

Research Article

Characterization of Thermostable Cellulase from *Bacillus licheniformis* PANG L Isolated from the Himalayan Soil

Manita Shyaula (),^{1,2} Sunil Regmi (),² Deegendra Khadka (),² Ram Chandra Poudel (),² Agni Dhakal (),² Devesh Koirala (),² Jaishree Sijapati (),² Anjana Singh (),¹ and Jyoti Maharjan ()²

¹Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal ²Nepal Academy of Science and Technology, Khumaltar, Lalitpur, Nepal

Correspondence should be addressed to Anjana Singh; anjanas67@gmail.com and Jyoti Maharjan; jyotimaharjan@yahoo.com

Received 18 April 2023; Revised 13 July 2023; Accepted 12 August 2023; Published 31 August 2023

Academic Editor: Giuseppe Comi

Copyright © 2023 Manita Shyaula et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

This study aimed to isolate, purify, and characterize a potential thermophilic cellulase-producing bacterium from the Himalayan soil. Eleven thermophilic bacteria were isolated, and the strain PANG L was found to be the most potent cellulolytic producer. Morphological, physiological, biochemical, and molecular characterization identified PANG L as *Bacillus licheniformis*. This is the first study on the isolation of thermostable cellulase-producing *Bacillus licheniformis* from the Himalayan soil. This bacterium was processed for the production of cellulase enzyme. The optimum conditions for cellulase production were achieved at 45°C after 48 h of incubation at pH 6.5 in media-containing carboxymethyl cellulose (CMC) and yeast extract as carbon and nitrogen sources, respectively, in a thermo-shaker at 100 rpm. The enzyme was partially purified by 80% ammonium sulphate precipitation followed by dialysis, resulting in a 1.52-fold purification. The optimal activity of partially purified cellulase was observed at a temperature of 60°C and pH 5. The cellulase enzyme was stable within the pH ranges of 3–5 and retained 67% of activity even at 55°C. Cellulase activity was found to be enhanced in the presence of metal ions such as Cd²⁺, Pb²⁺, and Ba²⁺. The enzyme showed the highest activity when CMC was used as a substrate, followed by cellobiose. The K_m and V_{max} values of the enzyme were 1.8 mg/ ml and $10.92 \mu g/ml/min$, respectively. The cellulase enzyme obtained from *Bacillus licheniformis* PANG L had suitable catalytic properties for use in industrial applications.

1. Introduction

Cellulose is a fibrous, tough, crystalline, and linear polymer of D-glucose units linked by β -1, 4-glycosidic bonds [1, 2]. It is a major component of plant material and the most abundant renewable source of energy [3, 4]. This cellulose is of major economic value in developing methods for successfully treating and using cellulosic wastes as cheap carbon sources [5]. Cellulases, a group of glycosyl hydrolases, can efficiently hydrolyze cellulose into fermentable sugar through the synergistic action of endoglucanase, exoglucanase, and β -glucosidase [6, 7].

Cellulases are used in a variety of industries, including food, brewing, pharmaceuticals, pulp and paper,

detergents, textiles, leather, waste treatment, feed, and agriculture [3, 8–10]. They are commonly produced by bacteria, archaea, prokaryotes, plants, animals, and fungi [11, 12]. Comparatively, bacterial species have higher growth rates, high enzyme thermostability, better expression systems, and resistance to extreme environments [13, 14].

Recently, thermophilic bacteria have attracted a lot of attention as a source of cellulolytic enzymes. The hydrolysis of cellulose by thermophiles has various advantages such as greater stability, increased specific activity, inhibition of microbial growth, and easier mass transfer [15–17]. Thermophilic cellulose-degrading bacteria have been isolated from diverse sources such as soil [18], compost systems [19], goldmines [20], wastewaters [21], and extreme environments such as hot springs [22, 23]. Several thermophilic *Bacillus* strains, including *B. vallismortis* RG-07 [24], *Bacillus* sp. SMIA-2 [14], *Bacillus* and *Geobacillus* strains [16], and *B. halodurans* CAS 1 [25], have been reported for cellulolytic activities.

The Himalayas are highly diversified regions and have multiple stress conditions such as temperature fluctuations, excessive UV exposure, nutrient scarcity, and low oxygen and air pressure [26–28]. The evolution of microorganisms in response to these pressures has given rise to their unique biochemical and physiological diversity with innovation and unique traits [29]. Therefore, the present study was conducted to isolate and optimize the medium for potential thermostable cellulase-producing bacteria and to purify and characterize the produced enzyme according to various parameters.

2. Materials and Methods

2.1. Isolation of Thermophilic Bacteria. The soil samples were collected from three different areas of the Solukhumbu District, Nepal, namely, Pangboche (altitude 3450 m, latitude 27°51.426 N, and longitude 86°47.640 E), Lobuche (altitude 4960 m, latitude 27°57.269 N, and longitude 86°48.89 E), and Makalu Barun National Park (altitude 3700 m 27°39.29 N and longitude 87°45.52 E). One gram of soil sample was suspended in 9 ml of sterile Milli-Q water and serially diluted under sterile conditions. The diluted cultures were evenly spread on nutrient agar (NA) plates and incubated at 55°C for 24 h. The pure cultures on the NA medium were transferred to a freshly prepared NA slant with 20% glycerol and stored at -20° C [30].

2.2. Assessment of Enzymatic Production. The pure isolates were streaked on CMC agar plates and incubated at 55°C for 24 h [31]. The plates were stained with 0.1% congo-red solution for 15 min and washed with 1 M NaCl for destaining [32]. The bacterial isolates were also screened for other industrially important enzymes like amylase, lipase, caseinase, pectinase, and gelatinase [33–36]. The cultures were inoculated in nutrient broth (NB) at 55°C. For enzyme production, 1 ml of the culture was inoculated in 10 ml of CMC broth and incubated overnight at 55°C under shaking conditions at 100 rpm [37].

2.3. Morphological, Physiological, and Biochemical Characterization of the Isolates. The selected cellulase-producing bacterial isolate was identified by performing Gram staining and several biochemical and carbohydrate utilization tests [38].

