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RESEARCH PAPERS

Biochemical properties and industrial potential of a thermostable, Alkali-tolerant cellulase from *Bacillus krulwichiae* BW4(3)

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Highlights

- Bacillus krulwichiae BW4(3) produces cellulase active at pH 11 and 50 °C
- The cellulase retains high activity under moderate saline conditions
- High substrate affinity and catalytic efficiency were demonstrated
- Mn^{2+}, K^{+}, and Mg^{2+} enhance cellulase activity and stability
- Soybean meal enables cost-effective cellulase production at scale

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KEYWORDS
Cellulase;
Bacillus krulwichiae BW4(3);
Lonar Crater;
Alkaliphiles and halophiles.

Abstract: A cellulase-producing bacterium, *Bacillus krulwichiae* BW4(3), was isolated from the hypersaline and hyperalkaline environment of Lonar Lake, India, and evaluated for its cellulase activity under various parameter and nutritional conditions. The cellulase works best at a pH of 11 and a temperature of 50°C, meaning it's highly tolerant of alkaline conditions and moderately tolerant of heat. Kinetic analysis revealed a Michaelis constant (Km) of 0.33 mM and a maximum velocity (Vmax) of 158.73 U/mL, suggesting high substrate affinity and catalytic efficiency. The cellulase showed optimal performance in the presence of 4% NaCl, confirming moderate halotolerance, and retained significant activity in organic solvents, particularly methanol and ethanol. The cellulase activity was markedly enhanced by Mn^{2+} and K^{+} ions, whereas Na^{+} and Ca^{2+} ions were less effective in stimulating its function. Yeast Extract was identified as the most effective nitrogen source, followed by peptone and beef extract, whereas inorganic nitrogen sources such as ammonium chloride were less favorable. These findings highlight the potential of *Bacillus krulwichiae BW4(3)* cellulase as a robust biocatalyst for industrial applications in alkaline, moderately saline, and solvent-rich environments, as well as for cost-effective enzyme production using plant-based protein substrates.

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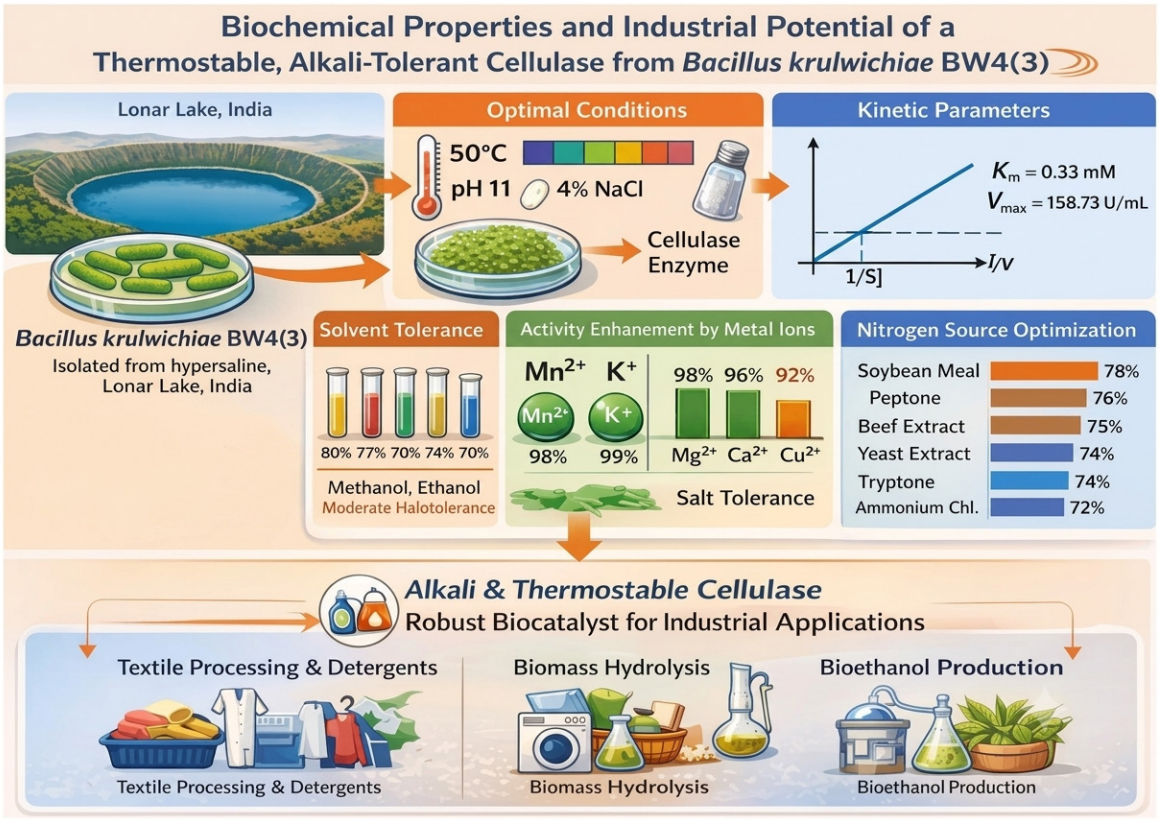
Graphical Abstract



Introduction

Cellulose is the most abundant renewable organic polymer on Earth, forming the primary structural component of plant cell walls and constituting a significant fraction of agricultural residues, forestry by-products, and industrial biomass waste. Structurally, cellulose is a linear ß-1,4-linked D-glucose polymer that assembles into a highly ordered crystalline microfibrillar matrix, making it resistant to enzymatic hydrolysis. Complete degradation of cellulose requires a synergistic enzymatic system composed of endo-ß-1,4-glucanases (EC 3.2.1.4), which randomly cleave internal ß-1,4 linkages, exo-ß-1,4-glucanases or cellobiohydrolases (EC 3.2.1.91), which release cellobiose units from chain ends, and ß-glucosidases (EC 3.2.1.21), which hydrolyze cellobiose to glucose (Bhat, 2000). The hydrolytic products can be fermented to bioethanol, biobutanol, organic acids, or converted into other value-added biochemicals, contributing to sustainable bioeconomy initiatives (Pal et al., 2024). While filamentous fungi such as *Trichoderma reesei* and *Aspergillus niger* have long been the industrial important for cellulase production, bacterial cellulases have gained increasing attention due to their faster growth rates, robustness, and ability to function under extreme physicochemical conditions, including high pH, elevated temperature, and high salinity (Horikoshi, 1999; Gupta et al., 2012). Bacteria also offer advantages in genetic manipulability and the secretion of enzyme for specific industrial applications. Lipase from fungi is applicable for food processing as well as biodiesel production while also applicable in pharmaceuticals and waste treatment because of adverse condition with organic solvents (Mahfoudhi et al., 2022; Morya et al., 2023).

