Isolation and identification of xylanase producing thermophilic bacteria from compost piles and optimization of xylanase production

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Thermostable xylanases are produced by thermophilic microorganisms. Their stability at high temperatures enable their widespread applications in commercial processes that include feed, food, beverages, textile, pulp, and paper industries. Xylanases can be used to replace conventional chemical bleaching agents such as chlorine compounds that are not friendly to the environment. The aim of this study was to isolate thermophilic xylanase producing bacteria from compost piles, and to optimize for high xylanase production. The research process involved the isolation of thermophilic bacteria from compost piles, qualitative screening for xylanolytic activity, and quantitative production of crude xylanases using Solid State Fermentation. Subsequently, temperature and pH were optimized for maximum xylanase activity. Identification of xylanases producing bacteria was determined by microscopic, biochemical characteristics, and molecular techniques. Selection results revealed that 7 out of 8 isolates had xylanolytic activity. Two isolates, G1 and H1 produced high crude xylanases activities, 12.21 U/mL and 10.85 U/mL, respectively. Optimization studies showed higher xylanase activities for isolates G1 (21.43 U/mL) and H1 (22.4 U/mL). The bacterial isolates were identified as *Bacillus pumilus* and *Bacillus safensis*. Further studies should be directed towards thermostable microbial enzymes for their potential to improve the quality of industrial products and research.

Keywords: xylanases; thermophilic bacteria; enzyme activity; industrial applications; compost piles.

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Introduction

Xylan is a major constituent of hemicellulose in plants [1]. Xylanases are hydrolytic enzymes that cleave the β-1, 4-glycosidic bonds of the xylan in the lignocellulosic materials to form xylose and xylo-oligosaccharides by the use of a mixture of hydrolytic enzymes including endo-β-1,4-xylanase and β-D-xylosidase [2]. These enzymes have attracted huge attention for their biotechnological applications. Pre-treatment of pulps before bleaching in the pulp and paper industry is the most significant industrial use of xylanases, which has been shown to save energy and improve yield and pulp properties in biomechanical pulping processes [3-5]. Application of xylanases in feed additives has resulted in improved nutritional properties of agricultural silages and digestion of nutrients in animals [6]. Xylanases have potential uses in the extraction and clarification of fruit juices, improvement of bakery products, and extraction of coffee and plant oils [7-9]. The use of xylanases in leather processing has improved the leather quality and has reduced environmental pollution [10]. Xylanases have also been applied in the saccharification of pre-treated lignocellulosic biomass for production of biofuels and other organic compounds [11].

Xylanases and associated debranching enzymes used in industries are produced by thermophilic microorganisms [12]. The microorganisms grow optimally at higher temperatures between 60-80°C [13]. The survival of thermophiles in compost piles results from high temperature of the central portion reaching up to 80°C [14]. Industries are in a continuous need of microorganisms that produce thermostable enzymes with high yield due to their characteristics and properties. These enzymes are able to withstand extreme conditions (high temperatures and alkaline or acidic pre-treatment) without denaturation [2, 15]. There is a limited use of xylanases produced by mesophilic organisms as these enzymes generally go through denaturation at temperatures above 55°C [16]. A 10°C increase in temperature on the industrial process doubles the rate of the reaction. Whilst, the amount of enzyme required is decreased [17]. At higher temperatures, thermostable enzymes have a longer half-life. Furthermore, higher temperatures above 60°C inhibit microbial growth and this results in reduced risk of contamination [18].

Xylanases have been produced from diverse genera and species of bacteria, actinomycetes, and fungi, including aerobes, anaerobes, mesophiles, thermophiles, and extremophiles [19-20]. Xylanases of microbial sources are preferred over those produced from plants and animals. They are structurally stable, easy to access, and can be easily genetically manipulated [21]. The production of xylanases from bacteria is more advantageous than other microorganisms due to fast growth rates [22]. The following bacterial genera are known to produce xylanases: *Bacillus*, *Cellulomonas*, *Micrococcus*, *Staphylococcus*, *Paenibacillus*, *Arthrobacter*, and *Microbacterium* [23-30]. A review by Mandal. [31] on microbial xylanases and their applications claimed that xylanases produced by bacteria are effective over a broad pH range (5-9) and temperature (35-60°C). The study by Chakdar *et al*. [32] also confirmed that maximum xylanase activity occurs over a wide pH range (6-9) and temperature (30-100°C).

