RESEARCH ARTICLE

Isolation and characterization of cellulolytic bacteria from Lubimbi hot Springs in Binga, Zimbabwe

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ABSTRACT

Cellulolytic bacteria have gained worldwide interest due to their ability to secrete thermostable enzymes with multiple uses. In this study, cellulolytic bacteria were isolated from local hot springs on carboxymethyl cellulose (CMC) agar and screened using 0.1% Congo red. Strains LB-4, LB-6 and LB-8 were found to have high cellulolytic indices of 1.8, 2.0 and 1.5, respectively. These strains were preliminarily identified based on morphology, gram's reaction, and biochemical characteristics. They were rod-shaped, stained blue and possibly belong to a genus of motile *Bacillus*. The isolates were further subjected to homology analysis of the 16S rRNA gene. BLAST search showed strains LB-4, LB-6 and LB-8 to be 99.13%, 98.26% and 98.91% identical to *Bacillus subtilis*, *Bacillus* sp., and *Bacillus licheniformis* in the NCBI GenBank, respectively. The cellulolytic activity of each strain was evaluated using submerged fermentation that was terminated by the dinitrosalicylic acid (DNS) method. The optimum cellulase activity of the bacteria strains were observed after 24 hr at pH 7 and 40ºC while utilizing 1% CMC as a carbon source and 1% yeast extract as a nitrogen source. It can be concluded that cellulolytic bacteria were screened and identified from Lubimbi hot springs and the cellulase activity of each strain was optimized for different fermentation conditions. These strains have potential use in the pre-treatment of crop residues for biogas production.

Key words: *Bacillus*, biogas, cellulose, homology, lignocellulose, pre-treatment

Introduction

Lignocellulose is the most abundant and cheap renewable resource on earth (Ge et al., 2018). The plant cell wall consists of 85-90% complex polymers of lignocellulose (Jung et al., 2015), which are made up of 40-50% cellulose, 15-25% hemicelluloses and 20-25% lignin (Zing et al., 2017). Cellulose is the major constituent of plant cell walls. It has a wide range of applications in agriculture, fuel and paper industry (Yang et al., 2014). In terms of bioenergy production, about 80% of cellulose can be converted into glucose (Sukhesh and Rao, 2018). However, cellulose has a water-insoluble crystalline structure that is covered by a heterogeneous layer of hemicelluloses and lignin. This limits its conversion into fermentable sugars. Pre-treatment is a promising strategy to improve the biodegradability of lignocellulose (Yang et al., 2014; Kasinath et al., 2021; Naik et al., 2021). Several attempts have shown the potential of chemical and physical methods to pre-treat lignocellulose. The limited utility of these methods has been due to high energy demand, the need for complex equipment and the release of inhibitory products (Yang et al., 2014; Kamusoko et al., 2019).

Biological pre-treatment is considered to be a better option than chemical and physical methods. It is an inexpensive and low-energy method that does not produce secondary pollution (Zheng et al., 2014; Kamusoko et al., 2019). Pre-treatment of cellulosic material using fungi, microbial consortium and enzymes is extensively reported in the literature (Zheng et al., 2014). Cellulases and hemicellulases are the most widely used enzymes for the pre-treatment of lignocellulosic substrates (Zheng et al., 2014). Cellulases are a complex structure of hydrolytic enzymes including cellobiohydrolase or exoglucanase (EC 3.2.1.91), endo β-glucanase (EC 3.2.1.4) and β-glucanase (3.2.1.21) that interact in a synergistic manner to convert cellulose into fermentable sugars. The enzymes are mainly produced by fungi, bacteria and actinomycetes (Yang et al., 2014;

Kamusoko et al., 2021). Cellulases of fungal origin are mainly exploited in food, feed, textiles, fuel and chemical industry. The limited growth of fungi coupled with the high costs of cellulase production is the main challenge of using cellulolytic fungi. Comparatively, cellulolytic bacteria have simple cultivation, rapid growth, short generation time and good potential applicability (Yang et al., 2014). Cellulolytic bacteria occupy multiple habitats including sewage, agricultural wastes, soil, hot springs, and guts of ruminants and insects. Genera consist of *Bacillus*, *Clostridium*, *Cellulomonas*, *Micrococcus*, *Alteromonas*, *Acetivibrio*, *Pseudomonas* and *Bacteriodes* (Kamusoko et al., 2021). A large research gap still exists in the diversity of novel bacteria strains with high cellulolytic capability.