Haloalkaliphilic bacteria from soda lakes are particularly promising sources of extremozymes. Soda lakes, such as those found in Africa's Rift Valley, Inner Mongolia, and India's Lonar Crater, are characterized by high alkalinity (pH 9–12) and salinity, conditions that mimic harsh industrial environments (Grant, 2006). Microorganisms inhabiting these ecosystems have evolved enzymes with high stability and activity under alkaline and saline conditions, making them excellent candidates for industrial processes such as textile desizing, pulp biobleaching, detergent formulation, leather processing, and biomass saccharification (Horikoshi, 1999; Ventosa et al., 1998). *Bacillus krulwichiae* is a Gram-positive, spore-forming, facultatively anaerobic, haloalkaliphilic bacterium first described by Nielsen et al. (2012) from highly alkaline environments. It is known for its metabolic versatility, including the production of industrially relevant hydrolases such as proteases, amylases, and cellulases that remain stable under extreme conditions. The present study focuses on cellulase production by *B. krulwichiae* BW4(3) isolated from Lonar soda lake, India, aiming to optimize the physicochemical parameters for enzyme production, characterize the effects of pH, temperature, salinity, nitrogen sources, organic solvents, and metal ions on activity, and determine the kinetic parameters (Km and Vmax) for its cellulase. The findings are expected to contribute to the development of industrially viable cellulases capable of functioning in extreme process environments.

Material and methods

Sample collection from Lonar Crater

Water samples were collected sterile bottles from Lonar crater. Enrichment of the sediment samples in Horikoshii B medium and streaking plate technique were used for the isolation of pure colony. The *Bacillus* culture was maintained on the same medium slant for further analysis while gycerol stock was prepared for the long duration storage.

Screening for cellulase activity

The primary screening involved using CMC agar plates with a specific formula. The medium contained 10.0 g of CMC, along with nutrients like yeast extract and several salts (NaCl, $(NH_4)_2SO_4$, K_2HPO_4, and $MgSO_4 \cdot 7H_2O$). The pH was set to 10.0 before the plates were sterilized. Overnight cultures of *Bacillus strain* BW4(3) were spot-inoculated and incubated at 37 °C for 48–72 h. After incubation, the plates were flooded with 0.1% Congo red solution for 15 min, rinsed with 1 M NaCl for 15 min, and examined for clearance zones indicative of cellulase activity (Teather & Wood, 1982).

Identification of bacteria by 16S rDNA sequencing and phylogenetic analysis

Genomic DNA from the *Bacillus* isolate was extracted using the standard phenol chloroform method. The partial 16S rRNA gene was amplified by polymerase chain reaction (PCR) employing universal eubacterial primers 16F27 (5′-CCAGAATTGATCMTGGCTCAG-3′) and 16R1525 (5′-TTCTGCAGTCTAGAAGGAGGTGWTCCAGCC-3′). The PCR conditions included an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at the primer-specific temperature, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The amplified products were purified by polyethylene glycol–NaCl precipitation and sequenced using an Applied Biosystems Model 3730 DNA sequencer (Foster City, CA, USA). The obtained 16S rRNA gene sequences (approximately 900 bp) were analyzed using the NCBI BLAST program to identify closely related taxa. Multiple sequence alignment was performed using CLUSTAL W (version 1.8), and a phylogenetic tree was constructed using the neighbor-joining method based on evolutionary distances in the MEGA software package.

Enzyme production via submerged fermentation

Submerged fermentation was carried out in 250 mL Erlenmeyer flasks containing 250 mL of production medium (per liter): 10.0 g CMC, 1.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, and 30.0 g NaCl, with pH adjusted to 10.0. The flasks were inoculated with 2% (v/v) of an overnight culture and incubated at 37 °C with shaking at 150 rpm for 72 h. The culture broth was centrifuged at 10,000 rpm for 15 min at 4 °C, and the supernatant was collected as the crude enzyme extract.

Cellulase assay

Enzyme activity was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959) using 1% CMC in 50 mM glycine–NaOH buffer (pH 10.0) as substrate. The reaction mixture (0.5 mL enzyme + 0.5 mL substrate) was incubated at the appropriate temperature for 30 min. Reducing sugars released were measured at 540 nm using glucose as a standard. One unit (U) of cellulase activity was defined as the amount of enzyme releasing 1 µmol of glucose equivalents per min under assay conditions.

Effect of pH

The effect of pH on cellulase activity can be determined by assaying enzyme activity across a pH range (7.0–12.0) using 1% carboxymethyl cellulose (CMC) as substrate and the DNS method to measure released reducing sugars. Buffers of equal ionic strength (50 mM) phosphate (pH 6–8), Tris–HCl (pH 8–9), and glycine–NaOH (pH 9–10.5) are used to maintain desired pH levels. The reaction mixture (1 mL CMC + 0.1 mL enzyme) is incubated at 50 °C for 10 min, after which 1 mL DNS reagent is added to stop the reaction. The mixture is boiled for 5 min, cooled, and absorbance measured at 540 nm. Glucose standards are prepared to generate a calibration curve for calculating enzyme activity in µmol glucose released per min per mL enzyme. The relative activity (%) is plotted against pH to find the optimum pH. For pH stability, the enzyme is pre-incubated in buffers of varying pH (without substrate) and residual activity measured at the optimum pH.

Effect of temperature

The effect of temperature on cellulase activity was studied by measuring enzyme activity at various temperatures while keeping pH and substrate concentration constant. The assay is usually performed using 1% carboxymethyl cellulose (CMC) in citrate buffer (pH 10.0). The effect of temperature on cellulase activity was studied by measuring enzyme activity at various temperatures (typically between 20 °C and 80 °C, for 10 mins). The amount of reducing sugar released is then determined using the 3,5-dinitrosalicylic acid (DNS) method, with absorbance measured at 540 nm. Enzyme activity (U/mL) is calculated from a glucose standard curve, and relative activity is expressed as a percentage of the maximum activity (100%). The optimal temperature corresponds to the point of highest activity, while loss of activity at higher temperatures indicates enzyme denaturation (Bhat, 2000; Lynd et al., 2002).

Effect of nitrogen sources

The effect of nitrogen sources on cellulase production was studied to determine which nitrogen sources best supports microbial growth and enzyme synthesis. In this experiment, a basal medium containing 1% carboxymethyl cellulose (CMC) as the carbon source was prepared, and various nitrogen sources were tested separately such as Yeast Extract, Beef extract, Peptone, Soyabean Meal and concentration was 1%.

