	-	-	+
6.	Vogues proskauer	+	+	-
7.	Citrate utilization	+	+	-
8.	Glucose	A	A	GA
9.	Sucrose	A	A	A
10.	Lactose	-	-	-
11.	Mannitol	A	A	-

Note: (-) Negative, (+) Positive, (G) Gas Production, (GA) Gas and Acid Production

RESULTS AND DISCUSSION

Isolation and screening of cellulase producing bacteria

Screening of the isolated bacteria from soil was performed and bacteria producing a clear zone around the colonies were considered having cellulolyic activity (Figure 1). Clear zone diameter for all cellulase producing isolates is shown in Figure 2. Most efficient cellulase producer having maximum zone of hydrolysis were PS-14, PS-09 and PS-05. Pure cultures of PS-14, PS-09 and PS-05 are shown in Figure 3. All the three isolates were found to be Gram-positive in nature. The morphology of all the isolates was found to be rod-shaped. Table 2 shows the result of biochemical tests.

Molecular identification of bacteria

The desired isolates were identified as: *Bacillus*

Table 2. Molecular identification of isolates.

S. No.	Accession number obtained from NCBI	Organism name
1.	LC778306	*Bacillus velezensis* PS-14
2.	LC778307	*Bacillus velezensis* PS-09
3.	LC778308	*Bacillus paramycoides* PS-05

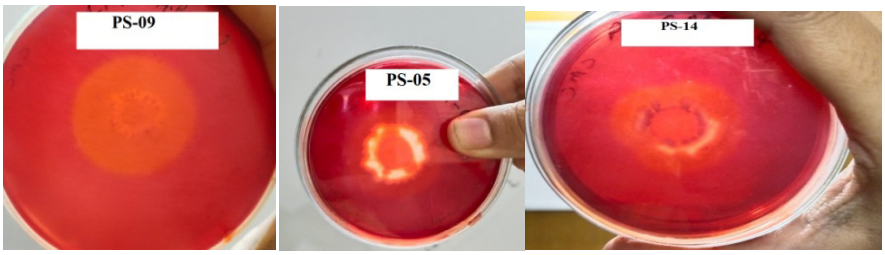

Fig. 1. Zone of hydrolysis by isolates

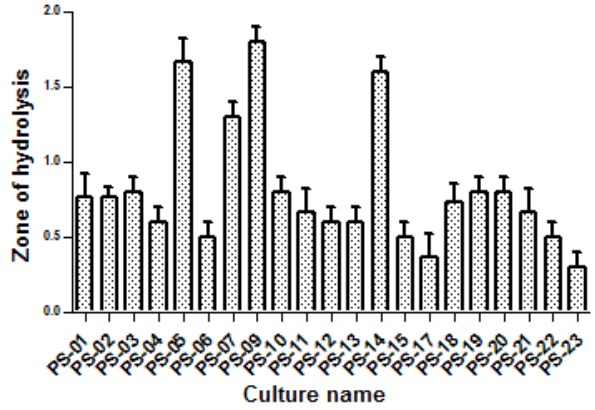

Fig. 2. Zone diameter for different isolates

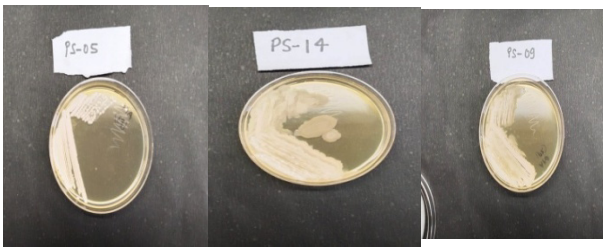

Fig. 3. Colony on Nutrient Agar

velezensis **PS-14,** *Bacillus velezensis* **PS-09** and *Bacillus paramycoides* **PS-05.** The nucleotide sequences obtained have been deposited in NCBI. Table 3 shows the molecular identification for all isolates. Phylogenetic tree of bacterial isolates from soil and their closest phylogenetic relatives are mentioned in Figure 4, 5 and 6.

Optimizing the production of cellulase

The efficient cellulase producers isolated were optimized for cellulase production using different production parameters.