2.4. Amplification of the 16S rDNA Gene Using PCR. The DNA was extracted and quantified by NanoDrop. The 16S rDNA gene was amplified by PCR from the genomic DNA of the strain using universal primer pair fD1

(5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACG GCTACCTTGTTACGACTT-3'). Amplification of DNA was carried out under the following conditions: denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 10 min. Amplified PCR products of bacterial isolates were analyzed by electrophoresis on 1% agarose gel at 80 V for 30 min, stained with ethidium bromide, and visualized in a gel documentation system [39]. The PCR products were purified using exonuclease shrimp alkaline phosphatase (Exo-SAP) kit protocol and then sequenced by using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, CA). The sequencing results were compared using the basic local alignment search tool (BLAST) on NCBI and 16S rRNA gene sequence homology evaluation carried out using GenBank data. A phylogenetic tree was constructed using MEGA 6.0 [40].

2.5. Optimization of Culture Conditions on Cellulase Activity. The optimization of various physicochemical parameters of growth conditions was carried out for maximum cellulase production. The effect of a single parameter was determined at a time by keeping the rest of the parameters constant. The major parameters and their effects on cellulase production were determined by measuring the incubation period (24-120 h), pH (3-11), temperatures (30-70°C), and various agitation speeds (static, 50, 100, 150, and 200 rpm). Various carbon sources tested included CMC, xylose, maltose, glucose, fructose, starch, sucrose, and cellobiose. Different types of nitrogen sources such as potassium nitrate, ammonium sulphate, tryptone, ammonium nitrate, ammonium chloride, peptone, yeast extract, and beef extract were examined for their effects on growth and enzyme production. For each step, the enzyme activity was assayed to determine the optimal yield [41].

2.6. Extraction of Crude Enzyme. The isolated bacterial strain was cultured in CMC broth and incubated at 45° C for 48 h under shaking conditions (100 rpm). The culture was centrifuged at 10,000 rpm for 10 min at 4° C, and the supernatant was used as a crude enzyme for cellulase activity assay and partial purification [22].

2.6.1. Cellulase Assay. Cellulase activity was determined by measuring the amount of reducing sugar liberated from CMC using the 3, 5-dinitrosalicylic acid (DNS) method [42]. The enzyme assay mixture was prepared by mixing $500 \,\mu$ l of crude enzyme solution with $500 \,\mu$ l of 1% (w/v) CMC dissolved in 0.1 M phosphate buffer at pH 7. The mixtures were incubated at 45°C for 15 min. The reactions were stopped by adding 1 ml of DNS reagent. All the mixtures were heated in boiling water at 100°C for 5 min for color development, and the optical density was measured at 540 nm. All of the cellulase assays were performed in triplicate. The enzyme activity was determined by using a calibration curve for glucose. One unit (U) of the enzyme activity is defined as the amount of enzyme that releases 1 μ mol of glucose per minute [43].

International Journal of Microbiology

2.7. Partial Purification of Cellulase. Partial purification of the crude enzyme was carried out by fractionation using ammonium sulphate (20–80%) followed by dialysis The crude enzyme was precipitated with ammonium sulphate overnight at 4° C in a magnetic stirrer and centrifuged at 10,000 rpm for 15 min at 4° C to collect the pellets. The pellets were resuspended in a small amount of 0.1 M phosphate buffer, pH 7, and dialyzed overnight for 24 h at 4° C against the same sample buffer by using snakeskin-pleated dialysis tubing [22]. Protein concentrations in the crude sample were estimated by using the biuret method with bovine serum albumin as a standard [44].

2.8. Characterization of the Enzyme

2.8.1. Effect of Temperature and pH on Enzyme Activity and Stability. The optimum temperature of the enzyme was determined by incubating the mixture of the enzyme and 1% CMC in 0.1 M phosphate buffer and pH 7 for 15 min at different temperatures ranging from 30° C to 90° C. The heat stability of the enzyme was determined by incubating the enzyme in 0.1 M phosphate buffer and pH 7 for 60 min at temperatures ranging from 30° C to 90° C for a period of 1 h. The residual activity of each sample for the hydrolysis of CMC was then calculated under assay conditions [22].

The optimum pH of the cellulase was determined by incubating the mixture of the enzyme and 1% CMC in the presence of buffers: 0.1 M acetate buffer (pH 3–5), 0.1 M phosphate buffer (pH 6–8), and 0.1 M glycine NaOH buffer (pH 9–11). The reaction mixtures were incubated at 60°C for 15 min. For the determination of pH stability, the enzyme was incubated in different buffers at different pH ranges (pH 3–11) for 1 h at 60°C [22].

2.8.2. Effect of Incubation Time, Various Metal Ions, Different Substrates, and Time Stability on Enzyme Activity. The optimum incubation time of the enzyme was determined by incubating the mixture of the enzyme and 1% CMC in 0.1 M phosphate buffer and pH 7 at optimum temperatures (60°C) for 15, 30, 45, 60, 75, and 90 min. The enzyme activity at each incubation time was monitored using the DNS assay [22]. The effect of various metal ions on the enzyme activity was determined by the presence of Na⁺, K⁺, EDTA, Mn²⁺, Ca²⁺, Ba²⁺, Fe²⁺, Fe³⁺, Zn²⁺, Mg²⁺, Ni²⁺, Co²⁺, Pb²⁺, and Cd²⁺ metal ions. The concentration of each metal ion was 10 mM in 0.1 M acetate buffer pH 5 [22].

The specificity of the cellulase substrate was determined by testing different substrates, namely, CMC, filter paper, and cellobiose substrate [45]. The enzymes were kept at 25°C, 4° C, and -20° C for 25 days, and the residual cellulase activities were measured at intervals of five days [46].

2.8.3. Enzyme Kinetics. The K_m and V_{max} values were determined by plotting velocity against substrate concentration CMC (5–30 mg/ml). The data were plotted and kinetic

constants were calculated. Calculations were also performed using the Lineweaver–Burk plot [47].

2.9. Data Analysis. All the measurements were conducted in triplicate, and the values were reported as mean \pm S.D. GraphPad Prism 8.4.3 and MS-Excel 2013 were used for data analysis and graphical illustrations.