Enzymatic processes in industries are specific, quick, and effective whilst saving raw materials, energy, chemicals, and/or water [33]. Developments in research on xylanase applications encompass finding novel enzymes and new microbial producers with higher specific activities and higher productivity [34]. The present study was aimed to isolate and identify thermophilic xylanases producing bacteria from compost piles and to optimize some of the contributing factors for high xylanase production.

Materials and Methods

Isolation of thermophilic bacteria

Compost pile samples were obtained from the residential area in Lapwing St, Three Rivers East, Vereeniging, South Africa (Global Positioning System: -26.662502, 28.026602). The 2.5-meter compost pile was built in 2014 and is made up of soil and plant materials. Samples were collected deep down (1-2 meters) within the compost pile. One gram of compost sample was diluted in 100 mL sterile saline water solution. From the stock solution, 1 mL of the sample was serially diluted into 9 mL sterile saline water solution up to 10^{-5} dilution. From each dilution mixture, 0.1 mL was taken and spread onto nutrient agar plates in triplicates. The plates were incubated at 50°C for 24 hours. Subsequently, countable colonies (dilution 10^{-4} and 10^{-5}) were purified by re-streaking onto new nutrient agar plates and incubated at 50°C for 24 hours.

Characterization of isolated bacteria

Colony morphology was observed under oil-immersion microscope. Morphotypes relative to cellular shapes, colonial elevation, colonial surface, and colonial pigmentation were selected and subjected to further tests. Basic biochemical tests were done to determine the major taxon of the morphotypes.

(1) Gram staining

Gram-staining as described by Smith and Hussey [35] was done on pure bacterial isolates.

(2) Catalase
Catalase production by bacterial isolates was determined by using 3% hydrogen peroxide (H_2O_2). A drop of 3% H_2O_2 was added to a loopful amount of bacterial smear on a microscope slide. Gas bubbles detected within 10 seconds indicated a positive reaction [36].

(3) Oxidase
Investigation of Cytochrome C oxidase was performed by using Bactident oxidase Test strips (Merck, Darmstadt, Germany). A 24-hour bacterial culture was applied onto the reactive surface of the test strip. The strip was observed for a color change [36].

(4) Carbohydrate fermentation
Carbohydrate fermentation was determined by using Phenol Red Lactose Broth containing phenol red, Durham tube, and peptone. Phenol red is a pH indicator which turns yellow below a pH of 6.8. Bacterial cultures were inoculated into broth and incubated at 37°C for 24 hours. After incubation, Lactose Broth were observed for color change and formation of bubbles was indicative of fermentation inside the Durham tube [36].

(5) Motility
Bacterial cultures were stabbed to the bottom of the SIM (Sulfide, Indole, Motility) media and incubated at 37°C for 24 hours. A diffuse cloud of growth away from the line of inoculation was an indication of a positive reaction [37].

(6) Hydrogen sulfide
Bacterial cultures were stabbed to the bottom of the SIM media and incubated at 37°C for 24 hours. A development of a black precipitate in the media indicated the positive hydrogen sulfide reaction [38].

(7) Indole test
Bacterial cultures were stabbed to the bottom of the SIM media and incubated at 37°C for 24 hours. Five drops of Kovac's reagent were added to the media. Appearance of bright red color within seconds was considered a positive reaction [36].

(8) Methyl red test
Glucose phosphate broth was inoculated with bacterial cultures and incubated at 37°C for 48 hours. Five drops of methyl red were added to determine the acidic ($pH < 5$) status of the medium. A red color development was seen as a positive reaction [36].

(9) Voges-Proskauer
Bacterial isolates were inoculated into glucose phosphate broth and incubated at 37°C for 48 hours. This was followed by addition of 5% naphthol and 40% Potassium hydroxide. The appearance of a red color was an indication of a positive reaction [36].