Each flask was inoculated with a cellulolytic microorganism and incubated under optimum conditions. The 24h old fresh culture was prepared and 0.5 mL broth culture were inoculated in CMC medium (100 mL) and incubated at 37°C for 48h. After incubation, culture filtrates were collected by centrifugation, prepared cellulase and activity was assayed using 1% CMC substrate and the DNS method for reducing sugar estimation at 540 nm were performed (Sadhu & Maiti, 2013; Immanuel et al., 2006).

Effect of organic solvents and metal ion on cellulose activity

The effect of organic solvents on cellulase activity was investigated. In this study, incubating the enzyme (1 mL) with various different organic solvents such as methanol, hexane, and chloroform (1%). The effect of metal ions on cellulase activity studied by incubating the enzyme (1 mL) with various metal ions such as Na, K, Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}(1%). (Ogino et al., 2000; Klibanov, 2001).

Effect of substrate concentration

The effect of substrate concentration on cellulose (1mL) activity evaluated by varying the concentration of carboxymethyl cellulose (CMC) in the enzyme assay while keeping other parameters constant such as pH (10), temperature (50°C), and incubation time (30 min). Typically, cellulase was incubated with CMC solutions ranging from 0.1 to 2.0% (w/v) in citrate–phosphate buffer (pH 10.0) at 50 °C for 30 min. The reducing sugars released were measured by the DNS method at 540 nm using glucose as a standard. Enzyme activity was calculated in units (µmol glucose released per min), and a plot of initial reaction rate (v_0) versus substrate concentration ([S]) was prepared.

Results and discussion

The phylogenetic analysis based on 16S rRNA gene sequences indicated that the taxonomic position of strain BW4(3) belong to the *B. krulwichiae* AB086897 (97%) and *Bacillus* sp. OS12 EF491962 (Figure 1). The highest similarity values with the sequences of obligately alkaliphilic microorganisms that can degrade benzoate and m-hydroxybenzoate were isolated to study the degradation of aromatic compounds in alkaline environments (Yumoto et al., 2003). The similarity of 97% or higher is typically sufficient to place an isolate within the *Bacillus* genus.

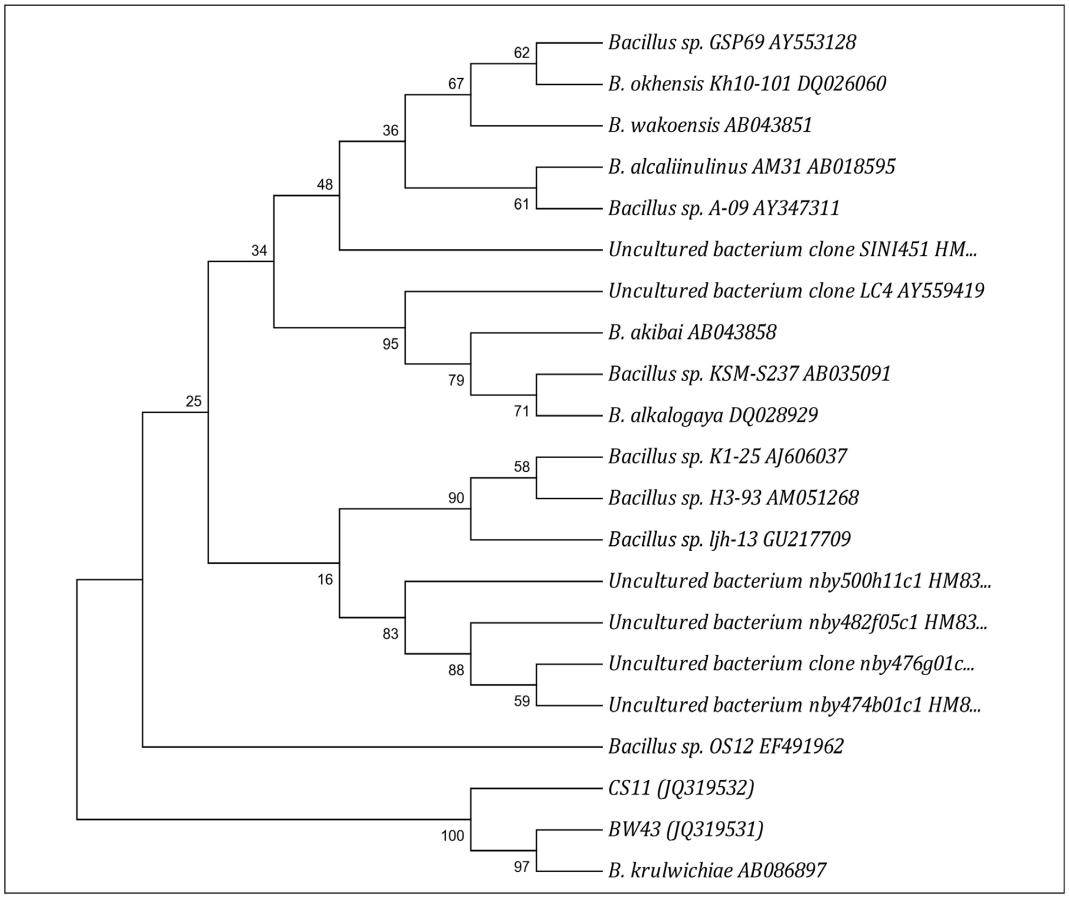


Figure 1. Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of Lonar lake isolates BW4(3) and some of their closest phylogenetic relatives. The tree was created by the neighbor-joining method. The numbers on the tree indicates the percentages of bootstrap sampling.

Effect of pH on cellulose activity

Enzyme activity showed a gradual increase from pH 7 (68% relative activity, ~88 U/mL) to pH 9 (73% relative activity, ~98 U/mL). At pH 10 and 11, activity plateaued near the maximum, with the highest value recorded at pH 11 (74% relative activity, ~101 U/mL). Beyond this optimum, a sharp decline was observed at pH 12, where relative activity dropped to 68% (~86 U/mL). Kaur et al., 2020 also obtained the same result for cellulase from CEL7 was optimum activity at alkaline pH 9. Similar results also observed to Sethi et al. (2013) and that cellulase activity to be optimum in the range pH 9.0-11.0. Yilmaz and Gurkok (2025), studied on production, purification and characterized extremozymes cellulase from *Bacillus pumilus* VLC7 from Soda Lake. They observed the optimum pH was found to be 9.0 and the lowest activity was revealed at pH 4.0 for cellulase. The cellulose production from *Bacillus subtilis* were found to be pH values ranging from pH 4.0 to pH 9.0 (Poovazhagi & Ramesh 2019). This indicates that *B. krulwichiae BW4(3)* cellulase was alkali-tolerant with peak performance in a slightly strong alkaline environment.