Lubimbi hot springs located in Binga, Matabeleland North of Zimbabwe are characterized by extremely high temperatures with an average of about 73ºC (Chikwama et al., 2022). Expectations are very high that these hot springs are perfect sources of novel thermotolerant bacteria with multifarious applications. However, the microbial diversity of Lubimbi hot springs is not yet fully studied. With this background, bacteria strains with hyper cellulolytic activity were screened from Lubimbi hot springs. The cellulolytic activity of the strains was also estimated and optimized under different submerged fermentation (SmF) conditions. The aim was to provide a hypothetical basis for the potential use of locally available cellulases for the pre-treatment of crop residues in biogas production.

Materials and Methods

Study site and sample collection

Cellulolytic bacteria were isolated from water samples collected from Lubimbi hot springs (18.4761° S, 27.3061° E). The hot springs are located in Binga District, Matabeleland North Province of Zimbabwe. Samples of water were collected at a depth of 20-50 cm in sterile 500 ml thermos flasks at four different sites. Temperature, pH, electrical conductivity and total dissolved solids were measured *in-situ* using a digital pH-686 meter (Juanjuan, China). These physico-chemical parameters varied from 71-82°C, 7.1-7.8, 1098-2058 µs cm^{-1} and 1023-1029 mg L^{-1}, respectively. Samples were carried to the Biotechnology Laboratory, Department of Biotechnology, Chinhoyi University of Technology and preserved at 4°C.

Isolation and screening of cellulolytic bacteria

Cellulolytic bacteria were isolated from hot spring water samples according to Kunasundari et al. (2016). One milliliter of a sample was serially diluted up to a dilution factor of 10^{-10}. A volume of 0.1 mL of the dilution (10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) was inoculated on carboxymethyl cellulose (CMC) agar: 1.36 g KH_2PO_4, 2 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 g $(NH_4)_2SO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 3 g CMC, 1 g yeast extract and 15 g agar in 1 000 mL. The inoculated plates were incubated at 37ºC for 5 days. After incubation, the plates were flooded with 0.1% Congo red for 20 min and counter-stained with 1 M NaCl for 15 min (Samira et al., 2011). The formation of a clear zone of hydrolysis around a colony indicated a positive test for cellulose degradation. Strains with the largest zone of clearance were isolated for repeated screening to obtain pure isolates as suggested by Naresh et al. (2019). The cellulolytic index was calculated using the following equation:

$$\text{Cellulolytic index} = \frac{Diameter\ of\ clearance\ zone - Diameter\ of\ bacteria\ colony}{Diameter\ of\ bacteria\ colony}$$

Morphological and biochemical identification of cellulolytic bacteria

The colony morphology of pure bacteria strains was checked using Gram staining. Biochemical characteristics such as motility, citrate utilization, indole, urease, catalase and starch hydrolysis tests were conducted to further identify the isolates. The tests were performed according to the standard protocols in Bergey's Manual of Systematic Bacteriology (Garrity et al., 2004).

16S rRNA sequencing of cellulolytic bacteria

The protocol for 16S rRNA sequencing was previously described by Kamusoko et al. (2021). Total genomic DNA was extracted from cellulolytic bacteria using a DNA extraction kit (Zymo Research, USA). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) in a PTC-200 Peltier Thermal Cycler (Bio-Rad/MJ Research, USA) using universal primers: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). After amplification, the PCR products were resolved on 1.5% agarose gel containing GR Green Nucleic Acid stain. The PCR amplicons were sequenced at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). Sequences were quality edited using BioEditversion 7.2 Software (Hall, 1999). Sequence relationships were determined by comparing with known representative sequences in the GenBank database by multiple sequence alignment using the Basic Alignment Search Tool (BLAST) algorithm of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov**).**

Preparation of crude enzyme extract from cellulolytic bacteria

Bacteria strains were grown in 1% CMC broth for the production of crude enzymes (Yang et al., 2014). A bacterial isolate was inoculated into a conical flask containing 150 mL of Luria Bertani (LB) broth + 1% CMC sterile medium and

SmF was performed by incubating in a shaker at 37°C for 48 hr at an agitation speed of 160 rpm. After incubation, the broth was centrifuged at 10 000 rpm for 10 min. The supernatant was collected and preserved at 4°C as the crude enzyme extract.

