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Isolation and identification of soil bacteria: Purification and functional characterization of celc

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Abstract
Microbial cellulases serve as important biocatalysts, distinguished by their unique characteristics and composition, and they have a wide range of applications across various industries. The research aimed to identify a bacterial strain with high cellulase production followed by purification of cellulase and its characterization. The highest cellulase producer strain designated as OKB3 was identified as *Bacillus amyloliquefaciens* by biochemical and molecular identification. The purification was achieved through ammonium sulfate precipitation and gel filtration chromatography. The purified monomeric enzyme had a 2.34 purification fold and a 33.34% recovery with 96 kDa molecular mass. This cellulase named CelC revealed the optimum activity at pH 6 at 55 °C and was highly stable at pH 5.5 to pH 6.0 at 0 °C to 4 °C. Such properties make CelC potentially valuable for industrial and biotechnological applications.

Keywords: Cellulase, purification, characterization

Introduction
Cellulase is a key enzyme complex that breaks down cellulose, an important component of plant cell walls, into glucose and other oligosaccharides, and is essential for many industrial processes, such as the management of waste, food manufacturing, and the production of biofuel [1]. Endoglucanases, exoglucanases (cellobiohydrolases), and β-glucosidases are the three primary categories of cellulases. Endoglucanases break down the cellulose's intrinsic linkages within the cellulose chain to create new chain ends, while exoglucanases target these ends to release cellobiose. β-glucosidases subsequently hydrolyze cellobiose to glucose [2].

Fungi, bacteria, and protozoa primarily produce cellulases, which are essential for the breakdown of lignocellulosic biomass into fermentable sugars. These enzymes support sustainable energy production and help reduce reliance on fossil fuels. Advances in genetic engineering and fermentation technologies have enhanced the economic feasibility of cellulase production. As a result, the global cellulase market is anticipated to increase significantly due to rising demand from sectors like biofuels and textiles [3].

A variety of microorganisms have been studied for their capacity to produce cellulase, with specific fungi such as *Aspergillus*, *Trichoderma*, and *Penicillium* particularly known for their cellulolytic capabilities [4]. Cellulase production has been reported for many fungal species, including *Aspergillus terrus* MS105, *Aspergillus fumigatus* MS16, and *Humicola insolens* MTCC 1433 families. However, low specific activity, decreased yields, and end-product-inhibition are some of the limitations that fungal cellulases frequently display [5]. Furthermore, fungal cellulases are less stable at high temperatures, which exhibits a problem as bulk industrial enzyme production generally exists under these conditions. Consequently, there is a growing interest in bacterial cellulase production, as bacteria can rapidly proliferate, express multiple enzyme complexes, and withstand extreme environments [6, 7, 8]. Typically, bacterial cellulases are produced constitutively, whereas fungal cellulases are inducible [9]. Common cellulolytic bacteria include Bacillus species, *Clostridium species*, *Thermobifida fusca*, *Thermonospora species*, *Streptomyces species*, *Ruminococcus albus*, *Thermobispora bispora*, *Erwinia chrysanthemi*, *Cellulomonas species*, and *Acetivibrio cellulolyticus* [10].

Important processes for studying enzymes are isolation, synthesis, and purification. Usually, the first step in isolation is to screen for microorganisms that can produce cellulases, frequently using agricultural waste as a carbon source. Bacteria isolated from agro-industrial waste and cow dung, for example, have demonstrated encouraging cellulolytic activity [11, 12].

Submerged fermentation offers advantages over solid-state fermentation, such as increased yields and improved control over environmental conditions. To improve enzyme production, SmF optimizes variables such as pH, temperature, inoculum size, and nutrient composition [11].

Purification is essential for obtaining high-quality enzymes suitable for industrial applications. Common methods include ammonium sulfate precipitation followed by chromatography techniques such as gel filtration or ion exchange chromatography. These processes can significantly increase the specific activity of the enzyme while reducing contaminants [13, 14].

The present study aims to isolate a highly efficient bacterial strain for cellulase production with purification and characterization of the produced enzyme.