(10) Citrate utilization test
Simmon Citrate agar slants were inoculated with bacterial isolates and incubated for 24 hours at 37°C. A color change from green to blue was an indication of a positive reactions [36].

Screening for xylanolytic activity
The xylanolytic activity was detected based on the clear zones of hydrolysis of xylan around bacterial colonies. Spot inoculation were done in nutrient agar plates (10 g xylan, 2 g yeast extract, 0.5 g NaCl, 0.015 g $CaCl_2$, 0.5 g Peptone, 0.5 g $MgSO_4$, and 20 g bacteriological agar. pH 7.0). The plates were incubated at 50°C for 24 hours. The plates were then flooded with 0.1% (w/v) Congo red staining solution for 15 minutes. The staining solution was washed with 0.1 M NaCl solution. Formation of clear zone of hydrolysis indicated xylanolytic activity of bacterial isolates [30].

Production of xylanase under Solid State Fermentation
The ability of bacterial isolates to produce xylanase under solid state fermentation (SSF) was determined by using wheat bran as a substrate. Ten (10) grams of wheat bran was moistened with sterile 18 mL of mineral salt solution. The mineral salt solution contained (6.6

g $MgCl_2.6H_2O$, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$. pH 6.7). Bacterial isolates were cultured in a 250 mL Erlenmeyer flask containing moistened wheat bran. These were incubated for 48 hours at 50°C. Subsequently, the solid substrate was removed and suspended into 50 mL of 50 mM phosphate buffer (pH 8.0), vortexed thoroughly to extract the xylanase. The sample was then centrifuged at 4,000 rpm for 10 minutes at 4°C to remove xylanase from the substrate. The supernatant was filtered through Whatman No. 1 filter paper. The clear filtrate was used as crude xylanase preparation [30, 44].

Determination of crude xylanase activity

Xylanase activity was measured by determining the release of reducing sugars from xylan using dinitrosalicylic acid (DNS) method [39]. The substrate assay consisted of 1% beechwood xylan (1.8 mL) in phosphate buffer (pH 6.5). Crude xylanase preparation (0.4 mL) was added to the assay and incubated at 50°C for 10 minutes. DNS solution (2 mL) was added to the reaction mixture, boiled for 10 minutes, and left to cool in cold water. Xylanases are able to overcome the restricted breakdown of xylan [40]. Seven positively screened bacteria isolates were determined for crude xylanase activity. Enzyme reaction contained positively screened enzymes, DNS, and a substrate. Enzyme Buffer containing DNS and a substrate without enzymes was used as a negative control. The absorbance was read against a blank (water) at 540 nm on T60-UV-visible Spectrophotometer (PG Instruments Ltd., Leicestershire, UK). Enzyme activity was analyzed in triplicates. According to McCleary and McGeough [41], one unit of xylanase activity is the amount of enzyme that releases 1 micromole of reducing sugar equivalent to xylose per minute under the stated assay condition. Enzyme activity was expressed as units of enzyme activity/mL of the bacterial medium.

The following formula was used to calculate the activity of the crude xylanase:

$$\text{Units/mL enzyme} = \frac{(\mu\text{mol of xylose liberated})(\text{dilution factor})}{(10)(0.4\ \text{mL})}$$

Where 10 is the time of essay in minutes and 0.4 mL is the volume (in mL) of enzyme used.

Effects of temperature and pH on crude xylanase activity

(1) Temperature

The optimum temperature for xylanase activity was determined by assaying the crude enzyme extract at various temperatures (40-70°C). The DNS method by Miller [39] was used to determine xylanase activity at pH 6.5 with 10 minutes' incubation time.

(2) pH

The optimum pH for xylanase activity was determined by assaying crude xylanase extract (0.4 mL) and 1.8 mL buffers containing 1% xylan. Different pH's were used for phosphate buffer (pH 6, 7, and 8) and Tris-HCL (pH 9 and 10). Xylanase activities were performed by the DNS method at the respective optimal temperature for each isolate.