The observed optimum at pH 11 suggests that the cellulase from *B. krulwichiae BW4(3)* was well suited for industrial processes requiring high-alkaline stability, such as textile bio-polishing, detergent formulations, and biomass saccharification in alkaline pretreatment liquors. Alkali tolerant cellulases are relatively rare in nature, but were often isolated from soda lakes, alkaline soils, and extremophilic microorganisms (Horikoshi, 1999; Sharma et al., 2017). The gradual increase in activity from neutral to alkaline pH indicates that the enzyme's active site conformation remains stable under deprotonated conditions, possibly due to enhanced ionic interactions and hydrophobic packing within the catalytic domain (Singh et al., 2016). The significant drop-in activity at pH 12 suggests that extreme alkalinity may disrupt tertiary structure by deamidation or peptide bond hydrolysis (Bhat & Bhat, 1997). Similar pH optima have been reported for *Bacillus halodurans* and *Bacillus clausii*. Cellulases, which also retain structural stability under high pH due to their amino acid composition enriched in acidic residues and higher proline content (Patagundi et al., 2014). Given *B. krulwichiae*'s natural habitat in alkaline environments, the cellulase's stability across a broad pH range is consistent with its ecological adaptation and indicates potential utility in biotechnological applications where conventional cellulases lose activity (Figure 2).

Effect of temperature on cellulose activity

At 40 °C, the cellulase exhibited ~72% relative activity (~94 U/mL). Activity increased with temperature, peaking at 50 °C with ~75% relative activity (~99 U/mL), indicating the optimal temperature for maximal catalytic efficiency. At 60 °C, activity remained high (~74% relative activity, ~98 U/mL), but beyond this point, a gradual decline was observed. At 70 °C and 80 °C, activity decreased moderately, reaching ~72% and ~71% relative activity respectively. At 90 °C and 100 °C, a pronounced decline occurred, with relative activity dropping to ~70% (~93 U/mL) and ~69% (~92 U/mL), suggesting heat-induced destabilization of the enzyme structure. Similar results also obtained Yilmaz & Gurkok (2025), enzyme showed optimum activity at 40 °C for 90 min. Poovazhagi and Ramesh observe Results that 37 °C was the most optimum temperature for cellulase activity (8.80 U/mL). The enzyme was the most active at desirable temperature of 20 °C. This conclude that the bacterial strain CEL7 is psychrophilic in nature (Kaur et al., 2020). The temperature profile indicates that *B. krulwichiae BW4(3)* cellulase was moderately thermotolerant, with an optimal activity at 50 °C. This is consistent with several *Bacillus*-derived cellulases adapted for alkaline environments, which typically show optima between 45–60 °C (Sharma et al., 2017; Patagundi et al., 2014). The retention of high activity up to 60 °C suggests a robust tertiary structure capable of resisting moderate thermal denaturation, likely due to increased disulfide bonds, salt bridges, and hydrophobic interactions stabilizing the catalytic domain (Vieille & Zeikus, 2001).

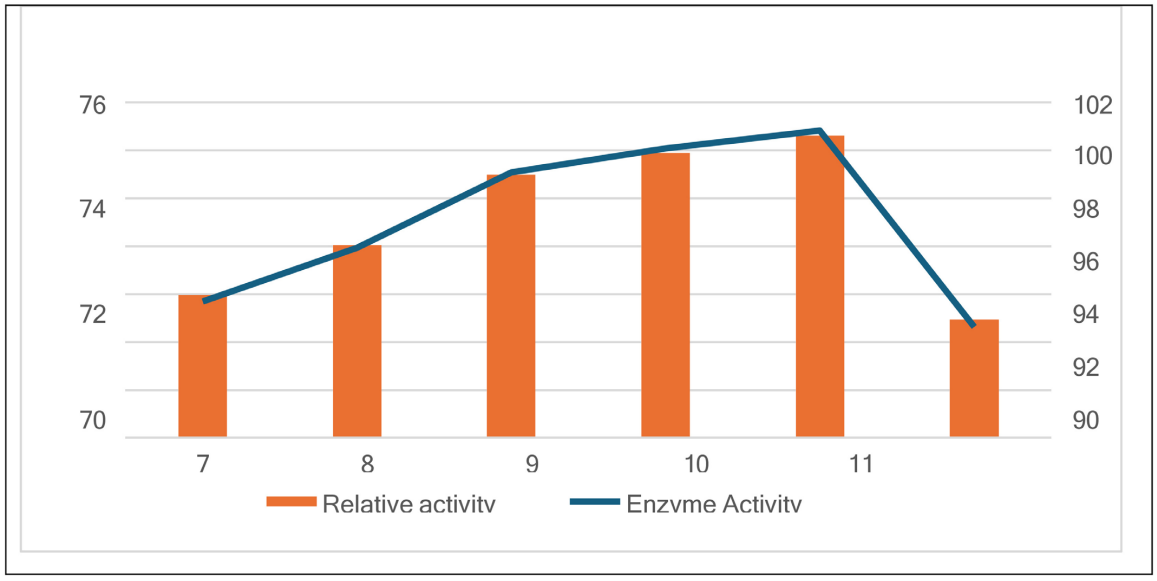


Figure 2. Effect of pH on Cellulase activity.

The decline in activity above 70 °C may be attributed to irreversible unfolding of the protein's active site, disruption of hydrogen bonding, and aggregation (Bommarius & Riebel, 2004). However, the residual activity (~92% of peak) even at 100 °C is noteworthy, indicating potential applicability in high-temperature industrial processes such as bioethanol production from lignocellulosic biomass, where pretreated substrates are often hot (Kuhad et al., 2011). In comparison to mesophilic cellulases, the thermostability of *B. krulwichiae BW4(3)* enzyme confers an operational advantage, as higher reaction temperatures increase substrate solubility, reduce viscosity, and minimize microbial contamination during industrial processing (Turner et al., 2007) (Figure 3).