Material and Method

Isolation

Sterile containers were used for the soil sample collection from various sites including gardens, fields, and livestock farms, and kept at 4 °C until they were processed further. Cellulase-producing bacteria were screened through serial dilution. For isolations of cellulolytic microbes, aliquots from each dilution were cultured on carboxymethyl cellulose (CMC) containing Luria Bertani (LB) agar plate and the samples were incubated for a duration of 24 hours at a temperature of 37 °C. After this incubation period, a solution of 0.1% Congo red was applied to the LB agar plates containing the bacterial isolates and allowed to stain for 15 min. After rinsing the plates with a 1 M NaCl solution to remove the extra dye, the hydrolysis zones were developed around the colonies that indicated cellulase activity. Further, the cellulose degradation efficiency screening was evaluated by culturing the isolates in Luria Bertani broth supplemented with 1% CMC and incubated for 20 h at pH 7.0, 37 °C, and 200 rpm. 2% seed culture was transferred to a production medium containing 1% CMC, 20 g/L peptone, 1 g/L $MgSO_4$, and 3 g/L KH_2PO_4 [15] incubated at 37 °C, 200 rpm and adjusted pH 7.0 for up to 120 h. To assay the enzyme activity, the supernatant was collected at every 24 h intervals for the screening of a highly efficient cellulase-producing strain.

Morphological and biochemical characterization of the isolate

After the isolation, a potential strain was identified through Gram stain, endospore staining, and motility test. For the biochemical analysis of an isolate, the HiCarbo Kit (KB009 A/KB009 B1/KB009 C) was used for carbohydrate profiling, and other biochemical experiments were carried out using the KB002™ HiAssorted kit (HiMedia Laboratories).

Molecular identification

The strain of cellulase producing organism was identified by molecular identification. The bacterial isolate was first inoculated in nutrient broth and incubated for 20 h. After incubation, the old culture was used for genomic DNA isolation and purification [16]. The isolated DNA was amplified by polymerase chain reaction (PCR) using universal primer "27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACGA-3′)" [17]. By aligning the DNA sequences with the GenBank database accessible at the NCBI (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/), the BLAST program [18] was used to identify specific differences in the compared sequences. The Neighbor-Joining method was used for the construction of a phylogenetic tree. Rapid annotation of the metagenome using Metagenomics RAST server 4.0.3 subsystem technology [19] was used to analyze the sequence.

Antibiotic susceptibility test

The test is employed to identify the resistance and sensitivity of bacterial species to various antibiotics. The disc diffusion technique is a widely used, effective, and reliable method for this purpose. In this study, Himedia octadisc (HiMedia Laboratories) was used for antibiotic susceptibility testing [20, 21]. Mueller-Hinton (MH) agar plates were prepared and subsequently inoculated with 100 µl of bacterial culture using the spread plate method. Octadisc were placed on the plates using sterile forceps and the samples were maintained at a temperature of 35 °C for a duration ranging from 24 to 48 h after the inhibition zone was measured.

Enzyme production

The selected isolate was grown in a defined seed medium described by Bhatt *et al*. [8]. For cellulase production, an optimized agricultural waste medium was used, containing; A fermentation medium composed of 6% mung straw, 2% maltose, 2% soybean cake, 0.5% sodium nitrate, 0.3% dipotassium hydrogen phosphate, and 0.2% magnesium sulfate heptahydrate was prepared. The medium was supplemented with 2% seed culture and incubated at 35 °C under continuous shaking at 125 rpm for 72 h. To enhance cellulase production, key process parameters including pH, temperature, inoculum size, and agitation speed were systematically evaluated following the methodology outlined by Bhatt *et al*. [22].

Enzyme and protein assay

Cellulase activity was measured using the 3,5-dinitrosalicylic acid (DNSA) assay, where 3,5-dinitrosalicylic acid reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid. The reaction yielded an orange color and measured at 540 nm.

For DNSA estimation, 0.05 mL enzyme aliquots were used. The reaction mixture consisted of incubating each enzyme aliquot with 1% CMC dissolved in 0.1 M phosphate buffer at pH 5.0 and 55 ºC, for 8 min [23]. Miller's approach was used to measure the amount of sugar liberated [24]. A unit of enzyme activity is the quantity of enzyme that degrades to produce one micromole of reducing sugar per minute. Using glucose, a standard curve was plotted. Four replicate samples were used for each enzyme assay.

The protein concentration in the supernatant was determined using Lowry's method, with bovine serum albumin (BSA) as the standard. 4.5 mL of alkaline reagent and 0.5 mL of Folin reagent were combined with 1 mL of the enzyme solution for each experiment. Following a 30 min incubation period,

protein content was quantified using a BSA standard curve, with absorbance measured at 750 nm.