3. Results

3.1. Isolation and Screening for Cellulase Production. Eleven thermophilic bacterial strains were isolated from the Himalayan soil. All the isolates produced cellulase, amylase, gelatinase, and lipase enzymes. Nine isolates produced caseinase, while pectinase was not reported in any of the isolates. The isolate coded PANG L showed maximum cellulase activities (0.044 ± 0.004 U/ml) and was processed further (Table 1).

3.2. Characterization and Identification of PANG L Bacterial Isolate. The morphological, physiological, and biochemical test results of PANG L are shown in Tables 2–4 as well as in Figure 1. The concentration of DNA for the PANG L was $269 \mu g/ml$ with purity (A_{260}/A_{280}) value of 1.79. The amplified PCR product was 1.5 Kb which was further purified and sequenced (Figure 2(a)). The 16S rDNA gene sequence of PANG L exhibited maximum homology (99%) with strain *B. licheniformis.* The 16S rDNA sequence was submitted to Gene bank with the accession number OQ455938. According to the phylogenetic tree, the isolate PANG L was closely related to the *B. licheniformis* strain ATCC 14580 (Figure 2(b)). Based on these results, the isolate was designated as *B. licheniformis* strain PANG L.

3.3. Optimization of B. licheniformis PANG L Culture Conditions and Enzyme Activity. The optimum incubation period, pH, temperature, agitation, carbon, and nitrogen sources were determined to improve the overall growth and enzyme production (Figure 3). The enzyme-producing ability of the isolate increased with the fermentation period up to $48 h (0.034 \pm 0.002 \text{ U/ml})$ thereafter, it declined (Figure 3(a)). The optimum enzyme production $(0.058 \pm 0.008 \text{ U/ml})$ was recorded at pH 6.5. A sharp decrease in the cellulase activity was observed below and above this pH (Figure 3(b)). The maximum cellulase activity $(0.083 \pm 0.001 \text{ U/ml})$ was found to be at 45°C (Figure 3(c)). The optimum agitation rate for higher cellulolytic enzyme production $(0.089 \pm 0.003 \text{ U/ml})$ was observed at 100 rpm while the production was the least at 200 rpm (Figure 3(d)). CMC was found to be the most suitable carbon source, which recorded a maximum enzyme activity of $(0.085 \pm 0.004 \text{ U/ml})$ followed by cellobiose $(0.072 \pm 0.008 \text{ U/ml})$ (Figure 3(e)). Among all nitrogen sources, the maximum cellulase activity $(0.103 \pm 0.005 \text{ U/ml})$ was observed when yeast extract was used as a source of nitrogen. Potassium nitrate $(0.012 \pm 0.002 \text{ U/ml})$ was found to be the least effective nitrogen source (Figure 3(f)).

TABLE 1: Quantitative screening of cellulase enzyme.

Isolates' codes	Enzyme activity (U/ml) mean±standard deviation
MBN A	0.028 ± 0.007
MBN B	0.030 ± 0.001
MBN CP	0.014 ± 0.008
MBN CW	0.013 ± 0.006
MBN DP	0.003 ± 0.007
MBN DW	0.042 ± 0.004
LOB P	0.019 ± 0.003
LOB W	0.029 ± 0.003
PANG P	0.016 ± 0.003
PANG W	0.026 ± 0.002
PANG L	0.044 ± 0.004

TABLE 2: Morphological and physiological characteristics.

Characteristics	B. licheniformis PANG L results	
Cell morphology		
Shape	Rod	
Colony		
Color	Pale	
Margin	Irregular	
Surface	Flat	
Consistency	Rough	
Light transmission	Opaque	
Gram's staining	Gram-positive	
Spore	Subterminal endospore	
Motility	Motile	
Anaerobic growth	Positive	
NaCl		
7% NaCl	Positive	
10% NaCl	Positive	
Growth at different temperature	30 to 65°C	

TABLE 3: Phenotypic characteristics.

Biochemical tests	<i>B. licheniformis</i> PANG L results
Catalase	Positive
Oxidase	Positive
Indole	Negative
Methyl red	Negative
Voges–Proskauer	Positive
Citrate utilization	Positive
Oxidative/fermentative	Positive
Urea hydrolysis	Negative
Nitrate reduction	Positive
H ₂ S production	Negative

3.4. Partial Purification of Cellulase. Ammonium sulphate precipitation of the crude enzyme was standardized, and the maximum activity was observed at 80% saturation. Therefore, 80% ammonium sulphate was used and no other concentration was applied. The crude enzyme exhibited a specific activity of 0.271 U/mg, whereas the ammonium sulphate precipitated and dialyzed enzyme showed a specific activity of 0.344 U/mg with 1.26 and 0.413 U/mg with 1.52-fold enhancement, respectively (Table 5).

TABLE 4: Substrate fermentation test.

Carbohydrate utilization test	<i>B. licheniformis</i> PANG L results	
Cellobiose	Positive	
Dextrose	Positive	
Dulcitol	Negative	
Esculin	Positive	
Fructose	Positive	
Cellulose	Positive	
Galactose	Negative	
Glucose	Positive	
Glycerol	Positive	
Glycogen	Negative	
Inositol	Negative	
Lactose	Positive	
Maltose	Positive	
Mannose	Positive	
Raffinose	Negative	
Starch	Positive	
Salicin	Positive	
Sorbitol	Negative	
Sucrose	Positive	
Xylose	Positive	

3.5. Partial Characterization of Cellulase Enzyme

3.5.1. Effect of Temperature and pH on Enzyme Activity and Stability. The optimum cellulase activity was observed at an incubation temperature of 60°C but gradually declined this temperature. Regarding the thermal stability of the cellulase enzyme, it retained 67% of its activity after preincubating at 55°C for 1 h. When the temperature was increased to 75°C, the activity reduced by 35% (Figure 4(a)). Based on the results of the pH activity and stability in Figure 4(b), it is observed that the maximum cellulase activity was maintained at pH 5 in 0.1 M sodium acetate buffer and the activity decreased as the pH increased towards alkalinity. The enzyme retained more than 70% of its activity over the pH range of 3–6. More than 50% of the activity of the cellulase was maintained at a broad pH range, ranging from pH 7 to 10 after 1 hr.