Km of cellulose

The enzyme activity increased rapidly with substrate concentration up to approximately 0.2 mM, after which the curve began to plateau, indicating substrate saturation of the active sites. The calculated Km value (~0.18 mM) (Figure 4) corresponds to the substrate concentration at which the reaction velocity reaches half of Vmax. The Vmax value was estimated at ~88 U/mL, representing the theoretical maximum catalytic rate under saturating substrate conditions. The relatively low Km value indicates a high affinity of *Bacillus krulwichiae BW4(3)* cellulase for its substrate, suggesting that the enzyme can operate efficiently even at

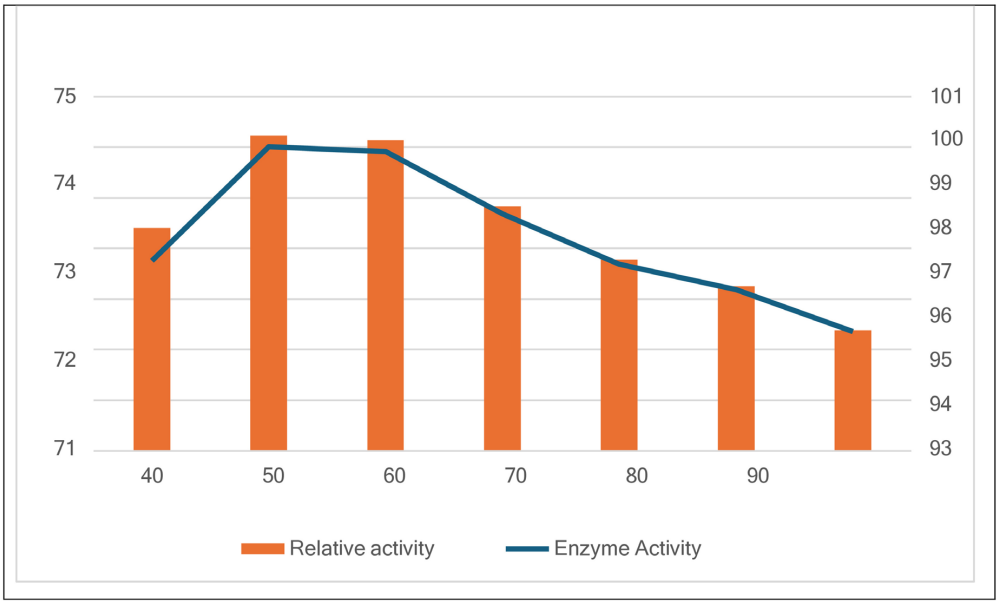


Figure 3. Effect of temperature on cellulase.

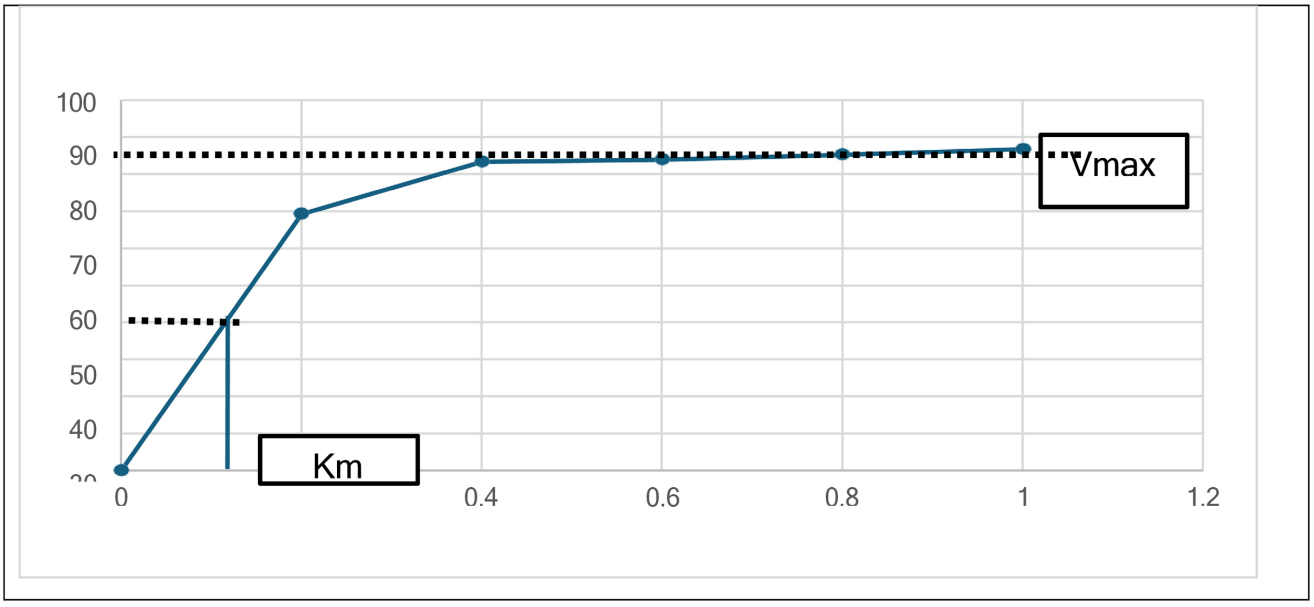


Figure 4. Michaelis Menten.

low substrate concentrations. Such high-affinity enzymes are advantageous in bioconversion processes where the substrate is present in limited amounts, such as in the degradation of lignocellulosic biomass post-pretreatment (Bhat & Bhat, 1997). The observed Vmax reflects the intrinsic catalytic capacity of the enzyme and, combined with the optimal pH (11) and temperature (50 °C) data, indicates potential utility in industrial saccharification processes. Comparatively, similar Km values have been reported for cellulases from alkaliphilic *Bacillus halodurans* and *Bacillus clausii* (Patagundi et al., 2014), supporting the conclusion that extremophilic *Bacillus* strains produce efficient biomass-degrading enzymes. The hyperbolic nature of the plot conforms to classic Michaelis-Menten kinetics, indicating a single-substrate, single-active-site catalysis without significant allosteric effects. At high substrate concentrations (>0.5 mM), the reaction velocity plateaued, suggesting no substrate inhibition within the tested range (Cornish-Bowden, 2012).

Lineweaver-Burk

The Lineweaver–Burk plot for cellulase from *Bacillus krulwichiae* BW4(3) showed a linear relationship between the reciprocal of substrate concentration (1/[S]) and the reciprocal of enzyme velocity (1/V), yielding the regression equation y=0.0021x+0.0063y = 0.0021x + 0.0063y=0.0021x+0.0063 with an R2R^2R2 value of 0.4888. From the y-intercept (0.0063), the maximum reaction velocity (Vmax) was calculated as approximately 158.73 U/mL, while the slope (0.0021) provided a Michaelis constant (Km) of about 0.33 mM (Figure 5). The moderate Km value suggests a reasonable substrate affinity, indicating that the enzyme can efficiently catalyze cellulose hydrolysis even at relatively low substrate concentrations. The comparatively high Vmax reflects strong catalytic potential, supporting the enzyme's applicability in industrial processes. However, the relatively low coefficient of determination indicates some variability in the data, possibly due to error amplification inherent in double-reciprocal transformations, especially at low substrate concentrations.