Purification of cellulase

The fermentation broth from 72 h cultures underwent centrifugation at a speed of 15,000 revolutions per minute for 15 min at a temperature of 4 °C. Following this process, the supernatant was collected and processed through ammonium sulfate precipitation, to which ammonium sulfate was subsequently introduced stepwise to reach a final saturation of 0–30%, 30–50%, and 50–80% with continuous stirring at 4 °C for 30 min. Centrifugation was used to obtain the precipitates under the same settings. Following each saturation stage, the enzymatic activity of the pellet and supernatant was assessed. After that, the pellet was reconstituted in a minimum of 50 mL of sodium acetate buffer (pH 5.5). To retain enzyme activity, all purification procedures were carried out at 4 °C [26].

After the precipitation, the enzyme solution was put onto a 30 x 600 mm Sephadex G-100 column that was previously adjusted with 50 mM sodium acetate buffer (pH 5.5). Following elution at a flow rate of 1.0 mL/min, the fractions were examined for enzyme activity and protein quantification.

Molecular weight determination

The molecular weight of the isolated enzyme was determined through polyacrylamide gel electrophoresis (PAGE), employing the Laemmli method as the analytical technique [27]. 12% resolving gel was prepared for the separation. A molecular weight marker kit from Genei Laboratories (Bangalore, India) was used as a reference. The protein bands were stained with 0.25% Coomassie Brilliant Blue R-250 in a solution containing methanol, acetic acid, and distilled water (3:1:6 ratio) and decolorized in the same solvent system.

A native PAGE zymogram was conducted utilizing a 12% resolving gel, incorporating 2 mL of a 0.5% (w/v) carboxymethylcellulose (CMC) solution. Electrophoresis was performed at 4 °C. To detect cellulase activity, the gel was incubated in a 1% (w/v) CMC solution at 4°C for 45 min to allow complete CMC diffusion. The gel was then incubated at 57 °C for 45 hours to allow enzyme activity. The gel was treated with 0.1% Congo red for staining and subsequently destained using 1 M sodium chloride (NaCl).

Temperature and pH effects on the stability and activity of purified enzyme

To examine the influence of pH on enzyme activity, pH 4.5 to pH 9.0 was used. The enzyme was incubated for 48 h at 35 °C with a pH 3.0 to pH 9.0 for determination of the residual activity. Tris–HCl (pH 8.0–9.0), sodium acetate (pH 3.0–6.0), and sodium phosphate (pH 6.0–8.0) were the buffer solutions utilized at 100 mM concentrations. The reactions were conducted at temperatures from 25 °C to 70 °C, and the effect of temperature on enzyme activity was observed at the optimum pH value. The enzyme solution was pre-incubated at 0 °C, 10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C and 70 °C to assess thermal stability for time intervals of 30, 60, 90, 120, 150 and 180 min.

Statistical analysis: Significant differences between experimental variables were analyzed using two-way ANOVA. The data were analyzed using GraphPad Prism version 5.01.

Results and Discussion

Screening of isolate for cellulase production

For this study, soil samples were collected from different locations. The microorganisms were cultured by serial dilution and plated on CMC-containing LB agar plates, resulting in the growth of 110 colonies. Among them, 26 colonies exhibited zones of clearance, confirming their ability to degrade cellulose by producing cellulase.

For a comparative analysis of cellulase production, the isolates were cultured in liquid media. Each cellulase producer was pre-cultured in seed media for 16 h, followed by inoculation of 2% seed culture in production media. Enzyme activity was measured at every 24 h intervals for 120 h. Among 26 strains, the highest cellulase producer strain designated as OKB3 (1.565 U/mL), showed the highest cellulase production within shorter incubation times and was able to grow under submerged fermentation conditions (Table 1).

Table 1: Cellulase activities of isolated colonies

Bacterial Isolate	Cellulase-activity (U/mL)	Bacterial Isolate	Cellulase-activity (U/mL)
Isolate 1	1.127	Isolate 14	0.895
Isolate 2	1.022	Isolate 15	0.614
Isolate 3	1.565	Isolate 16	0.712
Isolate 4	1.491	Isolate 17	1.426
Isolate 5	1.137	Isolate 18	1.173
Isolate 6	0.965	Isolate 19	1.125
Isolate 7	0.764	Isolate 20	0.825
Isolate 8	1.053	Isolate 21	1.079
Isolate 9	0.835	Isolate 22	0.348
Isolate 10	0.974	Isolate 23	0.478
Isolate 11	1.178	Isolate 24	0.862
Isolate 12	0.625	Isolate 25	0.566
Isolate 13	0.734	Isolate 26	1.315

Morphological and Biochemical Characteristics

Based on Gram's staining, the OKB3 was Gram-positive and rod-shaped morphology, indicating a Bacillus-like structure. The presence of spores was detected in OKB3. In addition, this strain showed motility and a scattered distribution pattern when observed microscopically.

