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ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF AN ALKALOPHILIC, COLD-ACTIVE CELLULASE FROM BACTERIA ISOLATED FROM BONIYAR REGION OF KASHMIR

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Abstract

Cellulases are abundantly found in microbes found in nature and play important roles in biotechnological industries. This enzyme aids in various process of breaking-down the cellulose in different commercially important products like antibiotics, compost, monomeric sugars and single-cell protein (SCP) essential for human use. These necessities have prompted us to isolate the cellulase producing from the unique and undisturbed region. The present study focusses on the isolation of potential alkaliphilic and psychrophile cellulase (CEL7) producing bacteria from Jammu and Kashmir. The enzyme CEL7 was found to be active at an optimum temperature of 20°C and pH 9.0. The metal ion Ag^{+} (5mM) and Ca^{2+} (2mM) enhanced the enzyme activity. On performing, 16S rRNA sequencing the cellulase producing bacteria showed high similarity with *Pseudomonas* sp.

Keywords: Alkalophilic, Brackish Water, Cellulase, Jammu and Kashmir, Psychrophile.

Introduction

Over-exploitation of non-renewable fossil fuels has increased the demand for energy (Kumar *et al.*, 2003; Ghazvini *et al.*, 2020; Chahartaghi *et al.*, 2019; Vand *et al.*, 2018; Kaur *et al.*, 2019) and it has prompted the exploration for cost-effective, eco-friendly and renewable alternative energy source (Gautam *et al.*, 2011; Bhardwaj *et al.*, 2014; Singh *et al.*, 2014; Sharma *et al.*, 2014; Singh *et al.*, 2015; Kotia *et al.*, 2018). Bioethanol obtained from lignocellulosic material is an effective alternative and sustainable renewable resource for clean fuel (Balat and Balat, 2009; Patel and Duran, 2017; Mannan *et al.*, 2017; Kumar and Kumar 2018). This approach will not only help in reducing the greenhouse gases but also relieve the pressure of the energy crisis (Kumar *et al.*, 2017; Chowdary *et al.*, 2019; Kotia *et al.*, 2017). Lignocellulosic biomass consists of hemi-cellulose, lignin and cellulose (Saini *et al.*, 2015; Sharma *et al.*, 2019; Rai *et al.*, 2019; Kumar *et al.*, 2015; Verma *et al.*, 2015; Singh *et al.* 2013; Churasia *et al.*, 2016; Kalra and Kumar, 2018). The recalcitrant structure of lignocellulosic material prevents enzymatic and microbial action. Therefore prior pre-treatment is required before enzymatic hydrolysis of lignocellulose to monosaccharides. NaOH (Sodium Hydroxide) pretreatment has been extensively studied to improve enzymatic hydrolysis and effectively remove the lignin from the biomass (Kucharska *et al.*, 2018). Even, alkaline pretreatment is effective approach but it is time-consuming. On comparing with acidic pretreatment, alkaline pretreatment liberates less sugar. Therefore, for subsequent enzymatic hydrolysis, pre-treatment of cellulosic material is utmost importance to obtain glucose which can be further converted into bioethanol by microbes (Zhang *et al.*, 2019; Arora *et al.*, 2015; Chilana *et al.*, 2015; Kaur *et al.*, 2014; Davinder *et al.*, 2017; Kumar *et al.*, 2020; Sangma *et al.*, 2019; Pramanik and Maji 2015; Pramanik and Padan, 2016).

