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Research Article

PRODUCTION AND CHARACTERIZATION OF THERMO-ALKALISTABLE XYLANASE FROM GEOTHERMAL SPRING ISOLATE

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ABSTRACT
Thermostable and alkalistable xylanase was screened from bacteria isolated from geothermal spring water of Unapdev, Maharashtra state, India. Maximum enzyme production was recorded 2.05 IU/ml after 48hrs at 40°C temperature under the shake-flask condition. Enzyme was partially purified by solvent precipitation followed by ammonium sulphate precipitation and desalting by dialysis and used for the characterization study. Enzyme activity was found to in wide range of pH and temperature with optimum at pH 9.0 and temperature 70°C. Media optimization studies showed highest enzyme production with 0.5% birchwood xylan, 0.5% yeast extract, 1.0% peptone and 0.3% NaCl. Higher xylanase production was achieved with wheat straw at 72hrs. Hydrolysis studies revealed complete degradation of birchwood xylan to xylose after 12hrs. The use of thermos table, alkalistable xylanase for enzyme assisted pulp bleaching could greatly reduce the need for pH and temperature readjustment, thus offering enormous technical and economic advantages.

Keywords*: Thermostable, Alkalistable, Geothermal Spring, Xylanase*

INTRODUCTION
Hemicellulose is a branched heteropolymer consisting of pentose sugar including, D-xylose and D-arabinose and hexose sugar i.e. D-mannose, D-glucose and D-galactose with xylose being most abundant (Kumar *et al.,* 2008). Enzymatic depolymerization of hemicellulose to monomer sugars needs the synergistic action of multiple enzymes, among them endo-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) collectively known as a xylanases play a vital role in depolymerizing xylan, the major component of hemicellulose. Xylanases (EC 3.2.1.8) are ubiquitous in nature and play a crucial role in xylan degradation by catalyzing the endohydrolysis of 1, 4-b-D-xylosidic linkages into short xylooligosaccharides (Biely *et al.,* 1985). Xylanases are widely used in manufacture of paper, paper product recycling, textile manufacture, baking, release of aroma and anti-oxidant molecules, and production of biopharmaceuticals, which are targeted at both selective and extensive modification of xylans, have provided an increased impetus to identify and obtain new xylanases with different specificities and properties (Ryan *et al.,* 2003). The use of hemicellulases including xylanases for delignification in the paper industry has been limited by the lack of large-scale availability of enzymes active at alkaline pH and elevated temperatures, which are the conditions prevailing in many pulp bleaching processes (Lundgren *et al.,*1994). Neutralization with acid and cooling of pulp for optimal activity are additional steps which add to the operational costs. The use of xylanases at various industrial processes is one of the sustainable approach.
Extremophiles are vital and novel source of enzyme for industrial applications (Niehaus *et al.,* 1999). Hot spring habitat is the natural sink of thermophilic microorganism, which can provide thermo-active enzymes. Exploitation of geothermal sites as source of extremophiles had been reviewed earlier (Haki and Rakshit, 2003). Various xylanase producing thermophilic bacteria including, *Paenibacillus ehemensis* (Singh *et al.,* 2010), *Alicyclobacillus sp.* (Bai *et al.,* 2010), *Geobacillus sp*. (Canakci *et al.,* 2007) and *Bacillus subtilis* (Sa-Pereira *et al.,* 2002) have been isolated from various geothermal spring and hot spring. Properties of thermophiles imply extremely important for industrial and biotechnological applications due to the fact that enzymes from such microorganisms remains active in harsh industrial conditions by retaining specific catalytic activity. Harnessing the power of enzymes for environmental

stewardship is of prime importance (Demarche *et al.,* 2011). The cost of enzymes is one of the main factors determining the economics of a process. Reducing the costs of enzyme production by optimizing the fermentation medium is the basic research for industrial application (Ghanem, 2000). The present study aimed to isolate the xylanolytic bacteria from geothermal spring and test the enzyme's optimum catalytic parameter. Enzyme characterization and the studies on the optimization of enzyme production were carried out.

MATERIALS AND METHODS

Sampling and Isolation of Microorganism

The water samples were collected from the hot spring of Unapdev, Jalgaon district, Maharashtra state, India. A 50mL water sample was passed through membrane filter assembly (0.45µm) for filtration using sterile syringe under aseptic conditions. Filter pad was inoculated aseptically into the sterile nutrient broth with pH 9.0 and incubated at 40°C for 24 hours.