The OKB3 identification was performed using a carbohydrate detection kit. OKB3 tested positive for multiple compounds, including fructose, maltose, xylose, and lactose. Positive results were also observed for dextrose, raffinose, sucrose, trehalose, and sodium gluconate. Additionally, the strain showed positivity for glycerol, salicin, mannitol, sorbitol, rhamnose, and cellobiose. Further positive reactions were noted for sorbose, ONPG, xylitol, D-arabinose, citrate, esculin, and malonate. On the other hand, the following sugars yielded negative reactions: Galactose, melibiose, L-arabinose, and mannose, along with inulin, dulcitol, and inositol, are among the tested compounds. Additionally, erythritol, α-methyl-D-mannoside, arabitol, melezitose, adonitol, and α-methyl-D-glucoside are also included in the analysis. These characteristics indicated the fundamental information about the selected bacterial strain's structural and functional diversity, which are important for further classification and biotechnological applications.

Molecular characterization

MG-RAST (Metagenomics Rapid Annotation Subsystem Technology) was used to analyze the genomic sequence of the bacterial strain with high cellulase production. This approach allowed the identification of the strain based on their taxonomic classification as derived from the distribution of taxonomic hits. The taxonomic breakdown for selected strain OKB3 is in the domain Bacteria represented at 99.92% and in the phylum Firmicutes (99.67%). It is classified in class Bacilli (97.47%) and in the order Bacillales (99.51%). It is in family Bacillaceae (99.29%) and is finally classified at genus level as *Bacillus* (99.20%).

The neighbor-joining approach was used to build the phylogenic tree, confirming the close relationship between strain OKB3 and *Bacillus amyloliquefaciens* strain YP6. The results suggest that strain OKB3 shares significant genetic similarities and is closely related to *Bacillus amyloliquefaciens* (Fig 1).

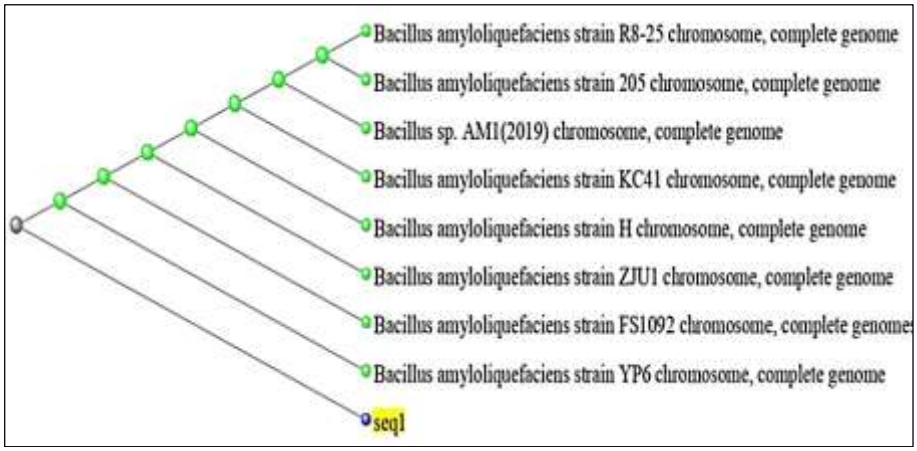

Fig 1: Phylogenetic tree of isolate 3

Antibiotic susceptibility test of strain OKB3

The susceptibility of strain OKB3 to a group of 14 antibiotics was tested using the inhibition zone method. The results showed that the strain was sensitive to 10 antibiotics and resistant to 4 (Table 2).

The antibiotic susceptibility profile of OKB3 shows broad susceptibility to a variety of antibiotics, particularly those with a wide range of action, such as ciprofloxacin and ofloxacin. The resistance of the strain to penicillin-G and ampicillin is remarkable, suggesting that beta-lactamase production or other resistance mechanisms may be present. The lack of sensitivity to colistin and erythromycin is a further indication of possible intrinsic or acquired resistance, which should be further investigated, particularly in relation to mechanisms of multidrug resistance.

The susceptibility profile of strain OKB3 ensures compatibility with common antibiotics, reducing the risk of contamination, while its resistance to important antibiotics increases stability, making it suitable for controlled fermentation in industrial applications.