Quantification of crude enzyme activity

Enzyme activity was estimated by the carboxymethyl cellulase (CMCase) activity assay according to Yang et al. (2014). About 1 mL of the culture supernatant was mixed with 1 mL of 1% CMC in 0.05M sodium acetate buffer (pH 5.5) and incubated in a water bath at 65ºC for 5 min. The reaction was terminated by adding 1.5 mL of dinitrosalicylic acid (DNS), subsequently boiled at 98ºC in a water bath for 15 min and cooled on ice. Reducing sugars were determined by measuring absorbance at 540 nm using a UV 1900i spectrophotometer (Shimadzu, Germany). One unit (U) of cellulase activity was measured as the amount of enzyme required to release 1 mole of glucose per min under standard assay conditions and it was expressed as units per milliliter (U/mL). All experiments were conducted in triplicate and data were presented as mean ± standard error.

Optimization of fermentation conditions for cellulase production

Cellulase production of the bacteria strains was optimized for carbon source, nitrogen source, incubation time, temperature and pH (Hussain et al., 2017). The SmF process was optimized using the one-variable-at-a-time approach. Incubation time was determined by inoculating 5 mL of overnight grown bacteria in 100 ml of 1% CMC followed by incubation at 37ºC for different periods of time (24 and 48 hr) at 120 rpm in a shaker. The effect of carbon source on cellulase activity was evaluated by inoculating bacteria in 1% CMC medium (pH 7) at four different concentrations (1, 2, 3 and 4%). Bacteria strains were incubated in 1% CMC media containing three different nitrogen sources (1% peptone, 1% yeast extract and 1% urea) to investigate the nitrogen requirements for cellulase production. Cellulase activity was optimized for pH by growing bacteria at four different pH conditions (3.0, 5.0, 7.0, and 9.0). The pH of the medium was adjusted using 1M HCl and 1M NaOH. The production media were then incubated at 37ºC for 24 hr. Temperature was studied by incubating bacteria culture in 1% CMC broth at various temperatures (20, 25, 30, 35, 40, 45, and 50ºC). After incubation, the wort was centrifuged at 10 000 rpm for 10 min. The CMCase activity was measured by adding 0.1 mL of the cell-free supernatant to 0.1 ml of 1% CMC in 10Mm sodium phosphate buffer at 37ºC for 60 min. About 1.5 mL of DNS reagent was added to the mixture, boiled for 15 min, and cooled in ice to terminate the reaction. The optical density was then measured at 540 nm using a UV 1900i spectrophotometer (Shimadzu, Germany).

Results and Disscusion

Isolation and screening of cellulolytic bacteria

Sustained efforts have been made to screen novel cellulolytic strains such as *Anoxybacillus flavithermus*, *B. subtilis*, and *B. amyloliquefaciens* for potential use in biofuel production (Hussain et al., 2017). Hot springs are a potential source of bacteria with high cellulolytic activity due to their high temperatures and alkaline conditions. Growing bacteria on a CMC medium, followed by Congo red staining is believed to be a rapid and simple method for isolation and screening of cellulolytic bacteria (Yang et al., 2014). Out of the total isolates grown on CMC-Na, only 11 were found to produce large clear zones around colonies (Table 1). The ability of bacteria to produce clear zones indicates their potential to degrade cellulose. The cellulolytic index of the studied bacteria isolates varied from 0.1-2.0. Isolate LB-6 showed the highest cellulolytic index whereas LB-9 had the lowest index. Among the 11 isolates, only three (LB-4, LB-6 and LB-8) reported high cellulolytic activity, with cellulolytic indices of 1.8, 2.0 and 1.5, respectively. Hence, LB-4, LB-6, and LB-8 were regarded as active cellulose degraders. The strains were subjected to morphological and biochemical identification, 16S rRNA sequencing, and evaluated for cellulase production. The maximum index (1.32) of cellulolytic bacteria screened from termites' guts (*Cryptotermes* sp.) was lower than the indices of LB-4, LB-6 and LB-8 (Peristiwati et al., 2018). The isolates showed lower cellulolytic indices than those isolated from tropical mangrove soil (Naresh et al., 2019). Ferbiyanto et al. (2015) reported higher cellulolytic indices in the range of 0.75-2.5 from termite gut of worker *Macrotermesgilvus*.