Group of cellulolytic enzymes are the ones which catalyze the hydrolysis of cellulosic wastes to monomeric glucose molecules through synergistic action of β-glucosidase, endoglucanase, and exoglucanase (Zhang *et al.*, 2017; Wang *et al.*, 2017; Kapoor *et al.*, 2019; Sharma *et al.*, 2017; Pooja and Tanay, 2019). Enzyme cellulase has attained the attention of many different industries like detergent, feed, food, leather, paper and textile (Raveendran *et al.*, 2018). Application of this enzyme in industries demands for exploration of stable enzyme which remains active at low temperature and high pH (Adrio and Demain, 2014). *Bacillus, Cellulomonas, Cellvibrio, Micrococcus* and *Pseudomonas* sp. have been reported as the active producer of cellulase enzyme (Sethi *et al.*, 2013; Nandimath *et al.*, 2016; Naresh *et al.*, 2019). Recently, numerous cellulolytic bacteria have been isolated from diverse habitats like soil, compost, and water (Gupta *et al.*, 2012).

This research was undertaken to isolate the potential alkaliphilic and psychrophile cellulase producing bacteria from Jammu and Kashmir. Varied culture conditions were assessed and optimization of these conditions was done to achieve the high enzyme activity and productivity was done for isolated strain. The isolated strain was then identified on the basis of the biochemical test and 16S rRNA sequencing.

Material and Methods

Sample Collection

The water sample was collected from Boniyar, Baramulla, J&K (Latitude 34.1008° N and longitude 74.2003° E). Sub-surface water was collected aseptically in sterilized one litre bottle by directly dipping the bottle into the surface water. The collected sample was transferred to the laboratory and kept at 4^{0}C for further analysis.

Isolation and Purification of Isolated strains

The water sample was serially diluted to 3-fold and 100µL of 3-times dilution sample was plated (in triplicate) on the carboxymethyl cellulose substrate-agar media. Then the CMC agar plates were kept overnight at 37°C for 24 hrs. After that, the plates were observed for colonies with distinct morphology and texture. Twelve different isolates were sub cultured to obtain the pure isolates.

Morphological and Biochemical Identification of Isolated Strains

Gram Staining and Biochemical tests (Indole, EMB agar, Catalase and Urease) were performed by following the standard protocols (Cappuccino and Sherman, 2005).

Screening for Cellulase-producing Bacterial Isolates

The selected twelve pure isolates were primarily screened for cellulase production by Congo Red staining-NaCl destaining for an hour. The plates were checked for the zone of hydrolysis. The secondary screening was done to check the localization of the enzyme. The positive cellulase isolates were first cultured overnight and then centrifuged to remove the growth media. The supernatant was collected, whereas the cell-pellet was mixed with lysis buffer and kept at 37°C for 2 hours. Then the lysate was spun for 40 minutes at 10,000 rpm to obtain the Cell-Free Extract (CFE).

The secondary screening was done by agar diffusion method on CMC agar plates, for which, four 5mm holes were punched on CMC plates under aseptic conditions. 50 µl of each cell-free extract (CFE), supernatant and lysis buffer (positive control) was added leaving one well empty (negative control) and the reaction was allowed overnight. Further, the plates were screened for localization of the enzyme by visualizing the zone of hydrolysis on staining with 1% Congo-Red and washing with 1N NaCl. One isolate (CEL7) showing the positive result for supernatant was selected for further analysis (Apun *et al*., 2000).

Biochemical Characterization of Cellulase Enzyme (CEL7)

Temperature Optima: The most suitable temperature was assessed by measuring the activity of the enzyme on varied temperatures (ranging 10-50°C). The tubes containing the enzyme extract (containing Buffer (300µl), 1% CMC (500µl) and enzyme (200µl)) was incubated at 10°C intervals in the range of 10-50°C for 30 minutes. Further, the tubes were boiled for 5 minutes, cooled and the activity was determined by standard DNSA method (Miller, 1959).

pH Optima: The optimum pH was assessed by measuring the activity of enzyme at different pH ranging from 3-11. The tubes containing 300µl of different buffers (100mM Citrate Buffer (pH 3 to 6), 100mM Phosphate Buffer (pH 7), 100 mM Tris-HCl Buffer (8 to 10) and 100mM Glycine-NaOH Buffer (pH 11), 500µl of 1% CMC and 200µl of enzyme were incubated for 30 minutes at 20^0C. Further, the tubes were boiled for 5 minutes at 100°C. The activity was determined by standard DNSA method (Miller, 1959).