Screening and Identification of Isolates

The strains were screened for xylanase activity using Bushnell-Haas (BH) medium pH 9.0 containing 0.5% Birchwood xylan (BX) as substrate. Xylanase producing strains were selected by flooding replica plates with 0.5% (w/v) Congo red for 15min followed by repeated washing with 1M NaCl for zone analysis (Gessesse and Gashe, 1997). Positive xylanase activity was detected by the presence of yellow halo against red background. Tentative identification of isolate was done using physiological and biochemical tests according to Bergey's Manual of Determinative Bacteriology. For xylanase production, the bacterial culture was cultivated in BH medium supplemented with 0.5% Birchwood xylan, 0.5% yeast extract, 1% peptone, 0.3% NaCl medium, pH 9.0 under shaking (120 rpm) conditions at 40°C. After 48hrs, the fermented broth was centrifuged and the cell free supernatant was subjected to purification.

Partial Purification of Xylanase

Fifty ml of cell-free supernatant was partially purified by precipitating with double volumes of cold acetone and double volumes of 95% cold ethanol respectively. The precipitate obtained after each step was centrifuged for 30 min at 10,000 rpm at 4°C and dissolve in 50ml of 50mM Glycine NaOH buffer (pH 9.0). Ammonium sulphate precipitation up to 80% saturation was carried out and incubated overnight at 4°C to precipitate the total proteins followed by centrifuged at 10,000g for 30min, precipitates dissolved in 5ml of Glycine NaOH buffer (pH 9.0) and dialysed against the 1000 times diluted same buffer for 24hrs and finally dissolved in 12ml of said buffer.

Growth Pattern and Xylanase Production

Optimum pH and temperature for the growth of the isolate was determined in liquid media by cell turbidity measurement at 600 nm using spectrophotometer. Growth profile with respect to the enzyme production was also studied by taking the sample at every 6 hours interval up to 72 hrs.

Optimization of Medium for Growth and Xylanase Production

Different concentration of yeast extract (0.00%, 0.25%, 0.50% and 1.00%), NaCl (0.00%, 0.15%, 0.30% and 0.60%), Birchwood Xylan (0.00%, 0.25%, 0.50% and 1.00%) and peptone (0.00%, 0.50%, 1.00% and 2.00%) were supplemented into the culture medium to optimize xylanase production. Xylanase activity was measured after growing the culture for 48hrs wherein concentration of one of the constituents was varied in culture medium.

Evaluation of Crude Carbon Source as Substrate

Wheat straw and sawdust were used as a carbon sources by replacing birchwood xylan at a final concentration of 1.0% w/v in the optimized medium. Inoculum was prepared by inoculating 10ml Bushnell-Haas medium containing xylan in a 100ml flask and incubating it on shaker 120rpm at 40°C for 24hrs. For xylanase production, the organism (5% inoculum) was cultivated in 200ml BH medium supplemented with birchwood xylan (pH 9.0) in 500ml flask under shaking at 120 rpm at 40 °C. After 48 hrs and 72 hrs, the fermented broth was centrifuged and the cell free supernatant was subjected to purification. Growth was estimated by measuring optical density of the culture broth collected at different time intervals at 600 nm.

Enzyme Assay

Xylanase activity was determined by a method described by Bailey *et al.*, (1992) using 1.0% (w/v) birchwood xylan in 50mM Glycine NaOH buffer (pH 9.0) after 5 min reaction time. The assay mixture containing 2.0ml substrate solution with 0.5ml suitably diluted enzyme solution in the buffer was incubated at 60°C for 5 min and reaction was stopped by addition of 3.0ml dinitrosalicylic acid (DNS) reagent followed by keeping at 90°C for 5min and absorbance read at 540 nm using spectrophotometer (Shimadzu UV1800). The amount of reducing sugar liberated was determined by the DNS method (Miller, 1959) using xylose as a standard. One unit (IU) of xylanase activity was defined as the amount of enzyme required to liberate one µmol of xylose per minute under the assay conditions.

Protein Estimation

The extracellular protein was precipitated with 10% (w/v) trichloroacetic acid at 4°C. After centrifugation at 10,000 rpm for 10min, the pellet was dissolved in 0.1M NaOH solution. The protein content was estimated by Lowry's method with bovine serum albumin as a standard (Lowry *et al.,* 1951).

Characterization of Xylanase

Effect of Temperature and pH on Xylanase Activity

The optimum temperature of xylanase activity was determined by incubating the enzyme at different time interval and temperatures ranging from 30°C to 70 °C, with 1% soluble birchwood xylan in 50mM Glycine-NaOH buffer at pH 9.0. The pH profile of xylanase was evaluated by incubating the enzyme in the presence of 1% birchwood xylan solubilized in appropriate buffers: i.e. 50mM Citrate-Na citrate buffer (pH 5.0 and 6.0), 50mM Tris-HCl buffer (pH 7.0 to 8.0), 50mM Glycine-NaOH buffer (pH 9.0 to11.0), the reaction was carried out at 60°C for 5min and the residual activity was estimated by standard assay procedure.