Table 1. *Cellulolytic activity of bacterial isolates from hot springs*

Isolate code	Clear zone diameter (mm)	Colony diameter(mm)	Cellulolytic index
LB-1	4.0	2.0	1.0
LB-2	2.0	1.0	1.0
LB-3	3.0	2.0	0.5
LB-4	2.8	1.0	1.8
LB-5	2.0	1.0	1.0
LB-6	3.0	1.0	2.0
LB-7	4.5	3.0	0.5
LB-8	5.0	2.0	1.5
LB-9	1.0	0.5	1.0
LB-10	4.5	2.0	1.3
LB-11	1.0	0.5	1.0

LB: Lubimbi

Morphological and biochemical identification of bacteria isolates

Figure 1 shows the morphological characteristics of the bacterial colonies. Using gram's reaction, microscopic observation showed all the strains (LB-4, LB-6 and LB-8) to stain blue. This revealed the strains to be Gram positive. Microscopically, the strains were rod-shaped and found to belong to a group of motile bacillus. The gram staining and biochemical characteristics of the three potential strains are shown in Table 2. According to morphological and biochemical characteristics, the strains belong to the genus *Bacillus.* Thermophilic *Bacillus* spp., with cellulolytic potential, have been previously isolated from hot springs and other harsh ecological habitats (Adiguzel et al., 2009; Yang et al., 2014; Hussain et al., 2017; Naresh et al., 2019).

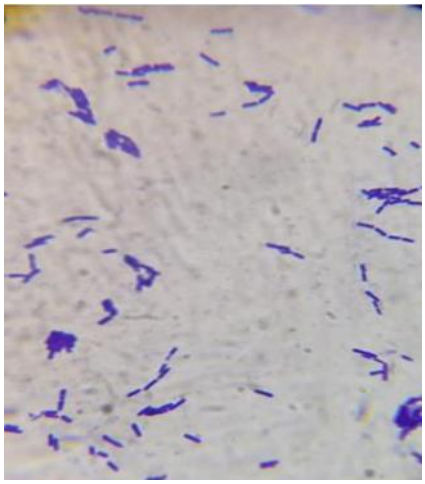

Figure 1. *Gram reaction of the bacterial strains.*

16 rRNA sequencing of cellulolytic bacteria

The 16S rRNA gene is a highly conserved region that encodes the 30S ribosomal subunit of bacteria and archaea.

Analysis of 16S rRNA is a precise and suitable way for the molecular taxonomy of prokaryotes (Ferbiyanto et al., 2015; Kamusoko et al., 2021). Amplification of 16S rRNA sequence of cellulolytic bacteria with universal primers 27F/1492R confirmed DNA amplicons of 1.5 kb in size. The findings are supported by previous work of Yang et al. (2014) and Ferbiyanto et al. (2015). Homology analysis reported a strong degree of similarity (>98%) between isolates LB-4, LB-6 and LB-8, and strains in the NCBI GenBank of *Bacillus subtilis*, *Bacillus* sp., and *Bacillus licheniformis*, respectively (Table 3). BLAST algorithm with a similarity threshold of 98.65 % is used to compare the 16S rRNA sequences with potential phylogenetic neighbors (Kim and Chun, 2014). Previous studies have demonstrated that thermophilic strains belonging to the genus *Bacillus* are capable of producing cellulolytic enzymes (Adiguzel et al., 2009; Yang et al., 2014; Hussain et al., 2017).