Effect of pH on cellulase activity

The enzyme activity is highly dependent on pH. The maximum enzyme activity was observed at pH 7 by PS-14, PS-09 and PS-05 with maximum enzyme activity, 3.28 IU/ml, 3.36 IU/ml and 3.23 IU/ml respectively (Figure 7). The 3D shape of the enzyme's catalytic sites must be preserved by pH and when pH values changes, the enzyme's ionic bonding changes because its functional form is lost, Abada *et al.* (2021). Islam and Roy (2019) isolated and characterized cellulase-producing bacteria from sugar industry waste, in their study *Paenibacillus* sp. showed highest cellulase activity at optimal pH 7.0. Goel *et al.* (2019) isolated bacteria with cellulolytic activity and identified the isolate as *Pseudomonas* and observed maximum activity at pH 7.

Effect of temperature on cellulase activity

PS-09, PS-14 and PS-05 when analysed for activity produced 3.26 IU/ml, 2.51 IU/ml and 2.44 IU/ml enzyme activity respectively at 40^0C (Figure 8). A decrease in activity at temperature above 40^0C was observe which may be because when temperature increases it inhibits the growth of microorganisms which in turn result in loss of activity, Rasul *et al.* (2015). Similar findings were observed by Lingouangou *et al.* (2022) shows that *Bacillus subtilis,*

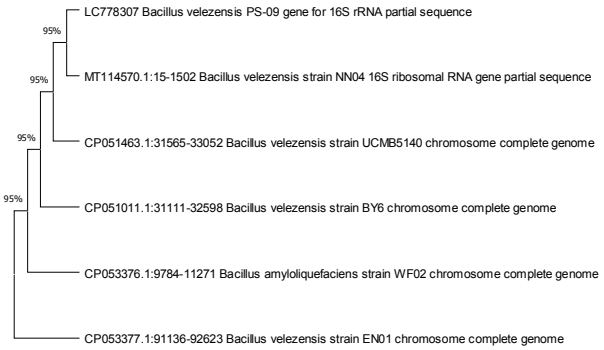

Fig. 4. The phylogenetic tree of PS-09 isolate.

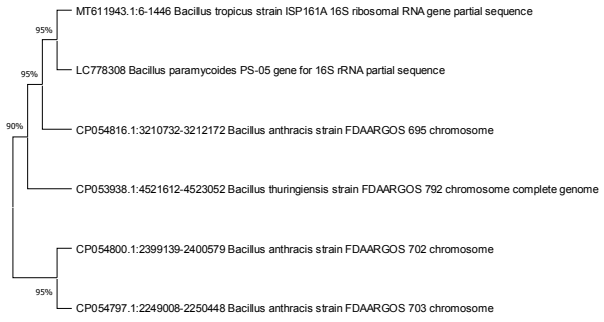

Fig. 5. The phylogenetic tree of PS-05 isolate.

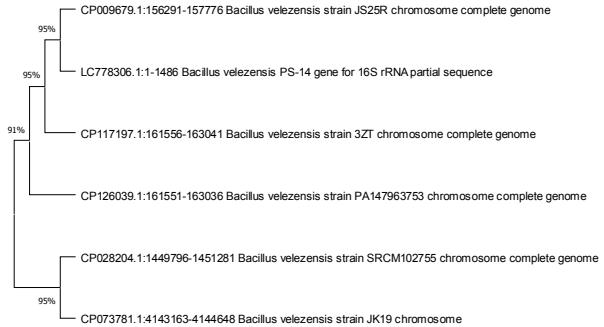

Fig. 6. The phylogenetic tree of PS-14 isolate.

Pantoea dispers and *Lysinibacillus fusiformis* gave maximum cellulase production at 40^{0}C. Hussain *et al.* (2021) isolated CBM21 and CSG8 they identified it as *Pseudomonas* sp. and revealed that maximum production was achieved at optimum temperature at 40 °C and 35 °C respectively.