Thermal Stability of the Crude Enzyme

The temperature stability of enzyme extract was determined by incubation at 50, 60, 70, 80 and 90°C for intervals of 5, 30 and 60 min at pH 9.0 and the residual enzyme activity was measured (Subramaniyan and Prema, 2000; Beg *et al.,* 2001).

Analysis of Hydrolysis Product of Birchwood Xylan

The standard assay mixture containing1% of birchwood xylan in Glycine-NaOH buffer and partially purified enzyme was incubated at 37°C. The enzyme reaction was stopped by heating at 100°C for 5min at different time intervals. The hydrolysis products were detected by thin-layer chromatography using TLC plates from Merck, India. The separation of the component was achieved using n-butanol:water:acetic acid (60:20:20) mixture as a solvent system. Compounds were detected by exposing to iodine vapours followed by heating at 80°C for 5min. D-xylose (1%) was applied as standard product and birchwood xylan (1%) as standard substrate in separate lanes.

RESULTS AND DISCUSSION

Sampling Site and Isolation of Bacteria

The temperature and pH of water samples during sampling were found 48ºC and 7.5 respectively. Bacteria were isolated by employing membrane filter technique. The eight isolates were selected according to differences in their colony morphology.

Screening and Identification of Isolate

Two out of total eight isolates showed clear halos demonstrating their ability to produce extracellular enzyme when subjected to further screening using B-H medium supplemented with 0.5% Birchwood xylan with pH 9.0. Xylanase activity was confirmed by Congo red staining followed by NaCl destaining of the plate yielding yellow halo against red background. Xylanase producing isolate UD2 with weak cellulase action was selected for detailed studies. The isolate was found Gram negative, short rod, facultative anaerobe, motile, oxidase and catalase positive. It utilized glucose, maltose and lactose, produced pink colonies on MacConkey's agar (Table 1). On the basis of these characteristics, the isolate UD2 was tentatively identified as *Moellerella wisconsensis* by referring to Bergey's Manual of Systematic Bacteriology and ABIS online identification system. However, molecular analyses based on

16S rDNA and DNA-DNA hybridization with related species has to be performed to find out their exact taxonomic position.

Table 1: Morphological and biochemical and characteristics of isolate

Tests		Results and observation
Shape		Rods
Gram stain		Gram negative
Motility		+
Spore stain		No spores
Pigmentation		No
Indole Production		-
Methyl Red		+
VogesProskauer		-
Citrate Utilization		+
Urea Hydrolysis		-
Nitrate reduction		+
Gelatin liquefaction		+
Catalase production		+
Oxidase		+
Phenylalanine Deaminase		-
Sugar fermentation test	Glucose broth	Acid only
	Maltose broth	+
	Mannitol broth	-
	Xylose broth	-
	Lactose broth	+
Triple sugar iron agar	Slant	Acidic
	Butt	Acidic
	CO_2	-
	H_2S	-
MacConkey agar	Pink colored colonies	

Growth Pattern and Xylanase Production

The time course of growth and the production of extracellular xylanase followed similar pattern. The growth and the production of extracellular xylanase increased up to 48 hours and decreasing with increased incubation time. Nearly 1.81 IU/mL activity was reported at 48hrs and OD of growth was 0.33 at 540 nm (Figure 1). So, maximum production was found in early stationary phase, which is similar to the earlier report (Banu *et al.*, 2005)

The optimum pH and temperature for growth of isolate was found to be 40°C to 50 °C and pH 9.0 respectively (Figure 2 and 3).