The kinetic parameters obtained by Lineweaver–Burk analysis confirm that *Bacillus krulwichiae BW4(3)* cellulase was an efficient biomass-degrading enzyme with a relatively high maximum turnover rate and a substrate affinity within the range of industrially relevant cellulases (Bhat & Bhat, 1997; Singh et al., 2016). While double-reciprocal plots are traditionally used for linearizing Michaelis–Menten kinetics, they can overemphasize errors at low substrate concentrations, potentially explaining the lower fit (R^2 = 0.4888) compared to nonlinear regression methods (Cornish-Bowden, 2012). Despite this limitation, the derived Vmax value was considerably high, indicating strong catalytic potential under optimal pH (11) and temperature (50 °C) conditions determined earlier.

Effect of NaCl on cellulose activity

The effect of NaCl concentration on cellulase activity of *Bacillus krulwichiae* BW4(3) the enzyme exhibited stable activity at lower NaCl concentrations (1–3%), with relative activity ranging between 74–76% (~91–93 U/mL). Activity increased sharply at 4% NaCl, reaching the maximum relative activity of ~82% (~100 U/mL), indicating that moderate salinity enhances catalytic performance. Beyond this optimum, activity declined progressively, dropping to ~77% at 5% NaCl and stabilizing around ~74% at higher salt levels (6–7%) (Figure 6). This pattern suggests that the enzyme is moderately halotolerant, with optimal performance under mildly saline conditions, likely due to enhanced structural stability and maintenance of hydration shells around the active site at moderate ionic strengths. However, excessive salt may disrupt enzyme–substrate interactions or induce conformational changes, reducing catalytic efficiency.

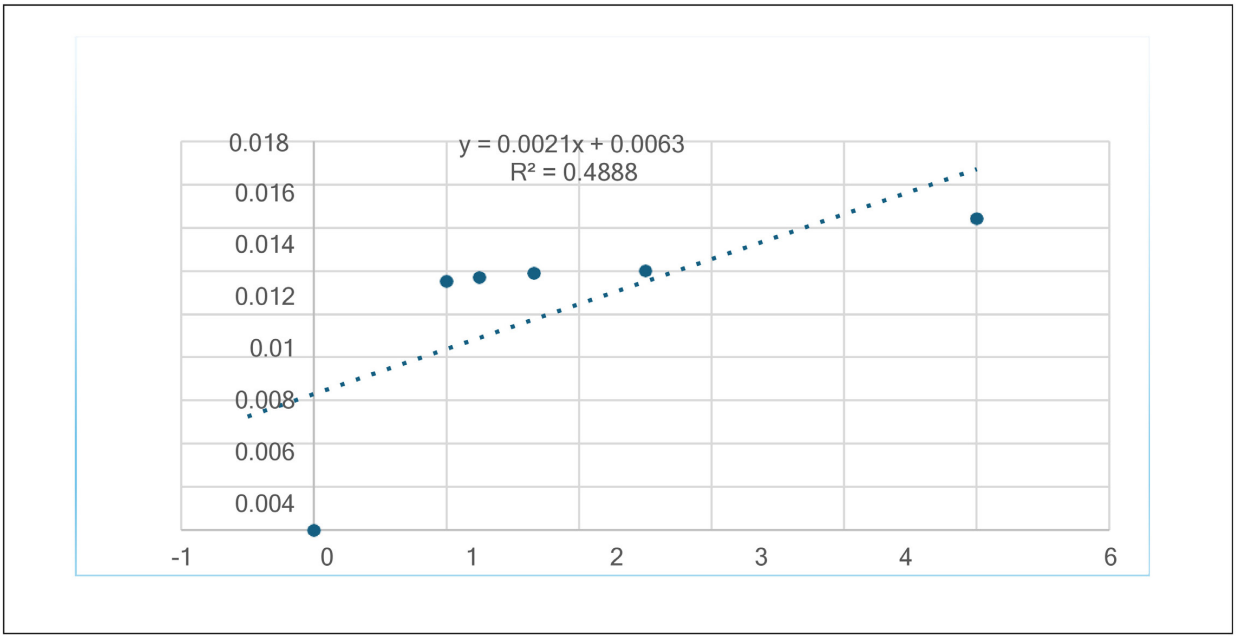


Figure 5. Lineweaver - Burk Plot.

Such halotolerant behaviour is common among enzymes from alkaliphilic and extremophilic *Bacillus* species, making them suitable for applications in saline or marine industrial environments. The NaCl tolerance profile of *B. krulwichiae* BW4(3) cellulase indicates that the enzyme is moderately halotolerant, with optimal activity at 4% NaCl (Figure 6). This enhancement at moderate salinity can be attributed to the stabilizing effect of salt ions on protein structure, which helps maintain the hydration shell around the enzyme and reduces conformational flexibility that could otherwise destabilize the active site (Ventosa et al., 1998). In such conditions, electrostatic interactions are often strengthened, leading to improved substrate binding and catalytic turnover (Margesin & Schinner, 2001). The observed decline in activity at NaCl concentrations above 4% suggests that excessive ionic strength may have inhibitory effects, such as disrupting hydrogen bonding within the enzyme's tertiary structure or altering the solvation layer, ultimately impairing enzyme–substrate complex formation (Oren, 2010). This trend aligns with reports on other halotolerant cellulases from extremophilic *Bacillus* strains isolated from soda lakes and saline soils, which typically show enhanced activity in moderate salt but reduced performance in hypersaline conditions (Sharma et al., 2017).

Effect of organic solvents on cellulose activity

The effect of various organic solvents on cellulase activity of *B. krulwichiae* BW4(3) is illustrated in the graph, with relative activity (%) shown by orange bars and enzyme activity (U/mL) by the blue line. Among the tested solvents, methanol supported the highest activity (~80% relative activity, ~101 U/mL), indicating that the enzyme retains stability and catalytic function in its presence. Chloroform and hexane showed a gradual reduction in activity (~77% and ~74% relative activity, respectively), suggesting moderate solvent tolerance. The lowest activity was recorded with acetone (~70% relative activity, ~94 U/mL), possibly due to its strong dehydrating effect and ability to disrupt the enzyme's tertiary structure. Interestingly, isopropyl alcohol and ethanol maintained activities (~76–77% relative activity) (Figure 7) comparable to chloroform, implying that these polar protic solvents cause less conformational disruption compared to acetone.