3.5.2. Effect of Incubation Time, Metal Ions, Substrates, and Storage Stability on Enzyme Activity. The optimum incubation time with the substrate 1% CMC in 0.1 M phosphate buffer, pH 7 was found to be 45 min. Further incubation for more than 45 min resulted in a gradual loss of the enzymatic activity (Figure 5(a)). The activity of the enzyme increased in the presence of Cd^{2+} followed by Pb²⁺and Ba²⁺, respectively, whereas the presence of manganese and calcium ions significantly decreased the activity of the enzyme (Figure 5(b)).

The enzyme showed the highest activity towards CMC (100%) and moderate activity towards cellobiose (77.48%) and the least activity towards filter paper (36.32%) (Figure 5(c)). The enzyme retained 72% of activity after storing at 25°C for 25 days, while 98.8% of the activity was recorded for the enzyme stored at -20° C. About 8% loss of activity was recorded at 4°C (Figure 5(d)).



FIGURE 1: *B. licheniformis* PANG L and its characteristics: (a) colonies of *B. licheniformis* PANG L on NA, (b) cellulase activity of *B. licheniformis* PANG L on CMC agar, (c) amylase activity, (d) biochemical test, (e) growth on 10% NaCl, (f) substrate fermentation test, and (g) esculin test.

3.5.3. Enzyme Kinetics. Kinetic analysis with CMC revealed the K_m and V_{max} values 1.8 mg/ml and 10.92 μ g/ml/min, respectively, by the Lineweaver–Burk plot (Figure 6).

4. Discussion

The demand for microbial cellulase enzymes is continuously increasing due to their tremendous importance in the bioenergy and bioprocessing industries. The use of novel cellulase from various thermophiles could improve thermophilic cellulase production in the industrial process [48]. The present study focused on the search for high cellulaseproducing thermophilic bacterial isolates from the Himalayan soil. The studies conducted by Marchant et al. [49] and Thakur et al. [50] reported thermophilic microorganisms from cold environments. Eleven isolates were able to grow at 55°C. Each thermophilic isolate produced thermostable hydrolytic enzymes such as lipase, cellulase, amylase, caseinase, and gelatinase. This showed that soil-derived bacterial isolates were the source of extracellular enzymes. Other studies have also reported hydrolytic activities in several thermophilic bacterial strains [51, 52]. Based on the quantitative cellulase assay, isolate PANG L exhibited higher cellulase activity among the isolated strains. The obtained cellulase activity was lower than that of other studies conducted by Ladeira et al. [14] and Kazeem et al. [53], indicating that the PANG L strain is a moderate cellulase producer. The morphological, physiological, biochemical,



FIGURE 2: Characterization of the B. licheniformis PANG L isolate: (a) visualization of PCR-amplified B. licheniformis PANG L 16S rDNA bands on agarose gel, lanes 1, 2, 3, 4, 5, 6, 7, and 8: sample, lane 9: negative control and lane M: DNA ladder (Takara Dye Plus of 250 bp) and (b) phylogenetic tree for B. licheniformis PANG L and other related strains. The tree was obtained using the 16S rDNA sequence retrieved from the database by using the neighbor-joining method. The bootstrap values were generated from 1000 replicates.





FIGURE 3: Optimization of *B. licheniformis* PANG L culture parameters and enzyme activity: effects of (a) incubation period, (b) pH, (c) temperature, (d) agitation, (e) carbon sources, and (f) nitrogen sources on cellulase production.

Purification step	Cellulase activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Purification fold
Crude	0.106 ± 0.003	0.39 ± 0.04	0.271	1
80% ammonium sulphate pool	0.179 ± 0.002	0.52 ± 0.03	0.344	1.26
Dialysis	0.112 ± 0.002	0.271 ± 0.05	0.413	1.52



FIGURE 4: Effects of temperature and pH on cellulase activity and stability (a) temperature and (b) pH.

and molecular characterization confirmed PANG L as *B. licheniformis.* In previous studies, *B. licheniformis* has been reported to produce cellulase enzymes, including *B. licheniformis* 2D55 [53], *B. licheniformis* AMF-07 [54], and *B. licheniformis* JK7 [55].

Optimization of fermentation conditions can improve cellulase production and play a significant role in an industrial bioprocess [56]. The fermentation process was carried out for up to 120 h. The results revealed that maximum enzyme production was obtained at 48 h. The slight reduction in the cellulolytic activity after 48 h might be due to either unavailability of nutrients or inhibition by toxic components present in the medium [57]. The study conducted by Shahid et al. [58] reported a similar trend in cellulase production by *Bacillus* sp. Temperature is a crucial factor that controls bacterial physiology and enzyme



FIGURE 5: Characterization of cellulase enzyme. Effects of (a) incubation time, (b) metal ions, (c) different substrates, and (d) storage stability.



FIGURE 6: Lineweaver–Burk double reciprocal plots of cellulase from *B. licheniformis* PANG L.

production ability [59]. The optimum cellulase production was obtained at 45°C. However, several other studies revealed that the cellulase production was achieved at 50°C by *Bacillus subtilis* K-18 [18] and at 40°C by *B. subtilis* [60]. In the case of pH, maximum cellulase production was observed at pH 6.5. A similar result was also found for *B. licheniformis* MVS1 by previous researchers [61].

Agitation is another factor that plays an important role in the transfer rate of nutrients and oxygen, the increased aerobic metabolism of microbes, and cell aggregate dispersion [62]. The current study showed that the maximum enzyme production was observed at an agitation rate of 100 rpm. At increased agitation, the enzyme may get inactivated due to the shearing of the bacterial cell [63]. The choice of the cheapest substrate is of great importance for the production of enzymes. CMC was found to be the best carbon source for the maximum production of cellulase, which is similar to other studies [64, 65]. This might be due to the activation of regulatory mechanisms responsible for higher cellulase production [48]. Organic nitrogen sources result in better cellulase production as compared to inorganic sources. This enhancement may be due to other nutrients and growth stimulators in the organic nitrogen source besides nitrogen [66]. The findings agreed with the results of Shahid et al. [58], who reported that organic nitrogen sources were more suitable for optimizing cellulase production by B. megaterium than inorganic sources. Through this successive selection of incubation time, temperature, pH, agitation, carbon, and nitrogen source, a 1.7-fold increase in the cellulase activity was obtained for the strain PANG L.