Molecular identification

The two bacterial isolates with the highest xylanase activities were subjected to molecular identification. Genomic DNA was extracted using a Cetyltrimethylammonium bromide (CTAB) method [42]. The DNA product was amplified by Polymerase Chain Reaction (PCR) on a C1000 Thermal Cycler (Bio-Rad laboratories, Hercules, California, USA). A PCR reaction mixture (final volume 25 µL) contained 12.5 µL double strength PCR Master Mix (0.4 mM dNTPs, 4 mM $MgCl_2$, and 0.05 U/µL Taq DNA polymerase (Fermentas Life Science, US)), 5.0 µL primer mix, 4 µL nuclease free water (Thermo Fisher, Waltham, Massachusetts, US) and 1 µL of genomic DNA (20-80 ng/µL). The following 16S rRNA bacterial gene sequence primers were used: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTGAGTTT-3') [43]. DNA of known pure bacterial isolate *Bacillus Cereus* ATCC 10876 was used as a positive control and PCR water as a negative control.

The PCR cycling conditions were as follows: initial denaturation at 95°C for 2 minutes, 30 cycles of

Table 1. Characterization and screening of xylanolytic activity on bacterial isolates.

Test	Bacterial isolates							
	A1	A2	B1	B2	E1	F1	G1	H1
Gram reaction	-	-	-	+	-	+	+	+
Catalase	+	-	+	-	-	-	+	+
Oxidase	+	-	-	-	-	-	-	-
Carbohydrate fermentation	+	+	+	+	+	+	+	+
Motility	+	-	+	-	+	+	+	+
Indole production	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-
Voges-Proskauer	+	+	+	+	+	-	+	-
Citrate utilization	-	-	+	-	+	+	+	+
Screening for xylanolytic activity	+	+	+	+	-	+	+	+

denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for 90 seconds and a final extension step at 72°C for 5 minutes. PCR result was determined by agarose gel electrophoresis. Positive PCR products (bands at expected length of 1,465 base pairs) were sent to Inqaba Biotech Company (Pretoria, South Africa) for sequencing. Obtained sequences were viewed and edited by using ChromasPro (Technelysium, South Brisbane, Australia). BLAST (http://www.ncbi.nlm.nih.gov/BLAST) was used to determine the identity of the sequences. Identified sequences were submitted to GenBank for accession numbers.

Statistical analysis

Microsoft Excel (2016) was used to determine averages and standard deviations.

Results and discussion

Characterization of thermophilic bacteria

Bacterial cultures were isolated at 50°C from compost piles. Eight morphotypes from microscopic exanimations were selected. Table 1 shows the results from conducted biochemical tests. The isolates were gram negative (A1, A2, B1, E1) and gram positive (B2, F1, H1, G1). Bubble formation on catalase test confirmed a positive reaction on four isolates (A1, B1, G1, and H1). The Bactident oxidase Test strips in oxidase test turned purple to confirm isolate A1 positive [36]. For carbohydrates fermentation, the liquid in the test tubes turned yellow and bubbles were formed inside the Durham tubes for all bacterial isolates. A drop in pH and a gas production results from the production of the acid and oxygen when lactose ferments in the media. For a positive indole test, indole reacts with Kovac's reagent to produce red coloured ring [36]. In the present study, there was no color change on the isolates. A black precipitate did not develop for all isolates in hydrogen sulphide test, thus a negative reaction [38]. Motility test was positive for most isolates (A1, B1, E1, F1, G1, H1). Positive citrate result on isolates (B1, E1, F1, G1, and H1) was established from the formation of a blue color change on the slants [36].

Screening for xylanolytic activity

A formation of a clear zone on xylan containing agar plates flooded with Congo red was observed on seven isolates (except for E1) (Table 1). Congo red was used as an indicator of polysaccharide hydrolysis. The isolate with the largest clear zone diameter was stipulated to exhibit the highest xylanase activity [29]. The results in the current study indicated that xylanases in bacterial isolates were able to degrade xylan in the agar medium.