Table 2: Antibiotic susceptibility test

Antibiotic	Concentration	Zone of Inhibition	Susceptibility
Cephalothin (Ch)	30 mcg	+	Susceptible
Clindamycin (Cd)	2 mcg	+	Susceptible
Co-Trimoxazole (Co)	25 mcg	+	Susceptible
Erythromycin (E)	15 mcg	-	Resistant
Gentamicin (G)	10 mcg	+	Susceptible
Ofloxacin (Of)	1 mcg	+	Susceptible
Penicillin-G (P)	10 units	-	Resistant
Vancomycin (Va)	30 mcg	+	Susceptible
Ampicillin (A)	10 mcg	-	Resistant
Ciprofloxacin (Cf)	10 mcg	+	Susceptible
Colistin (Cl)	10 mcg	-	Resistant
Nitrofurantoin (Nf)	300 mcg	+	Susceptible
Streptomycin (S)	10 mcg	+	Susceptible
Tetracycline (T)	30 mcg	+	Susceptible

Purification of *Bacillus amyloliquefaciens* OKB3 cellulase

Cellulase was purified by precipitation using ammonium sulfate, which was subsequently followed by column chromatography. Precipitation was extended to three different steps for maximum recovery of the protein. Initially, 0-30% ammonium sulfate saturation was used, and the pellet presented 306.21 U/mL of cellulase activity. Later, 50% ammonium sulfate precipitation resulted in an activity of 340.29 U/mL in the pellet, showing that cellulase remained in the supernatant. Lastly, at 80% ammonium sulfate saturation, the maximum recovery was obtained in the pellet (431.71 U/mL), thus, efficient precipitation of the target protein occurred.

Further purification by Sephadex G-100 column chromatography showed that cellulase activities occurred in fractions 22, 38, and 48 respectively named CelA, CelB, and CelC (Fig 2). The purification of CelC resulted in a 2.34-

fold increase in purity and a recovery of 33.34% of the initial cellulase activity with 2194.65 U/mg specific activity (Table 3). The molecular weight of CelC was further confirmed by SDS-PAGE and zymogram analysis. Zymogram analysis of the native form and SDS-PAGE analysis under denatured conditions revealed a single active band with molecular weights of 96 kDa (Fig 3). This monomeric structure closely resembles cellulase from *Artemia salina* [28].

Table 3: A table summarizes the purification of CelC

Steps	Total enzyme activity (U)	Total Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture supernatant	480.56	0.514	934.94	100	1
AS (80%)	431.71	0.389	1109.79	89.83	1.186
Gel filtration	160.21	0.073	2194.65	33.34	2.34

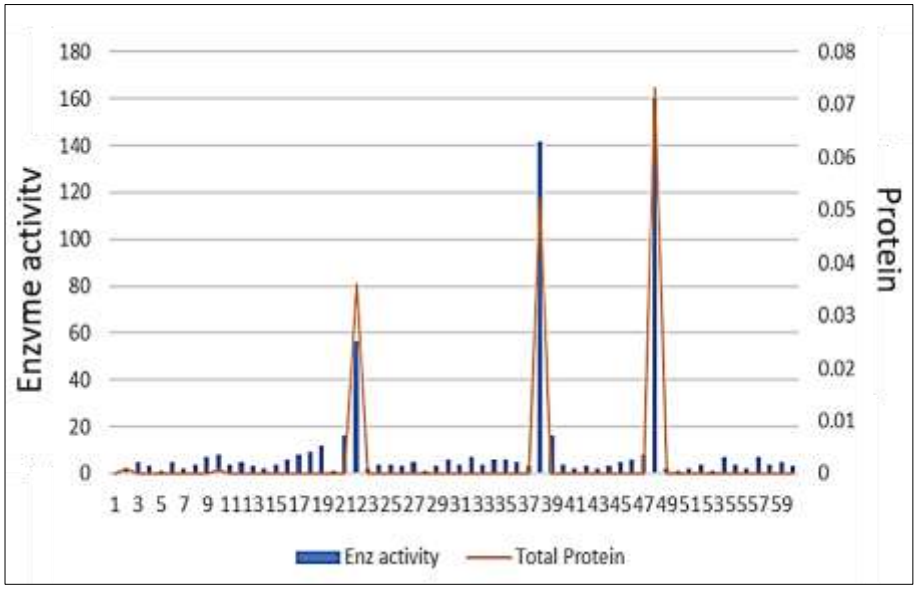

Fig 2: Fraction pattern of purified cellulase synthesized by *Bacillus amyloliquefaciens* strain OKB3