Effect of incubation period on cellulase activity

The major peak of activity was observed at 48 h which was 3.50 IU/ml by PS-09, 3.46 IU/ml by PS-14 and 3.40 IU/ml by PS-05 and thereafter gradually decreases (Figure 9). On incubating above 48 hr a decrease in activity was observed by all three cultures which may because on further incubation there is a decrease in nutrients of media which

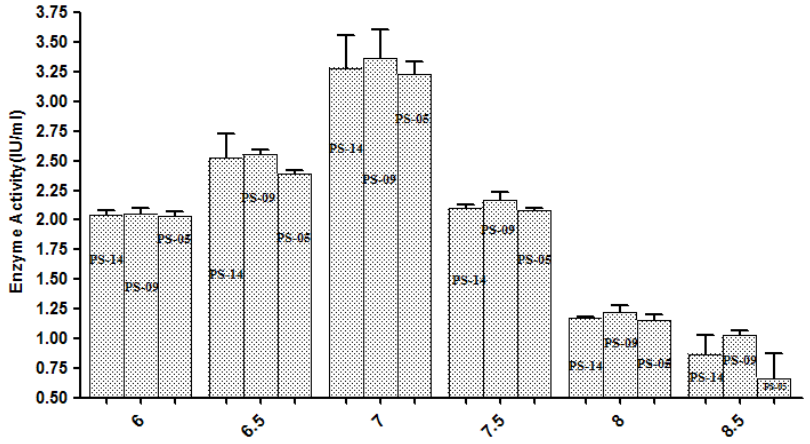

Fig. 7. Effect of pH

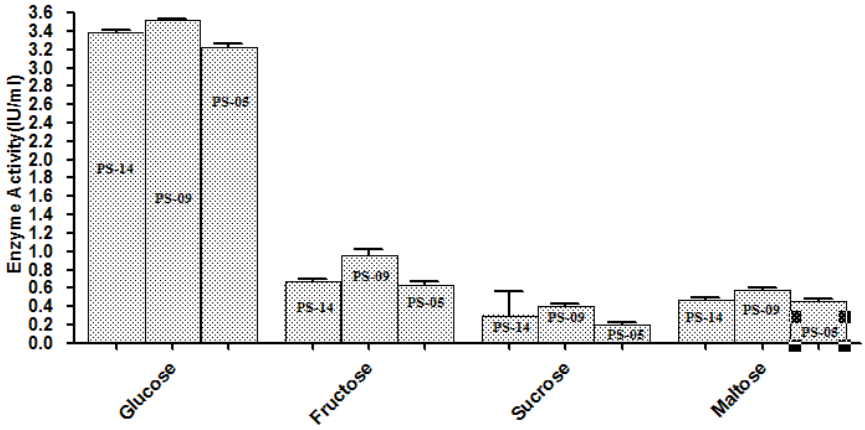

Fig. 8. Effect of temperature

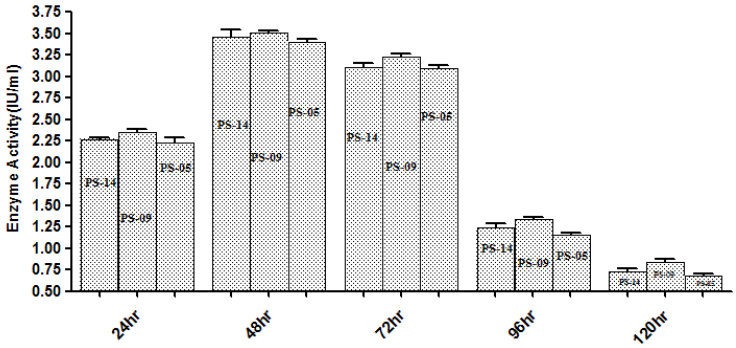

Fig. 9. Effect of Incubation Period

ultimately leads to cell death as reported by Arrifin *et al.* (2006). Our data presented are in accordance with Poly *et al*. (2022) who isolated *Pseudomonas* sp. from rumen fluid of cattle and reported maximum production at 48 h of incubation. Rasul *et al.* (2015) isolated *Bacillus* sp. SM-3M8 from soil and waste molasses of sugar industry & reported maximum activity at 40 ^{0}C.