Metal-Ion Complementation: The optimum metal ion cofactor for the enzyme was determined by carrying out the reactions in the presence of 2mM/5mM metal ions. Ag^+, Ca^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Hg^{2+} and Mg^{2+}. All the tubes were incubated for 30 minutes at 20^0C at pH 9.0. Further, the tubes were boiled for 5 minutes at 100°C. The activity was determined by standard DNSA method (Miller, 1959; Seo *et al*., 2013; Naresh *et al*., 2019).

Molecular Identification and Characterization of selected strain (CEL7): The isolate CEL7 showing positive result after the primary and secondary screening was cultivated in CMC media for 24 hours at 37°C. The genomic DNA of CEL7 was isolated by following the protocol as in (Green and Sambrook, 2018). The extracted genomic DNA was visualized on 0.8% agarose gel electrophoresis. The amplification of the extracted DNA was done by 16S rRNA ribotyping. 16S rRNA universal primers (452F: 5'-GACTGGGGTGAAGTCGTAAC-3' and 452R: 5'-TGGCTGGGTTGCCCCATTCGG-3') were used for amplification. The PCR reaction mixture consisted of 1 µl DNA, 5 µl of Master-mix (2X), and 1 µl of each primer (10picomole). The 36 cycles were programmed in a thermal cycler as follow: where initial denaturation was done at 94ºC for 3 minutes, rest 35 cycles followed the program in the following order: 94ºC for 1 minute, 42ºC/45ºC/50ºC/55ºC respectively for 45 seconds, 72ºC for 1 minute 30 seconds and at the end the final extension was done at 72ºC for 5minutes. After that, it was held at 4ºC. The PCR products obtained were then visualized on 1% agarose electrophoresis.. The best amplicon was sequenced at Eurofins Genomics India Pvt Ltd., Bangalore, India and the analysis of sequencing results was done by using BLAST tool of GenBank.

Results and Discussion

Isolation and Purification of Isolated strains

The cellulolytic bacteria were isolated from the water sample by spreading the 3-fold dilution of the sample on CMC agar media. A total of 12 distinct colonies were selected on the basis morphology. Then, the pure culture of the selected twelve colonies (CEL1-CEL12) were obtained by sub-culturing on CMC agar media.

Morphological and Biochemical identification of isolated strains

The pure colonies were subjected to gram staining and different biochemical tests following the standard procedures. The result of the biochemical tests are listed in Table 1.

Table 1: Gram Staining and Biochemical Testing of Cellulase-producing Bacteria (Negative (Neg) and Positive (Pos)

Sample	Gram Staining	Indole Test	EMB Agar Test	Catalase Test	Urease Test
CEL1	G-	++	--	+++	--
CEL2	G-	--	--	+	--
CEL3	G-	--	--	--	--
CEL4	G-	--	--	+	--
CEL5	G-	+	--	++	+
CEL6	G-	--	--	++	--
CEL7	G-	++	--	+++	+
CEL8	G-	+++	--	++	+
CEL9	G-	++	+	++	--
CEL10	G-	--	+	+++	--
CEL11	G-	--	--	++	--
CEL12	G-	--	--	+	--

Primary and Secondary Screening for Cellulase-producing Bacterial Isolates

The primary screening of pure isolates CEL1-CEL12 was evaluated for the zone of hydrolysis through congo red staining-NaCl destaining. Among the 12 pure isolates, only one isolate (CEL7) showed the positive result. The secondary screening was done to confirm the localization of the enzyme localization via well diffusion method. The CMC agar plates containing the enzyme were incubated at 37^{0}C overnight. The enzyme localization was confirmed by measuring zone of hydrolysis around the well. The CEL7 in secondary screening showed measurable zone of hydrolysis. This suggested that the enzyme synthesized by the remaining strains were very low.