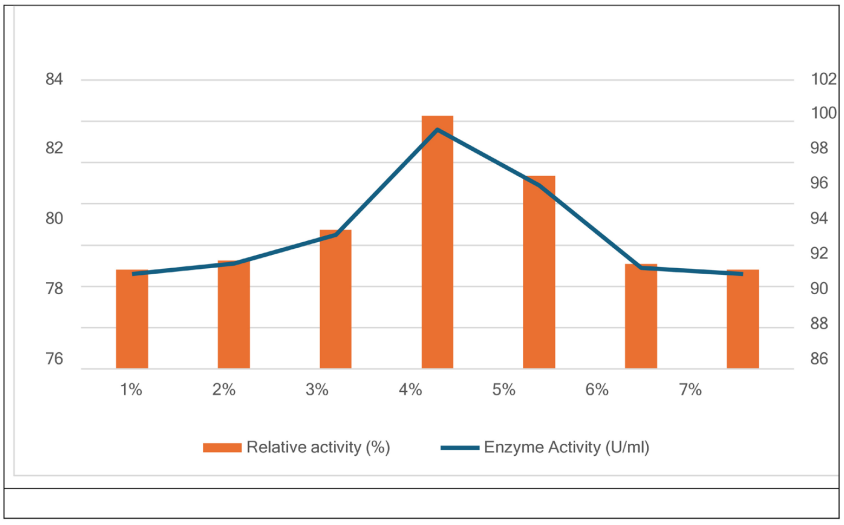


Figure 6. Effect of NaCl on Cellulase activity.

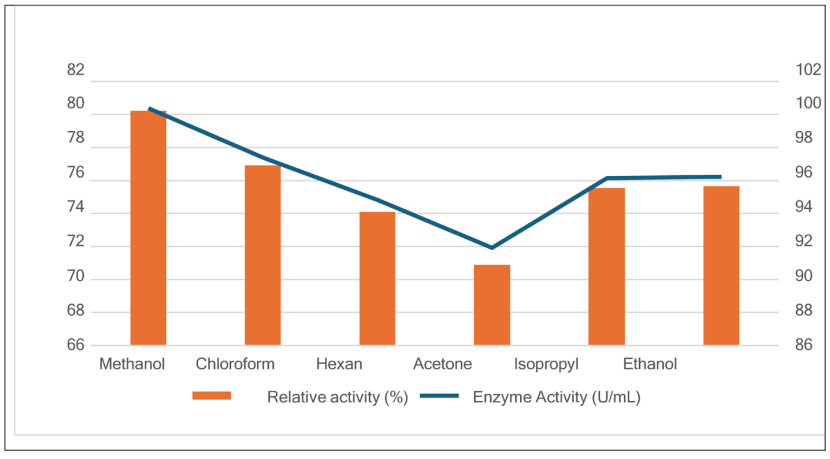


Figure 7. Effect of Solvent on cellulase.

Overall, the enzyme demonstrates a reasonable degree of organic solvent tolerance, an important property for industrial biocatalysis where enzymes may be required to function in mixed aqueous-organic phases, such as in biotransformations, synthesis of bio-based chemicals, or pretreatment of lignocellulosic biomass in solvent-containing systems. The superior stability in methanol and ethanol suggests that *B. krulwichiae BW4(3)* cellulase could be particularly suitable for applications in bioethanol production and other alcohol-rich processes. The solvent tolerance profile of *B. krulwichiae* BW4(3) cellulase indicates that the enzyme maintains appreciable catalytic activity in the presence of various organic solvents, with methanol showing the most favourable effect (~80% relative activity, ~101 U/mL). This suggests that low-molecular-weight alcohols may stabilize the enzyme's conformation, potentially through preferential hydration and reduced flexibility of the active site (Klibanov, 2001). Ethanol and isopropyl alcohol also supported relatively high activity, which is significant for processes such as bioethanol production, where residual alcohol levels could otherwise inhibit less robust enzymes. In contrast, acetone caused the greatest reduction in activity (~70% relative activity), likely due to its strong polarity and ability to strip essential bound water from the enzyme's hydration shell, resulting in denaturation (Zaks & Klibanov, 1988). Similarly, chloroform and hexane caused moderate reductions in activity, possibly by altering the hydrophobic microenvironment or partially penetrating into the enzyme's hydrophobic core, thereby disrupting tertiary structure (Margesin & Schinner, 2001). The observed tolerance to a broad range of solvents suggests that this cellulase is structurally resilient, a feature common in enzymes from alkaliphilic and extremophilic bacteria. Such solvent-stable enzymes are highly valued in non-aqueous biocatalysis, including esterification, transesterification, and depolymerization reactions in low-water systems, as well as in pretreatment of lignocellulosic biomass using alcohol-based solvents.

Effect of metal ions

The effect of various metal ions on cellulase activity of *B. krulwichiae* BW4(3) revealed that K^+ and Mn^{2+} produced the highest stimulation, both reaching ~98% relative activity with enzyme activity around 100 U/mL. Mg^{2+} also enhanced activity (~97% relative activity), whereas Cu^{2+} maintained high but slightly reduced activity (~96% relative activity). In contrast, Na^+ and Ca^{2+} showed lower effects, with relative activities of ~90% and ~92%, respectively (Figure 8). The stimulatory effect of K^+ and Mn^{2+} suggests their role in stabilizing the catalytic conformation of the enzyme or interacting with specific residues at the active site to enhance substrate binding and turnover. Mn^{2+} is known to promote structural rigidity and optimize orientation of catalytic residues in many glycosyl hydrolases, while K^+ can influence ionic strength and maintain protein hydration layers (Turner et al., 2007). Similarly, Mg^{2+} often contributes to stabilizing enzyme–substrate complexes by coordinating with negatively charged residues, which may explain its positive impact here. The slight reduction in activity with Cu^{2+} could be due to its redox-active nature, which may cause partial oxidative modification of amino acid side chains at higher local concentrations (Mathew & Abraham, 2004). The lower activities observed with Na^+ and Ca^{2+} may be attributed to their inability to induce favourable conformational changes, or in the case of Ca^{2+}, potential interference with flexible loop regions critical for catalysis (Sharma et al., 2017). These findings suggest that supplementing reaction mixtures with Mn^{2+} or K^+ could be a practical strategy to enhance the catalytic efficiency of *B. krulwichiae BW4(3)* cellulase in industrial applications, especially in biomass hydrolysis processes where such ions are often naturally present or can be added cost-effectively.