Optimization of fermentation conditions for cellulase production

Effect of incubation time on cellulase activity

Figure 2 compares the CMCase activity of LB-4, LB-6 and LB-8 strains after 24 and 48 hr of incubation. The work indicates that LB-4 and LB-6 attained optimum cellulase productivity of 0.14 and 0.20 U/ml, respectively, after 24 hr. However, strain LB-8 maintained a steady state of CMCase activity (0.13 U/mL) between 24 and 48 hr. Similarly, Hussain et al. (2017) reported a maximum CMCase activity from *B. subtilis* BTN7A, *B. amyloliquefaciens* SA5 and *B. megaterium* BMS4 after 24 hr, whereas cellulase productivity of *B. flavithermus* BTN7B did not change after 24 and 48 hr. Contrary to our findings, Pramanik et al. (2021) reported maximum cellulase productivity after 72 hr from *B. pseudomycoides*. A decline in cellulase productivity with increasing incubation time may be attributed to the

Table 2. *Gram staining and biochemical characteristics of bacteria isolates.*

Isolate	Gram's reaction	Motility	Catalase	Indole	Urease	Citrate	Starch hydrolysis	Triple sugar
LB-4	+	+	+	-	+	+	+	+
LB-6	+	+	+	-	+	+	+	-
LB-8	+	+	+	-	+	+	+	-

+: positive test -: negative test

Table 3. *Affiliations of 16S rRNA gene sequences of cellulolytic bacteria isolated from hot springs.*

Isolate	Query cover	*e* Value	Maximum identity	Identity in BLAST	Accession number
LB-4	100%	0	99.13%	*Bacillus subtilis*	CP032865.1
LB-6	100%	0	98.26%	*Bacillus* sp.	MF355364.1
LB-8	100%	0	98.91%	*Bacillus licheniformis*	MT642943.1

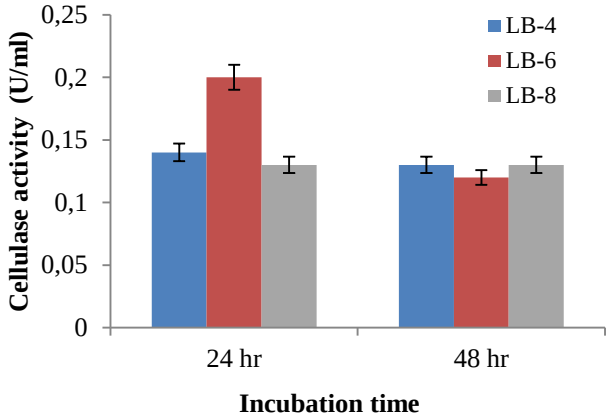

Figure 2. *Effect of incubation time on cellulose activity.*

exhaustion of nutrients and accumulation of toxins (Hussain et al., 2017).

Effect of pH on cellulase activity

Cellulolytic bacteria occupy habitats with diverse pH conditions. The pH determines how specific substrates are metabolized and affects the cellulolytic potential of bacteria. This study examined how cellulolytic bacteria performed under varying pH from 3.0 to 9.0. All test strains (LB-4, LB-6 and LB-8) reported the lowest cellulase activity (0.15-0.16 U/mL) at pH 3, whereas the highest cellulase activity (0.17-0.19 U/mL) was found at pH 7 (Figure 3). These findings are in agreement with CMCase activity of *B. Pseudomycoides* (Pramanik et al., 2021), *B. subtilis* BTN7A, *B. amyloliquefaciens* SA5, *B. megaterium* BSM4, *B. flavithermus* BTN7B (Hussain et al., 2017) and *B. coagulans* (Romsaiyud et al., 2009). However, Acharya and Chaudhary (2011) reported optimum CMCase activity at pH 9 from *B. subtilis* isolated in hot springs while Yang et al. (2014) observed maximum CMCase activity at pH 5.5 from *B. subtilis* BY-2 isolated from pig's intestine. Varying pH of the