The effect of temperature on the cellulase activity was determined at different temperatures ranging from 30°C to 90°C. The maximum enzyme activity was observed at 60°C indicating that the enzyme is moderately thermostable. The findings were also consistent with those obtained from Bacillus strains M-9 [67], Bacillus subtilis [68], and B. megaterium BM05 [58] lower than that for B. vallismortis RG-07 65°C [24] and B. licheniformis JK7 70°C [55]. The concern of thermal inactivation of the enzyme is often experienced in industrial processes [46]. Hence, enzyme stability is a critical issue on an industrial scale. The thermal stability of the cellulase indicated that the enzyme retained 67% activity up to 55°C. Therefore, it was concluded that the enzyme was moderately thermostable and could have a promising industrial application. In the case of pH, the optimal enzyme activity was found at pH 5 representing the acidophilic nature of the enzyme. The same trend was also obtained from Bacillus strain M-9 [67]. On the contrary, Kim et al. [69] reported that Bacillus sp. HSH-910 was optimally active at alkaline pH. The enzyme showed good stability toward acidic pH ranges of 3-6. Bischoff et al. [70] reported that cellulase from B. licheniformis was more stable under acidic conditions. The optimum incubation time with the substrate was found to be 45 min and declined thereafter. The decrease in the cellulase activity beyond 45 min may be due to the thermal denaturation of the enzyme.

Metal ions can form complexes with proteins and other molecules related to enzymes and act as donors or acceptors of electron as structural regulators [71]. The cellulase activity was stimulated in the presence of metal ions such as Pb²⁺, Cd²⁺, and Ba²⁺. The partial inhibition was observed in Ni^{2+} and Zn^{2+} . In accordance with the study conducted by Gaur and Tiwari [24], there is partial inhibition of cellulase observed in the presence of Ni²⁺ and Zn²⁺. However, the activity was strongly inhibited by Mn²⁺ and Ca²⁺ in the study reported by Bacillus strain [64]. The enzyme exhibited a high activity toward CMC. The substrate specificity of CMC indicates that the enzyme might be an endo- β -1, 4-glucanase [14]. Islam and Roy [41] also found the same characteristics of enzymes produced by Paenibacillus sp. The K_m and V_{max} values were found to be 1.8 mg/ml and 10.92 µg/ml/min, indicating that the enzyme has a high affinity towards CMC due to its low K_m value. The results differ from some earlier studies in which the K_m value was higher at 7.2 mg/ ml [45] and 1.923 mg/ml [24]. The important and desirable quality for industrial applications is the capacity to retain enzymes at room and/or refrigerated temperatures without significant loss of activity. From this study, it was observed that the enzyme was stable at room temperature for less than 10 days after which a slight decline was observed. The enzyme was stable at -20°C. This implies that the room temperature was less suitable for enzyme storage, resulting in a decrease in the enzyme activity.

However, Islam et al. [72] reported that 68% of activities were retained at room temperature after 28 days for cellulase from a *Bacillus* sp.

5. Conclusions

A cellulase-producing bacterium, *B. licheniformis* strain PANG L, showed an optimum activity at a temperature of 60°C and pH 5, with good stability at pH ranges of 3–5, indicating its thermo-acidic nature. Considering its stability at elevated temperatures and acidic conditions, the cellulase from *B. licheniformis* strain PANG L could be desirable for bioconversion processes and industrial applications. Besides cellulase, *B. licheniformis* strain PANG L also produced important hydrolytic enzymes for various substrates such as lipids, proteins, and starch indicating its potential for various industrial applications.

Abbreviations

BLAST:	Basic local alignment search tool
BSA:	Bovine serum albumin
CMC:	Carboxymethyl cellulose
DNS:	3, 5-dinitrosalicylic acid
EXO-SAP:	Exonuclease shrimp alkaline phosphatase
MEGA:	Molecular evolutionary genetics analysis
NA:	Nutrient agar
NB:	Nutrient broth
NCBI:	National Center for Biotechnology Information.

Data Availability

Data related to the tables, graphs, and calculations are included in the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest concerning the research, authorship, and/or publication of this article.

Authors' Contributions

MS performed laboratory procedures, data analysis, methodology, and manuscript writing. SR was involved in methodology and supervision. JM conceptualized the study and was involved in data interpretation, supervision, and critical revision of the manuscript. JS and AS were involved in revision of the manuscript. DK and RCP were involved in DNA sequencing and revision of the manuscript. AD and DK collected the soil samples used in this study. All authors read and approved the final manuscript.

Acknowledgments

We thank Nepal Academy of Science and Technology, Khumaltar, Lalitpur, Nepal, for providing facilities to accomplish this research. We would also like to thank Central Department of Microbiology, Tribhuvan University.