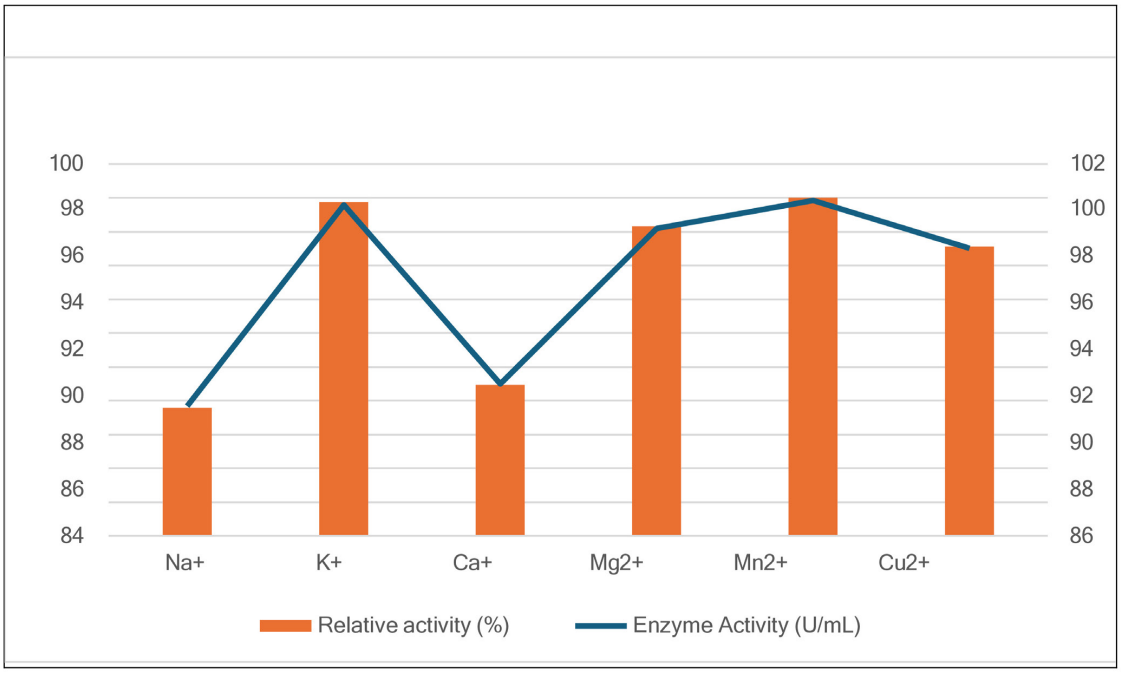


Figure 8. Effect of Metal Ion on cellulase activity.

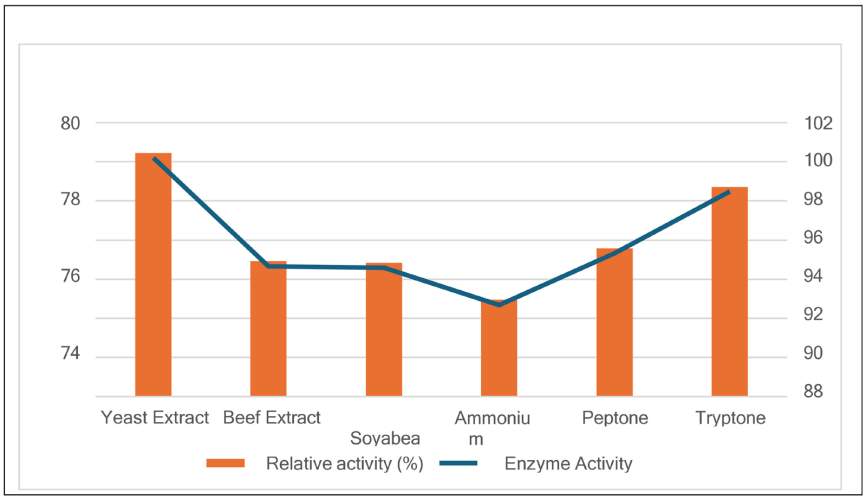


Figure 9. Effect of Nitrogen source on cellulose Production.

Effect of nitrogen sources

The effect of different nitrogen sources on cellulase production by *Bacillus krulwichiae* BW4(3) showed that soybean meal supported the highest enzyme activity (~78% relative activity, ~101 U/mL), followed by peptone (~76% relative activity, ~96 U/mL) and beef extract (~75% relative activity, ~94 U/mL). Yeast extract and ammonium chloride both showed lower activities (~73–74% relative activity, ~92–93 U/mL), while tryptone also produced moderate activity (~74% relative activity, ~92 U/mL) (Figure 9). The superior performance with soybean meal indicates that complex organic nitrogen sources rich in amino acids, peptides, and growth factors favour cellulase synthesis in *B. krulwichiae BW4(3)*. Soybean meal is also known to contain trace elements, vitamins, and slowly degradable proteins that can enhance enzyme yield by sustaining microbial growth over an extended period (Immanuel et al., 2006). The relatively high activity observed with peptone and beef extract further supports the preference of this strain for complex nitrogen sources over simple inorganic salts. In contrast, ammonium chloride, a simple inorganic nitrogen source, yielded comparatively lower activity, possibly due to rapid assimilation that may repress enzyme synthesis through catabolite repression mechanisms (Singhania et al., 2010). Yeast extract, despite being rich in vitamins and amino acids, showed only moderate stimulation, which might be linked to its composition not providing the optimal balance of nutrients for maximal cellulase production in this organism. The ability of *B. krulwichiae* BW4(3) to utilize multiple nitrogen sources, while showing a clear preference for plant-derived protein-rich substrates, suggests its potential for cost-effective cellulase production using agro-industrial byproducts such as soybean meal in large-scale applications.

Conclusion

The study demonstrates that *B. krulwichiae* BW4(3), isolated from the extreme alkaline and saline environment of Lonar Lake, produces a highly stable and efficient cellulase with promising industrial applicability. The enzyme's optimal activity at pH 11 and 50 °C, coupled with its moderate halotolerance (maximum at 4% NaCl) and solvent stability, underscores its robustness for use in harsh processing conditions. Kinetic parameters (Km = 0.33 mM, Vmax was 158.73 U/mL) confirm high substrate affinity and catalytic efficiency. The enhancement of activity by Mn^{2+}, K^{+}, and Mg^{2+}, along with the superior performance of soybean meal as a nitrogen source, provides avenues for process optimization and cost-effective production. Overall, the adaptability of *B. krulwichiae BW4(3)* cellulase to alkaline, saline, and solvent-rich environments positions it as a strong candidate for applications in textile, detergent, bioethanol, and biomass conversion industries.

Conflict of interests

On Behalf of all author, Dr. Vishal Dhundale Declare no conflict of interest

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