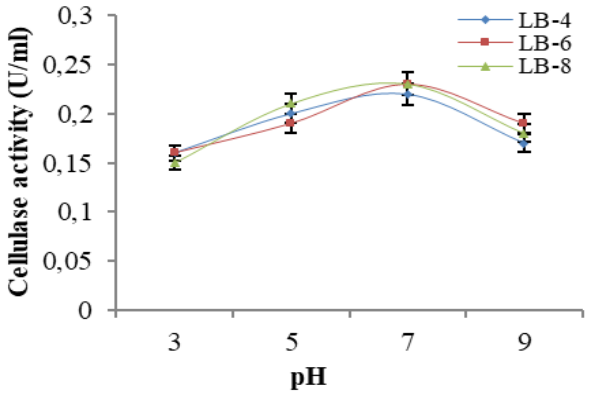

Figure 3. *Effect of pH on cellulase activity.*

fermentation medium has been reported to alter the ionic properties of the enzyme's active site causing damage to the three-dimensional structure and reducing its affinity towards the substrate (Naresh et al., 2019).

Effect of carbon source on cellulase activity

In the present study, the effect of different concentrations of the carbon source on cellulase fermentation was investigated. Results indicated that the carbon utilization efficiency of the strains decreased with rising CMC concentration (Figure 4). The maximum CMCase activity (0.11 U/mL) was reported from isolate LB-6 at 1% CMC. This shows that strain LB-6 could efficiently utilize CMC as a carbon source compared to other strains. Isolate LB-8 exhibited the lowest CMCase activity of 0.02 U/ml at 4% CMC. Overall, the CMCase activity of all the strains (LB-4, LB-6 and LB-8) was found to be optimum at 1% CMC concentration. This work is supported by several authors (Sakthivel et al., 2010; Behera et al., 2016; Islam & Roy, 2018). According to Yang et al. (2014), cellulases are inducible enzymes that require cellulose-containing substrate as a carbon source for fermentation. The most favorable carbon source for cellulase production is CMC medium (Das et al., 2010).

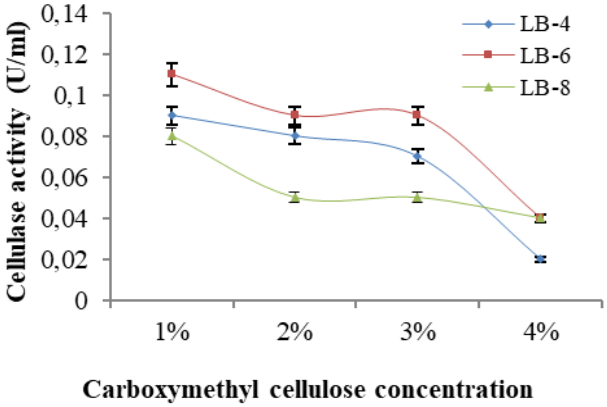

Figure 4. *Effect of carbon source on cellulose activity.*

Effect of nitrogen source on cellulase activity

Nitrogen plays a significant role as the main building block for proteins and nucleic acids in microbial cells. The effect of varying a nitrogen source on SmF of bacteria strains was evaluated (Figure 5). All the strains optimally produced cellulases whilst utilizing 1% yeast extract as the sole nitrogen source. Strain LB-8 reported the highest cellulase activity (0.19 U/mL) suggesting that it could efficiently exploit yeast extract as a nitrogen source. Yeast extract is enriched with nitrogen, vitamin B complex, and trace mineral nutrients that could have promoted cellulase production (Jach et al., 2022). The results are in agreement with the works of Sakthivel et al. (2010) and Pramanik et al. (2021) who

obtained maximum cellulase activity from yeast extract. However, all the strains (LB-4, LB-6 and LB-8) recorded the lowest cellulase activity when 1% urea was used as a nitrogen source with LB-4 having the lowest cellulase activity of 0.01 U/mL. Degradation of an inorganic nitrogen source (urea) might have lowered the pH of the medium, thus affecting cellulase production (Yang et al., 2014). Contrarily, ammonium sulfate which is an inorganic salt was found to be the best source of nitrogen for cellulase production. This could be due to the direct incorporation of ammonium sulfate in protein synthesis (Sethi et al., 2013).