References

- P. Gupta, K. Samant, and A. Sahu, "Isolation of cellulosedegrading bacteria and determination of their cellulolytic potential," *International Journal of Microbiology*, vol. 2012, Article ID 578925, 5 pages, 2012.
- [2] D. Mohan, C. U. Pittman, and P. H. Steele, "Pyrolysis of wood/biomass for bio-oil: a critical review," *Energy & Fuels*, vol. 20, no. 3, pp. 848–889, 2006.
- [3] M. K. Bhat, "Cellulases and related enzymes in biotechnology," *Biotechnology Advances*, vol. 18, no. 5, pp. 355–383, 2000.
- [4] D. Klemm, B. Heublein, H. P. Fink, and A. Bohn, "Cellulose: fascinating biopolymer and sustainable raw material," *Angewandte Chemie*, vol. 44, no. 22, pp. 3358–3393, 2005.
- [5] K. Shanmugapriya, P. S. Saravana, and M. Manoharan, "Screening and partial purification of cellulase from cellulase producing bacteria," *International Journal of Advanced Biotechnology Research*, vol. 3, no. 1, pp. 509–514, 2012.
- [6] L. R. Lynd, P. J. Weimer, W. H. van Zyl, and I. S. Pretorius, "Microbial cellulose utilization: fundamentals and biotechnology," *Microbiol Mol Biol Rev.*, vol. 66, no. 4, pp. 739–777, 2002.
- [7] T. M. Wood, "Properties of cellulolytic enzyme systems," *Biochemical Society Transactions*, vol. 13, no. 2, pp. 407–410, 1985.
- [8] M. K. Bhat and S. Bhat, "Cellulose degrading enzymes and their potential industrial applications," *Biotechnology Advances*, vol. 15, no. 3–4, pp. 583–620, 1997.
- [9] M. Karmakar and R. R. Ray, "Current trends in research and application of microbial cellulases," *Research Journal of Microbiology*, vol. 6, no. 1, pp. 41–53, 2011.
- [10] R. C. Kuhad, R. Gupta, and A. Singh, "Microbial cellulases and their industrial applications," *Enzyme Research*, vol. 2011, Article ID 280696, 10 pages, 2011.
- [11] E. A. Bayer, H. Chanzy, R. Lamed, and Y. Shoham, "Cellulose, cellulases and cellulosomes," *Current Opinion in Structural Biology*, vol. 8, no. 5, pp. 548–557, 1998.
- [12] V. S. Santhi, A. Gupta, S. Saranya, and S. R. D. Jebakumar, "A novel marine bacterium *Isoptericola* sp. JS-C42 with the ability to saccharifying the plant biomasses for the aid in cellulosic ethanol production," *Biotechnology Reports*, vol. 1-2, pp. 8–14, 2014.
- [13] M. Anbar and E. A. Bayer, "Approaches for improving thermostability characteristics in cellulases," *Methods in Enzymology*, vol. 510, pp. 261–271, 2012.
- [14] S. A. Ladeira, E. Cruz, A. B. Delatorre, J. B. Barbosa, and M. L. L. Martins, "Cellulase production by thermophilic *Bacillus* sp. SMIA-2 and its detergent compatibility," *Electronic Journal of Biotechnology*, vol. 18, no. 2, pp. 110–115, 2015.
- [15] M. Maki, K. T. Leung, and W. Qin, "The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass," *International Journal of Biological Sciences*, vol. 5, no. 5, pp. 500–516, 2009.
- [16] G. Rastogi, A. Bhalla, A. Adhikari et al., "Characterization of thermostable cellulases produced by *Bacillus* and *Geobacillus* strains," *Bioresource Technology*, vol. 101, no. 22, pp. 8798– 8806, 2010.
- [17] L. Viikari, M. Alapuranen, T. Puranen, J. Vehmaanperä, and M. Siika-Aho, "Thermostable enzymes in lignocellulose hydrolysis," *Advances in Biochemical Engineering*, vol. 108, pp. 121–145, 2007.

- [18] M. Irfan, Q. Mushtaq, F. Tabssum, H. A. Shakir, and J. I. Qazi, "Carboxymethyl cellulase production optimization from newly isolated thermophilic *Bacillus subtilis* K-18 for saccharification using response surface methodology," *AMB Express*, vol. 7, no. 1, pp. 29–9, 2017.
- [19] W. J. Lu, H. T. Wang, S. J. Yang, Z. C. Wang, and Y. F. Nie, "Isolation and characterization of mesophilic cellulosedegrading bacteria from flower stalks-vegetable waste cocomposting system," *Journal of General and Applied Microbiology*, vol. 51, no. 6, pp. 353–360, 2005.
- [20] G. Rastogi, G. L. Muppidi, R. N. Gurram et al., "Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA," *Journal of Industrial Microbiology and Biotechnology*, vol. 36, no. 4, pp. 585–598, 2009.
- [21] S. K. Tai, H. P. P. Lin, J. Kuo, and J. K. Liu, "Isolation and characterization of a cellulolytic *Geobacillus thermoleovorans* T4 strain from sugar refinery wastewater," *Extremophiles*, vol. 8, no. 5, pp. 345–349, 2004.
- [22] L. Potprommanee, X. Q. Wang, Y. J. Han et al., "Characterization of a thermophilic cellulase from *Geobacillus* sp. HTA426, an efficient cellulase-producer on alkali pretreated of lignocellulosic biomass," *PLoS One*, vol. 12, no. 4, pp. e0175004–e0175016, 2017.
- [23] P. Yadav, S. Korpole, G. S. Prasad, G. Sahni, J. Maharjan, and L. Sreerama, "Morphological, enzymatic screening, and phylogenetic analysis of thermophilic bacilli isolated from five hot springs of Myagdi, Nepal," *Journal of Applied Biology & Biotechnology*, vol. 6, no. 3, pp. 1–8, 2018.
- [24] R. Gaur and S. Tiwari, "Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07," *BMC Biotechnology*, vol. 15, no. 1, p. 19, 2015.
- [25] N. Annamalai, M. V. Rajeswari, S. Elayaraja, and T. Balasubramanian, "Thermostable, haloalkaline cellulase from *Bacillus halodurans* CAS 1 by conversion of lignocellulosic wastes," *Carbohydrate Polymers*, vol. 94, no. 1, pp. 409–415, 2013.
- [26] B. Stres, W. J. Sul, B. Murovec, and J. M. Tiedje, "Recently deglaciated high-altitude soils of the Himalaya: diverse environments, heterogenous bacterial communities and longrange dust inputs from the upper troposphere," *PLoS One*, vol. 8, no. 9, Article ID 76440, 2013.
- [27] N. K. Arora and H. Panosyan, "Extremophiles: applications and roles in environmental sustainability," *Environmental Sustainability*, vol. 2, no. 3, pp. 217-218, 2019.
- [28] N. Hassan, M. Rafiq, M. Hayat, A. A. Shah, and F. Hasan, "Psychrophilic and psychrotrophic fungi: a comprehensive review," *Reviews in Environmental Science and Biotechnology*, vol. 15, no. 2, pp. 147–172, 2016.
- [29] K. Dhakar and A. Pandey, "Microbial ecology from the Himalayan cryosphere perspective," *Microorganisms*, vol. 8, no. 2, p. 257, 2020.
- [30] H. N. Akmar, I. Asma, B. Venugopal, L. Y. Latha, and S. Sasidharan, "Identification of appropriate sample and culture method for isolation of new thermophilic bacteria from hot spring," *African Journal of Microbiology Research*, vol. 5, no. 3, pp. 217–221, 2011.
- [31] A. K. Ray, A. Bairagi, K. Sarkar Ghosh, and S. K. Sen, "Optimization of fermentation conditions for cellulase production by *Bacillus subtilis CY5 and Bacillus circulans* TP3 isolated from fish gut," *Acta Ichthyologica et Piscatoria*, vol. 37, no. 1, pp. 47–53, 2007.