Production of xylanase from isolates under Solid State Fermentation (SSF)

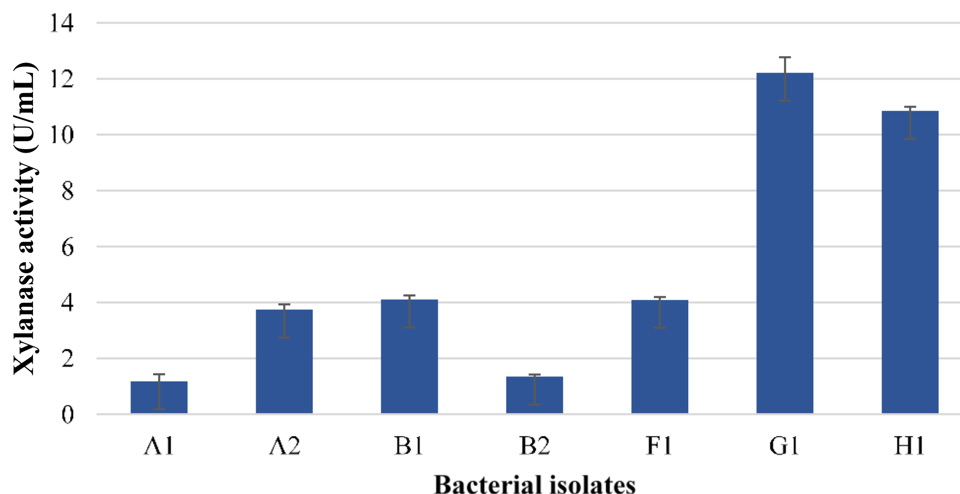

Figure 1. Xylanase activity at 50°C (pH 6.5) of the crude enzyme extracted from the bacterial isolates.

Xylanase activity from the bacterial isolates ranged from 1.18 U/mL to 12.21 U/mL within 10 minutes of incubation time at 50°C and pH 6.5 (Figure 1). Isolate H1 and G1 showed the highest enzyme activity, 10.85 U/mL and 12.21 U/mL, respectively. SSF is used in industrial production of enzymes. It is characterised by the absent or near absent aqueous phase that provides natural habitat for microbial growth. The low moisture level in the system makes it easier to control contamination. Moreover, the technique produces high yield at reduced energy and capital [44]. Wheat bran is used as a substrate in SSF to induces xylanase synthesis from xylan degradation. It is a carbon and nitrogen source for bacteria in producing xylanases [44]. Several studies have reported on the use of SSF for the production of xylanase from wheat bran [2, 44-46].

Temperature and pH on crude xylanase activity

Enzyme activity is the most vital factor in studying enzyme characteristics [47]. In the current study, temperature and pH were optimized on the two isolates (H1 and G1) to determine maximum crude xylanase activity. Optimization of process parameters could result in increased xylanase production yield [6].

(1) Temperature

Different orientations of enzyme structures at various temperatures affect xylanase activity [34]. The maximum xylanase activity for isolates G1 (17.89 U/mL) and H1 (16.73 U/mL) were observed at 60°C and 55°C, at pH 6.5 (Figure 2). Beyond the two respective optimal temperatures, enzyme activity was minimal. In a study conducted by Uday *et al*. [34], high xylanase activity was observed with an increase in temperature. However, further increases from a certain high temperature, xylanase activity decreased. These findings were also seen in studies by Wahyuntari *et al*. [48], Khandeparker *et al*. [49], and Irfan *et al*. [50] with maximum xylanase activity at temperatures (40-60°C).