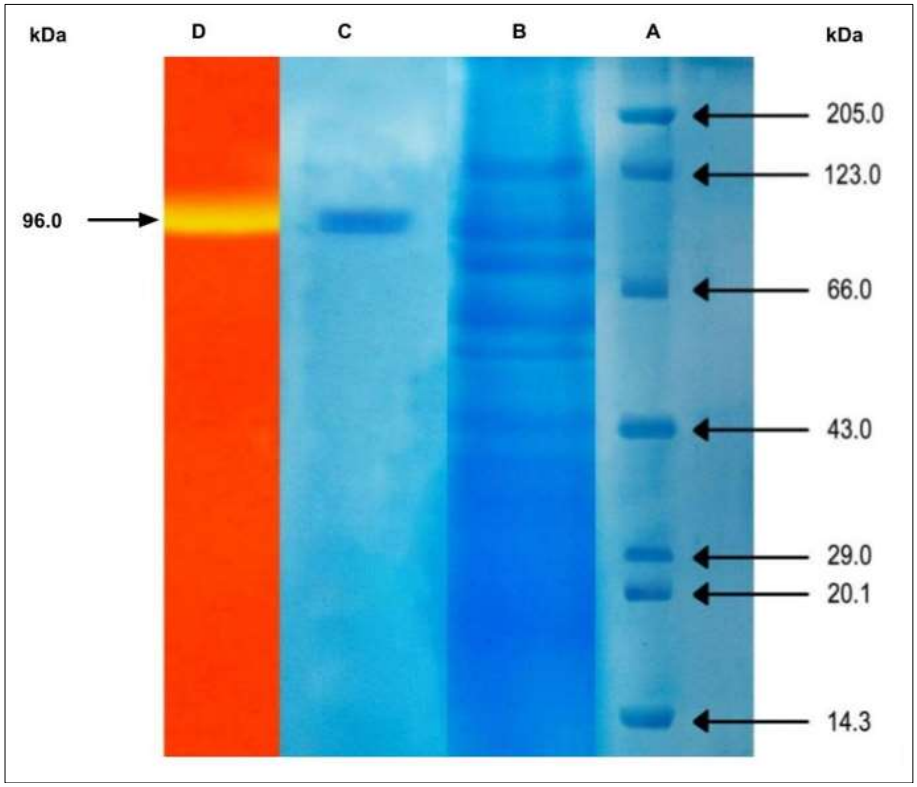

Fig 3: Molecular weight determination: Lane A: Molecular size marker (kDa), Lane B: Crude enzyme, Lane C: Purified enzyme (CelC) on SDS PAGE, Lane D: Purified enzyme (CelC) on Zymogram.

Effects of pH on relative and residual activity of purified cellulose: CelC was analyzed to determine their optimum pH for the highest activity of enzyme. In terms of pH, the enzyme performed the highest activity at a slightly acidic

value of 6.0, within a range of 4.5 to 9.0 (Fig 4A). However, at pH beyond 6.0, their efficiency dropped drastically, suggesting that these cellulases produced by strain OKB3 are not well suited to alkaline environments. These results are in agreement with those reported for cellulases from *Bacillus subtilis* YJ1 and *Aspergillus fumigatus* JCM 10253 [29, 30]. The cellulases also showed remarkable stability under slightly acidic conditions, especially at pH values between 5.5 and 6.0. Outside this range, the enzymes became significantly less effective, with activity decreasing by 50% at pH 9.0 (Fig 4B).