Effect of carbon source on cellulase activity

Both simple and complex sugars were studied for cellulase producing ability of isolates. Results obtained showed that among the simple sugars glucose showed the highest enzyme activity. Fructose and maltose also showed some enzyme activity but sucrose was least suited. However, glucose was found to be best carbon source showing 3.52 IU/ml, 3.38 IU/ml & 3.23 IU/ml by PS-09, PS-14 and PS-05 respectively (figure 10). Since glucose is a sugar that may be easily metabolized, it provides the highest level of productivity. The results obtained are well in accordance with Hussain *et al.* (2021) which revealed that glucose was best carbon source for CBM21 (*Bacillus* sp.) and CSG8 (*Pseudomonas* sp.). Duza and Mastan (2015) reported that glucose showed maximum enzyme production by *O.anthropi*-YZ1 (TS16MCN) and *B.anthracis* (TS5SRP).

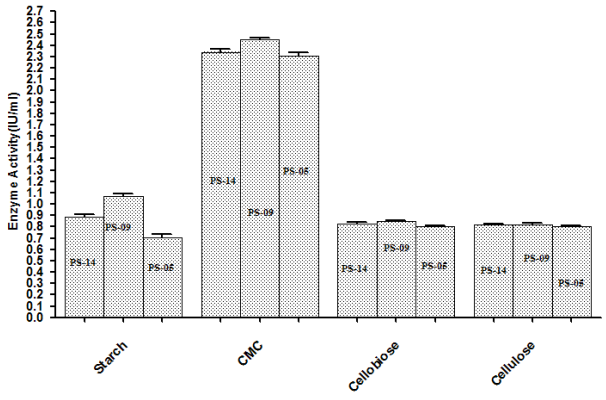

Fig. 10. Effect of simple sugar

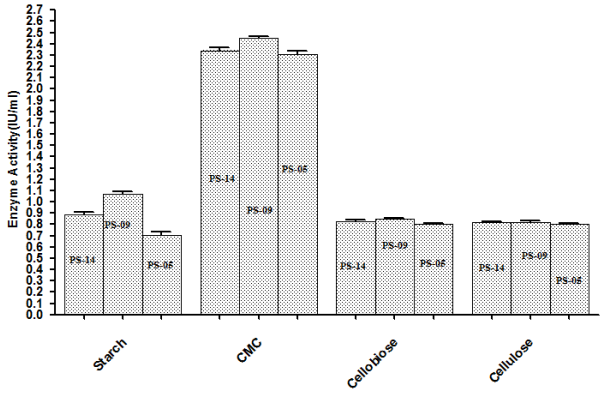

Fig. 11. Effect of complex sugar

Among complex sugars, CMC showed the maximum activity when used as a carbon source (figure 11). PS-14, PS-09 and PS-05 gave maximum production of cellulase on CMC and it was 2.34 IU/ml, 2.45 IU/ml and 2.30 IU/ml respectively. Starch and other polysachharides showed less activity when compared to CMC. Since CMC show less complexicity when compared to other polysaccharides and gets easily assimilated by the isolated bacteria, Wood and Bhatt (1988). Similar results were reported by Premlatha *et al.* (2015) wherein *Enhydrobacter* sp. ACCA2 showed CMC as best source. Lokhande and Pethe (2017) showed maximum cellulase production from *B. thuringiensis* using CMC.

CONCLUSION

Soil is the richest ecosystem and is regarded as a favourable site for the presence of microorganisms that produce different hydrolytic enzymes. Cellulolytic bacteria are widely present in soil. The aim of present investigation was to isolate and screen the cellulase producing bacteria from soil sample. Three best cellulase producers were found during our investigation as they gave maximum zone of hydrolysis. All three isolates were found to be Gram positive, rod shaped and belonged to *Bacillus* species based on their molecular identification. The optimum parameters for our three isolates were also studied to determine the optimal physico-chemical parameters. It revealed that *Bacillus velezensis* PS-14, *Bacillus velezensis* PS-09 and *Bacillus paramycoides* PS-05 showed highest activity at pH 7, 40^0 for 48 hr incubation. All three bacteria utilized glucose and CMC as the best carbon source. It may be concluded that the isolated species can produce cellulase when peptone and NH_4Cl among organic and inorganic as the sole nitrogen source and an optimal level of 2% inoculum size was needed for maximum enzyme activity. Cellulases find a diverse use at industrial scale. Cellulase helps to hydrolyze the lignocellulosic biomass that is an important aspect in biofuel production.

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Conflict of interest - The authors declare no conflict of interest.

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