- [32] R. M. Teather and P. J. Wood, "Use of Congo redpolysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen," *Applied and Environmental Microbiology*, vol. 43, no. 4, pp. 777–780, 1982.
- [33] B. T. Abd-Elhalem, M. El-Sawy, R. F. Gamal, and K. A. Abou-Taleb, "Production of amylases from *Bacillus amyloliquefaciens* under submerged fermentation using some agroindustrial by-products," *Annals of Agricultural Science*, vol. 60, no. 2, pp. 193–202, 2015.
- [34] S. Ahlawat, B. Battan, S. S. Dhiman, J. Sharma, and R. P. Mandhan, "Production of thermostable pectinase and xylanase for their potential application in bleaching of kraft pulp," *Journal of Industrial Microbiology and Biotechnology*, vol. 34, no. 12, pp. 763–770, 2007.
- [35] S. S. Balan, R. Nethaji, S. Sankar, and S. Jayalakshmi, "Production of gelatinase enzyme from *Bacillus* spp isolated from the sediment sample of Porto Novo Coastal sites," *Asian Pacific Journal of Tropical Biomedicine*, vol. 2, no. 3, pp. S1811–S1816, 2012.
- [36] E. Mobarak-Qamsari, R. Kasra-Kermanshahi, and Z. Moosavi-Nejad, "Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa* KM110," *Iranian Journal of Microbiology*, vol. 3, no. 2, pp. 92–98, 2011.
- [37] S. Banerjee, T. K. Maiti, and R. N. Roy, "Production, purification, and characterization of cellulase from *Acinetobacter junii* GAC 16.2, a novel cellulolytic gut isolate of *Gryllotalpa africana*, and its effects on cotton fiber and sawdust," *Annals of Microbiology*, vol. 70, no. 1, pp. 28–16, 2020.
- [38] L. M. Prescott, J. P. Harley, and D. A. Klein, *Microbiology*, W. Brown Publishers, Dubuque, IO, USA, 2 edition, 1993.
- [39] W. G. Weisburg, S. M. Barns, D. A. Pelletier, and D. J. Lane, "16S ribosomal DNA amplification for phylogenetic study," *Journal of Bacteriology*, vol. 173, no. 2, pp. 697–703, 1991.
- [40] K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, "MEGA6: molecular evolutionary Genetics analysis version 6.0," *Molecular Biology and Evolution*, vol. 30, no. 12, pp. 2725–2729, 2013.
- [41] F. Islam and N. Roy, "Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses," *BMC Research Notes*, vol. 11, no. 1, pp. 445-446, 2018.
- [42] G. L. Miller, R. Blum, W. E. Glennon, and A. L. Burton, "Measurement of carboxymethylcellulase activity," *Analytical Biochemistry*, vol. 1, no. 2, pp. 127–132, 1960.
- [43] T. K. Ghose, "Measurement of cellulase activities," *Pure and Applied Chemistry*, vol. 59, no. 2, pp. 257–268, 1987.
- [44] A. Bianchi-Bosisio, Proteins- Physiological Samples, Encyclopedia Of Analytical Science, Amsterdam, The Netherlands, 2004.
- [45] R. Rawat and L. Tewari, "Purification and characterization of an acidothermophilic cellulase enzyme produced by *Bacillus subtilis* strain LFS3," *Extremophiles*, vol. 16, no. 4, pp. 637– 644, 2012.
- [46] A. R. Shah and D. Madamwar, "Xylanase production under solid-state fermentation and its characterization by an isolated strain of Aspergillus foetidus in India," World Journal of Microbiology and Biotechnology, vol. 21, no. 3, pp. 233–243, 2005.
- [47] H. Lineweaver and D. Burk, "The determination of enzyme dissociation constants," *Journal of the American Chemical Society*, vol. 56, no. 3, pp. 658–666, 1934.