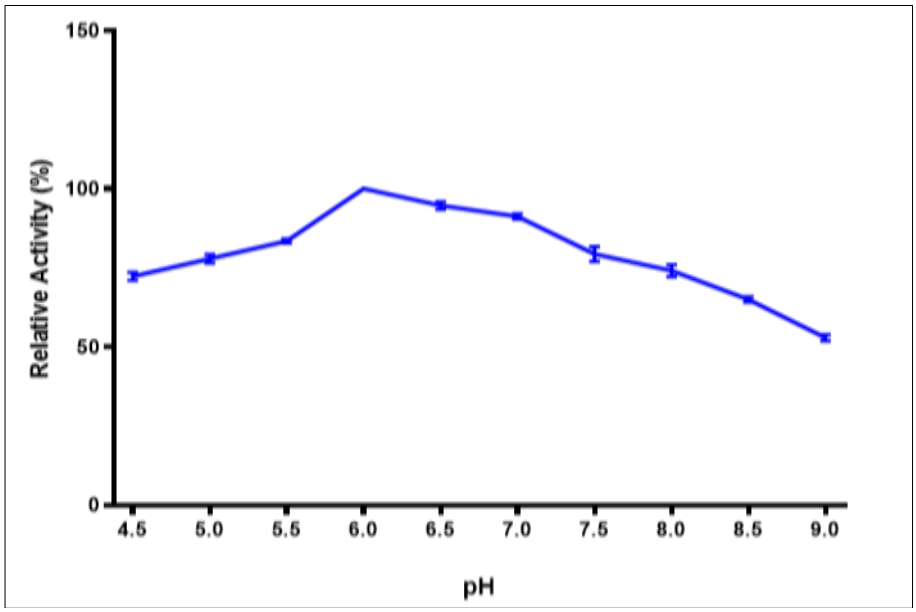

Fig 1A: Relative activities of pH on purified cellulase were measured according to the standard assay with buffer solutions at 0.1 M as follows: sodium acetate, pH 3.5–6.0; sodium phosphate, pH 6.0–8; Tris–HCl, pH 8–9.

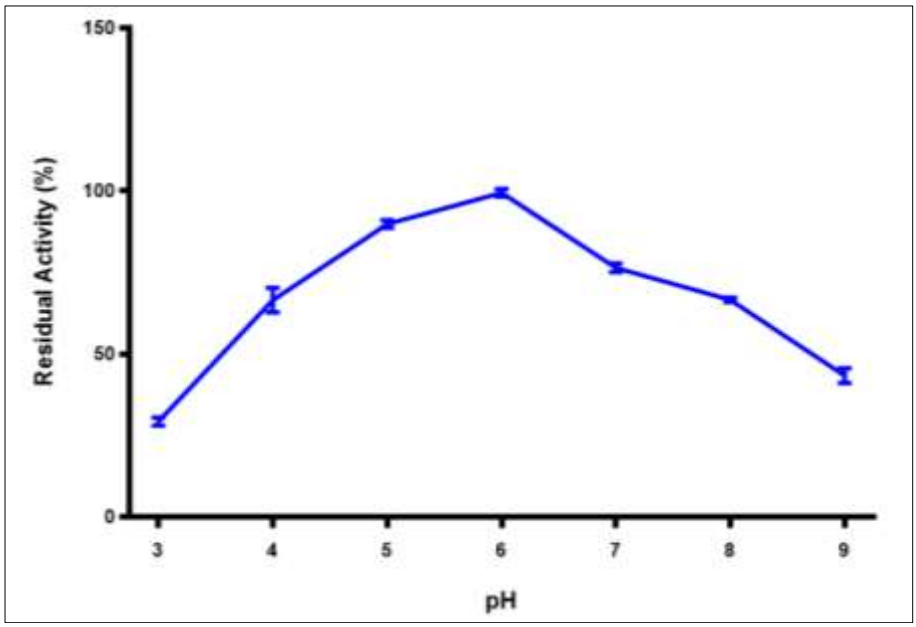

Fig 4B: Residual activity of purified cellulase at pH range of 3.0 to 9.0

Effects of temperature on relative and residual activity of purified cellulase

Temperature significantly influences the activity of enzymes, affecting their efficiency and overall functionality. Cellulases showed their highest activity at 55 ˚C when tested in a range of 25 ˚C to 70 ˚C (Fig 4C). Exceeding this temperature, whether higher or lower, resulted in a significant decrease in activity. This decline is probably due to the enzymes losing their structure and function, a process known as protein denaturation. Regmi *et al.* [31] studied thermostable cellulase that showed the highest activity at the optimum temperature of 60 °C and retained 67% of its activity even at 55 ˚C. To test the stability, the purified enzyme was pre-incubated at different temperatures ranging from 0 ˚C to 70 ˚C. They remained highly stable at lower temperatures (0 ˚C to 4 ˚C) after 180 min incubation, but their activity decreased with increasing heat and prolonged exposure (Fig 4D).

These results confirm the optimum conditions as pH 6.0 and 55 ˚C for maximizing cellulase activity. In addition, their stability under acidic conditions makes them particularly useful for applications in cellulase hydrolysis, as they reduce the need for frequent pH adjustments..