(2) pH

The changes in pH alter the three-dimensional structure of the enzyme protein and affect enzyme activity [51]. Crude xylanase enzyme activities for isolate G1 was 21.43 U/mL at pH 9.0 and optimal temperature at 60°C. Maximum xylanase activity of 22.4 U/mL was also observed at pH 8.0 at optimum temperature (55°C) for isolated H1 (Figure 3). These results indicated that alkaline pH has a great influence on the xylanase activity. Kamble and Jadhav [44] reported optimal pH of 8.0 for maximum xylanase production. In a study conducted by Yadav *et al*. [52], an optimal pH of 8.0 was

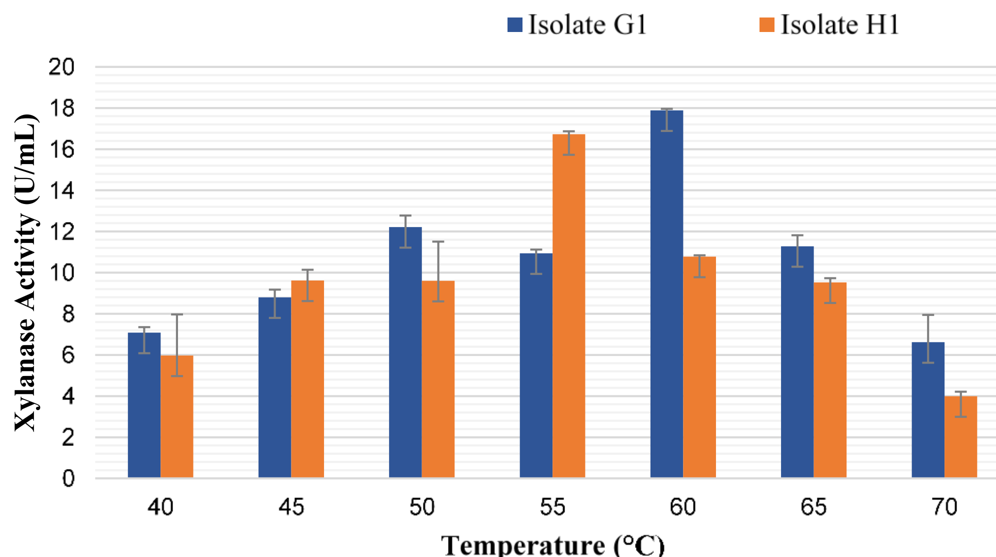

Figure 2. Effects of temperature on crude xylanase activity at pH 6.5.

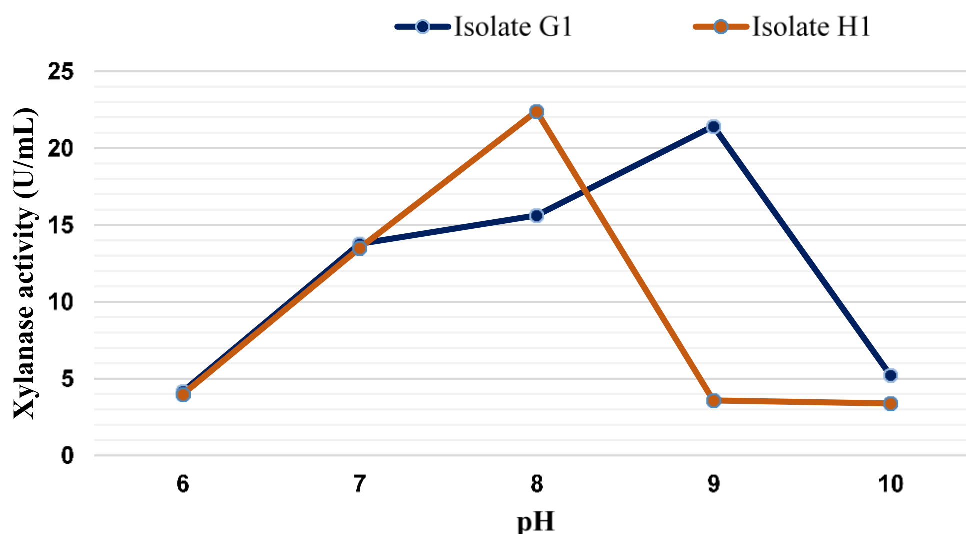

Figure 3. Effects of pH on crude xylanase activity at 60°C (G1) and 55°C(H1).

determined for maximum activity of thermostable alkaline xylanase from *Anoxybacillus kamchatkensis* NASTPD13 isolated from Paudwar Hot Springs. Such enzymes with high activity in alkaline pH (pH 8.0-11) are desirable in many industrial processes such as pulp and paper industries. Xylanase with optimal activity in alkaline pH destroy coloring impurities, a pre-treatment bleaching process that enhance brightness and whiteness of pulp for a preferable white paper product. Xylanase is used as a replacement to conventional bleaching agent, chlorine. Chlorine is not environmental friendly as it produces toxic and mutagenic chronoorganic compounds [53-55].