- [48] C. Aarti, A. Khusro, and P. Agastian, "Carboxymethyl cellulase production optimization from *Glutamicibacter arilaitensis* strain ALA4 and its application in lignocellulosic waste biomass saccharification," *Preparative Biochemistry & Biotechnology*, vol. 48, no. 9, pp. 853–866, 2018.
- [49] R. Marchant, I. M. Banat, T. J. Rahman, and M. Berzano, "The frequency and characteristics of highly thermophilic bacteria in cool soil environments," *Environmental Microbiology*, vol. 4, no. 10, pp. 595–602, 2002.
- [50] V. Thakur, V. Kumar, S. Kumar, and D. Singh, "Diverse culturable bacterial communities with cellulolytic potential revealed from pristine habitat in indian trans-Himalaya," *Canadian Journal of Microbiology*, vol. 64, no. 11, pp. 798– 808, 2018.
- [51] S. Alrumman, Y. S. M. Mostafa, S. Al-Qahtani, and T. H. T. Taha, "Hydrolytic enzyme production by thermophilic bacteria isolated from Saudi hot springs," *Open Life Sciences*, vol. 13, no. 1, pp. 470–480, 2018.
- [52] B. T. Mohammad, H. I. Al Daghistani, A. Jaouani, S. Abdel-Latif, and C. Kennes, "Isolation and characterization of thermophilic bacteria from Jordanian hot springs: *Bacillus licheniformis and Thermomonas hydrothermalis* isolates as potential producers of thermostable enzymes," *International Journal of Microbiology*, vol. 2017, Article ID 6943952, 12 pages, 2017.
- [53] M. O. Kazeem, U. K. M. Shah, A. S. Baharuddin, and N. A. Abdul Rahman, "Enhanced cellulase production by a novel thermophilic *Bacillus licheniformis* 2D55: characterization and application in lignocellulosic saccharification," *Bioresources*, vol. 11, no. 2, pp. 5404–5423, 2016.
- [54] F. Azadian, A. Badoei-dalfard, A. Namaki-Shoushtari, and M. Hassanshahian, "Purification and biochemical properties of a thermostable, haloalkaline cellulase from *Bacillus licheniformis* AMF-07 and its application for hydrolysis of different cellulosic substrates to bioethanol production," *Molecular Biology Research Communications*, vol. 5, no. 3, pp. 143–155, 2016.
- [55] J. K. Seo, T. S. Park, I. H. Kwon, M. Y. Piao, C. H. Lee, and J. K. Ha, "Characterization of Cellulolytic and Xylanolytic Enzymes of *Bacillus licheniformis* JK7 Isolated from the Rumen of a Native Korean Goat," *Asian-Australasian Journal* of *Animal Sciences*, vol. 26, no. 1, pp. 50–58, 2013.
- [56] S. Singh, V. S. Moholkar, and A. Goyal, "Optimization of carboxymethylcellulase production from *Bacillus amyloliquefaciens* SS35," *3 Biotech.*, vol. 4, no. 4, pp. 411–424, 2014.
- [57] H. Ikram-ul, H. Uzma, S. Kiran, M. Mohsin Javed, A. Sikander, and M. A. Qadeer, "Cotton saccharifying activity of cellulases by *Trichoderma harzianum* UM-11 in shake flask," *International Journal of Botany*, vol. 1, no. 1, pp. 19–22, 2004.
- [58] Z. H. Shahid, M. Irfan, M. Nadeem, Q. Syed, and J. I. Qazi, "Production, purification, and characterization of carboxymethyl cellulase from novel strain *Bacillus megaterium*," *Environmental Progress & Sustainable Energy*, vol. 35, no. 6, pp. 1741–1749, 2016.
- [59] F. Shah and S. Mishra, "In vitro optimization for enhanced cellulose degrading enzyme from *Bacillus licheniformis* KY962963 associated with a microalgae *Chlorococcum* sp. using OVAT and statistical modeling," *SN Applied Sciences*, vol. 2, p. 1923, 2020.
- [60] S. Sethi, A. Datta, B. L. Gupta, and S. Gupta, "Optimization of cellulase production from bacteria isolated from soil," *ISRN Biotechnology*, vol. 2013, Article ID 985685, 7 pages, 2013.

- [61] S. Acharya and A. Chaudhary, "Optimization of fermentation conditions for cellulases production by *Bacillus licheniformis MVS1 and Bacillus* sp. MVS3 isolated from Indian hot spring," *Brazilian Archives of Biology and Technology*, vol. 55, no. 4, pp. 497–503, 2012.
- [62] F. Mantzouridou, T. Roukas, and P. Kotzekidou, "Effect of the aeration rate and agitation speed on β-carotene production and morphology of *Blakeslea trispora* in a stirred tank reactor: mathematical modeling," *Biochemical Engineering Journal*, vol. 10, no. 2, pp. 123–135, 2002.
- [63] I. Giavasis, L. M. Harvey, and B. McNeil, "The effect of agitation and aeration on the synthesis and molecular weight of gellan in batch cultures of *Sphingomonas paucimobilis*," *Enzyme and Microbial Technology*, vol. 38, no. 1–2, pp. 101–108, 2006.
- [64] S. Sadhu, P. Saha, S. K. Sen, S. Mayilraj, and T. K. Maiti, "Production, purification and characterization of a novel thermotolerant endoglucanase (CMCase) from *Bacillus* strain isolated from cow dung," *SpringerPlus*, vol. 2, no. 1, p. 10, 2013.
- [65] A. Thakkar and M. Saraf, "Application of statistically based experimental designs to optimize cellulase production and identification of gene," *Natural Products and Bioprospecting*, vol. 4, no. 6, pp. 341–351, 2014.
- [66] A. Mathew, A. N. Eldo, and A. G. Molly, "Optimization of culture conditions for the production of thermostable polygalacturonase by *Penicillium* SPC-F 20," *Journal of Industrial Microbiology and Biotechnology*, vol. 35, no. 9, pp. 1001–1005, 2008.
- [67] B. K. Bajaj, H. Pangotra, M. A. Wani, P. Sharma, and A. Sharma, "Partial purification and characterization of a highly thermostable and pH stable endoglucanase from a newly isolated *Bacillus* strain M-9," *Indian Journal of Chemical Technology*, vol. 16, no. 5, pp. 382–387, 2009.
- [68] W. A. Malik and S. Javed, "Biochemical characterization of cellulase from *Bacillus subtilis* strain and its effect on digestibility and structural modifications of lignocellulose rich biomass," *Frontiers in Bioengineering and Biotechnology*, vol. 9, Article ID 800265, 2021.
- [69] J. Y. Kim, S. H. Hur, and J. H. Hong, "Purification and characterization of an alkaline cellulase from a newly isolated alkalophilic *Bacillus* sp. HSH-810," *Biotechnology Letters*, vol. 27, no. 5, pp. 313–316, 2005.
- [70] K. M. Bischoff, A. P. Rooney, X. L. Li, S. Liu, and S. R. Hughes, "Purification and characterization of a family 5 endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis*," *Biotechnology Letters*, vol. 28, no. 21, pp. 1761–1765, 2006.
- [71] J. F. Riordan, "The role of metals in enzyme activity," Annals of Clinical Laboratory Science, vol. 7, no. 2, pp. 119–129, 1977.
- [72] M. Islam, P. K. Sarkar, A. K. M. Mohiuddin, and M. Suzauddula, "Optimization of fermentation condition for cellulase enzyme production from *Bacillus* sp," *Malaysian Journal of Halal Research*, vol. 2, no. 2, pp. 19–24, 2019.