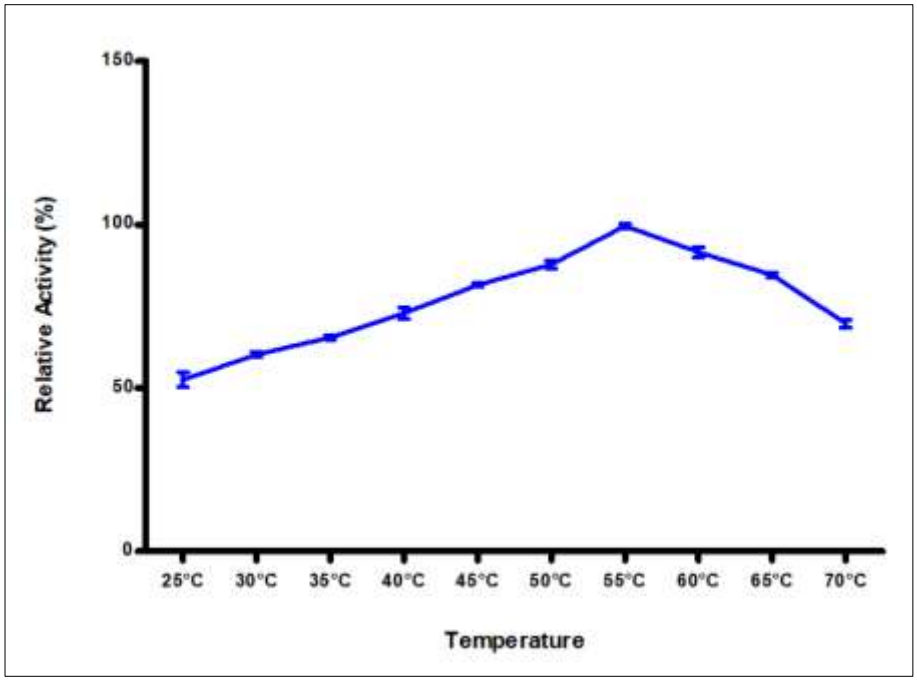

Fig 4C: Relative activity of purified cellulase on temperature ranging from 25 ˚C to 70 ˚C.

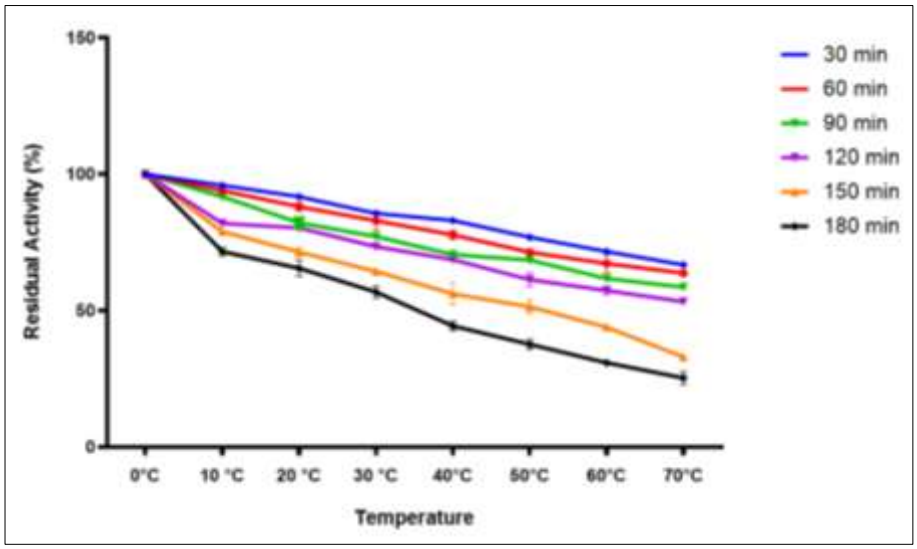

Fig 4D: Residual activity of purified cellulase at temperatures 0 ˚C, 10 ˚C, 20 ˚C, 30 ˚C, 40 ˚C, 50 ˚C, 60 ˚C, and 70 ˚C at a different time interval.

Fig 4: The effect of pH and temperature on the activity of the purified CelC

Conclusion

This research presents the findings of the production of an acidothermophilic CelC enzyme from a newly identified strain *Bacillus amyloliquefaciens* OKB3. Its stability at lower temperatures suggests that the enzyme can be stored for extended periods and used in enzymatic reactions. Its monomeric structure (96 kDa) also indicates the structural and functional properties of the enzyme. These results reveal the ability of the enzyme to perform low-cost bioconversion of an agricultural by-product and suitable to utilize in fuels and value-added products and it may have substantial ecological and economic impact on industrial biotechnological processes.

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