Molecular identification

Bacterial DNA was extracted and the 16S rRNA gene fragment was amplified using PCR technique. Positive PCR products were seen as

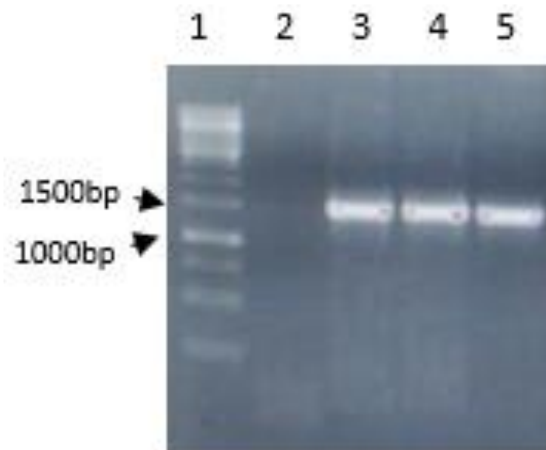

Figure 4. An image of 1% agarose gel indicating amplified 16s rRNA gene fragments. Lane 1: 1 kb molecular marker; lane 2: non-template control (PCR water); lane 3: positive control (bacterial DNA - *Bacillus Cereus* ATCC 10876); lanes 4 and 5: unknown bacterial DNAs.

bands on the gel electrophoresis (Figure 4). The bands from unknown bacterial isolates were at expected amplicon sizes (1,465 base pairs) for primers (27F, 907R) [43]. Blast results from sequences sent to Inqaba Biotech revealed high gene sequence similarity (≥99%) between isolates in the present study and those from NCBI GenBank database. The isolates were identified as *Bacillus pumilus* and *Bacillus safensis*. Sequences of the species were assigned accession numbers MT080691 and MT080692. The biochemical test results (Table 1) on the two identified isolates were gram-positive, rod-shaped, catalase positive, metabolised carbohydrates by fermentation and were motile. The mentioned traits conformed to characteristics of the bacterial genus *Bacillus* [56-58]. However, the determined morphological and biochemical tests in the current study are inconclusive to identify to species level [58]. Molecular techniques as conducted in the present study are used to supplement morphological and biochemical tests in bacterial identification [59]. Xylanase activity has been previously determined from similar *Bacillus* species. In a study by a Gaur *et al*. [60], the bacterial species *Bacillus vallismortis* showed maximum xylanase activity (3,768 U/mL) at 55°C and pH 7.0. Geetha and Gunasekaran [61] determined activity on purified xylanase on *Bacillus pumilus*. The maximum activity was 755.81 U/mL at 60°C and pH 6.5. Several studies have reported on other xylanase producing *Bacillus* species [29, 30, 50].

Conclusion

Environmental pollution by chemical operations has triggered the use of energy efficient, economically viable, and environmental friendly enzymes. The present study demonstrated that thermophilic bacteria are potential sources of xylanase. These xylanase producing isolates *Bacillus pumilus* and *Bacillus safensis* produced maximum xylanase activities at their respective optimal temperatures and pH. The optimized characteristics of enzymes are vital for the activity and stability in biotechnological applications. The extreme thermo and alkaline tolerance properties of the isolates are quite suitable for industrial uses such as detergent, food, pharmaceutical, leather, agriculture, and kraft pulp prebleaching processes. They could be used alternatively for commercial strains. Further studies are needed for purification of thermostable microbial enzymes and optimization of other parameters such as incubation times and moisture content. This will enhance enzyme production and improve the quality of industrial products and research.

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