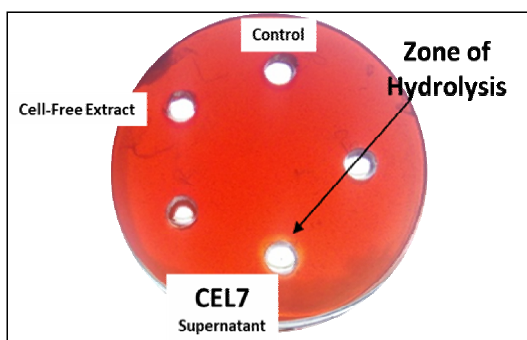

Fig. 1 : 1% CMC-agar plate showing zone of hydrolysis in the supernatant of CEL7 culture.

Biochemical Characterization of Enzyme Activity (CEL7)

Temperature Optima: The temperature optimum was assessed by performing the standard DNSA method at varying temperatures (10-50°C), followed by quantification by glucose standard curve (Fig. 2A). The enzyme was the most active at an optimum temperature of 20°C (Fig. 2B). This showed that the CEL7 is psychrophilic in nature.

pH Optima: The optimum pH was determined by carrying out cellulase assay in different buffers of varying pH 3-11. The enzyme showed the maximum activity at an optimum pH of 9.0. The best pH was found to be (value IU/mL) the alkaline pH of 9.0. (Fig. 2C). This showed that the CEL7 was alkalophilic in nature. This result was found to be similar with findings as in (Sethi *et al.*, 2013) and supports that cellulase activity to be active in the range pH 9.0-11.0.

Metal-Ion Complementation: Changes in enzyme activity in presence or absence of different metal ions with different concentrations (2mM/5mM) was assessed. It was found that Ag^+ (5mM) and Ca^{2+} (2mM) metal ions increased the enzyme activity whereas Co^{2+}, Fe^{2+}, Hg^{2+} and Mg^2 were found to decrease the activity of the enzyme (Fig. 2D).

Fig. 2 : Biochemical Characterization of CEL7 cellulase. A: Glucose-Standard Curve; B: Temperature Optima; C: pH Optima; D: Metal-ion Complementation

Isolation and Molecular characterization of selected strain

The extracted DNA of CEL7 was of good quality as it was confirmed by running it on the 0.8% agarose gel. After that extracted DNA of CEL7 was subjected to amplification with 16S rRNA universal primers. The sample showed an amplicon of 1.5 Kb at 45°C similar to result comprehended in the literature (Dhanjal *et al*., 2017). The sequence result obtained were analysed with BLASTn tool provide by NCBI. On analysis was BLASTn, it showed the similarity with *Pseudomonas* sp., *Pseudomonas chengduensis, Pseudomonas toyotomiensis, Pseudomonas pseudoalcaligenes, Pseudomonas oleovorans, Pseudomonas indoloxydans*, and *Pseudomonas mendocina*. To get a better insight of diversity, the phylogenetic tree was NCBI option.

Conclusion

The alkaliphilic and psychrophilic strain exhibiting cellulolytic activity was isolated from Boniyar, Baramulla, Jammu and Kashmir. The enzyme (CEL7) obtained from the isolated strain showed an optimum activity at temperature 20°C and pH 9.0

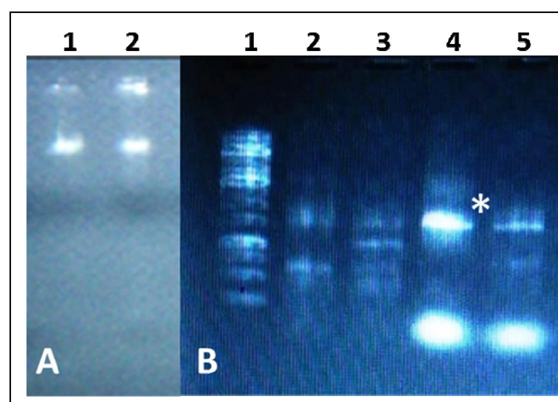

Fig. 3: Molecular Characterization of CEL 7. A: 0.8% Agarose Gel showing DNA Extraction from CEL7 (Well 1: Lambda DNA; Well 2: CEL7 DNA); B: 1% Agarose Gel showing PCR amplification of 16S rRNA from CEL7 (Well 1: 1kb DNA Ladder; Well 2: Annealing at 42ºC; Well 3: Annealing at 45ºC; Well 4: Annealing at 50ºC; Well 4: Annealing at 55ºC)