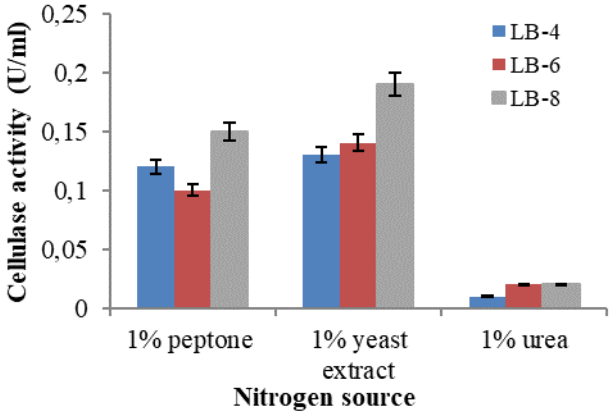

Figure 5. *Effect of different nitrogen source on cellulase activity.*

Effect of temperature on cellulase activity

The enzyme activity of the isolates was studied at various temperatures ranging from 20 to 50ºC. All the isolates showed effective cellulase production from 35 to 45°C with the highest activity at 40°C (Figure 6). The temperature might have affected exoenzyme secretion by altering the physical properties of the microbial cell membrane. Isolate LB-6 reported the highest cellulase activity of 0.32 U/mL whereas the lowest activity of 0.01 U/mL was observed from isolates LB-4 and LB-8. The optimum temperature of 40°C was also reported from cellulase fermentation of several bacteria species (Sethi et al., 2013; Gozan et al., 2018). Pramanik et al. (2021) also observed the highest cellulase activity (2.24 U/mL) from the fermentation of *B. pseudomycoides* at 40°C. Generally, the most frequently used temperature for bacteria fermentation is 37ºC (Hussain et al., 2017; Gozan et al., 2018).

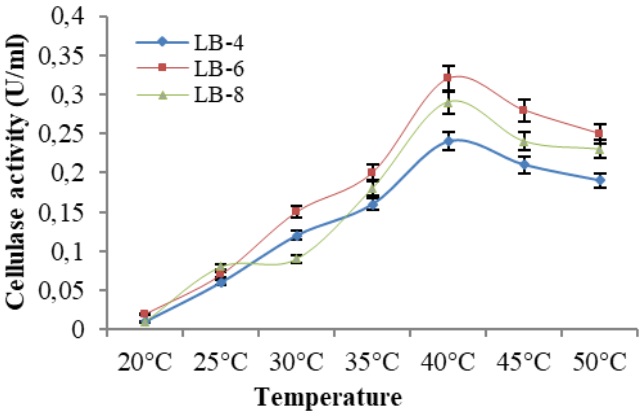

Figure 6. *Effect of temperature on cellulase activity.*

Conclusions

Hot springs are an ideal source of many thermotolerant cellulolytic bacteria with a wide range of applications. In this study, three bacteria strains (LB-4, LB-6 and LB-8) with high cellulolytic activity were isolated on a culture-enrichment medium. All the strains were found to belong to a genus of motile *Bacillus* on the basis of morphological and biochemical characterization. Using 16S rRNA homology analysis, strains LB-4, LB-6 and LB-8 were identified as *B. subtilis*, *Bacillus* sp., and *B. licheniformis*, with similarities of 99.13%, 98.26%, and 98.91% in the NCBI GenBank, respectively. The optimum fermentation conditions of *B. subtilis* LB-4, *Bacillus* sp. LB-6 and *B. licheniformis*LB-8 for cellulase production were at pH 7, 1% yeast extract, 1% CMC and 40ºC after 24 hr. The three bacteria isolates can be scaled up to commercial production under these optimal fermentation conditions. However, more research still needs to be done to further optimize the strains for inoculum size, medium additives, aeration rate, etc., and evaluate their ability to degrade CR in biogas production.

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