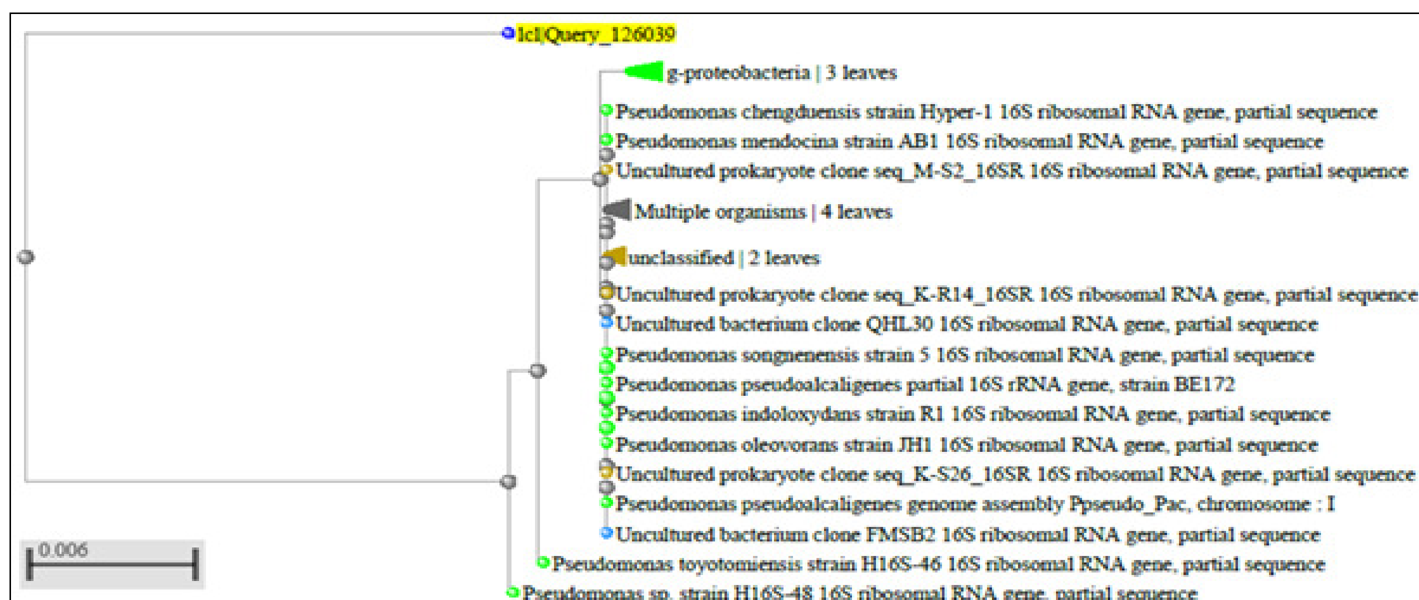

Fig. 4: Phylogenetic Tree for CEL7

The Ag^{+} (5mM) and Ca^{2+} (2mM), metal ions were found enhance the enzyme activity. Cellulase produced by the isolated strain has great potential in the field of biotechnology as well as industries. Further, this bacterial strain can also be used in consortia with *Saccharomyces cerevisiae* for bioethanol production. It can also be genetically modified to achieve the functionality even under the harsh conditions of more alkaline pH to synthesize a potent enzyme with wide-spectrum of industrial applications.

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Conflict of Interest

The authors declare no conflict of interest

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