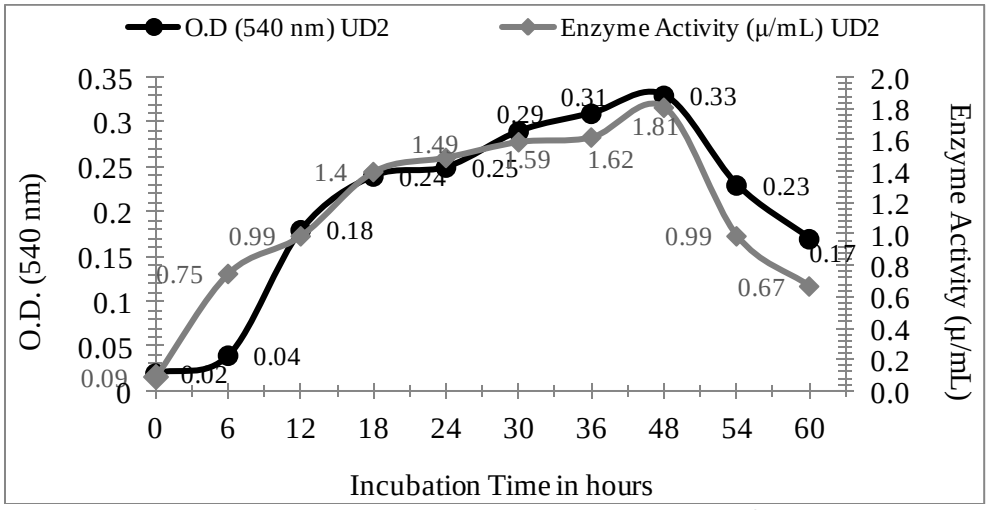

Figure 1: Growth pattern and enzyme production of the isolate

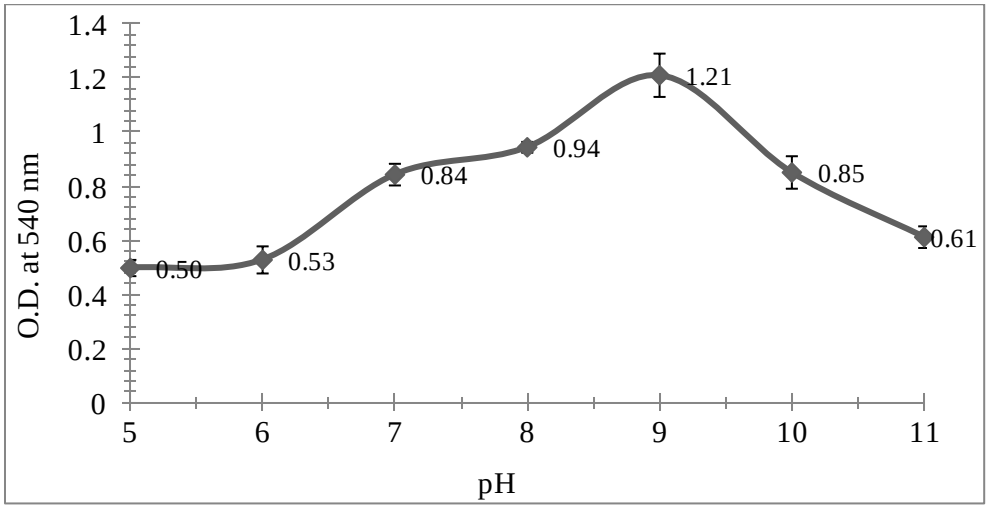

Figure 2: Growth of the isolate at the various pH scale

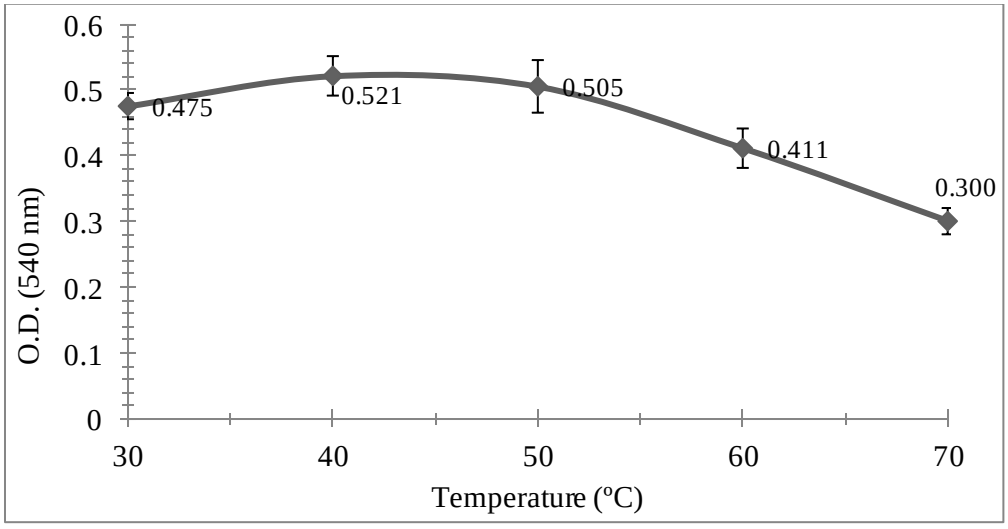

Figure 3: Growth of the isolate at the different temperature scale

Optimization of Medium for Growth and Xylanase Production

Optimization was done by selecting the one parameter at a time. Isolate showed highest xylanase production when the growth medium was supplemented with 0.5% yeast extract, 1% peptone, 0.3% NaCl and 1% Xylan at 37°C after 48 hours of incubation (Table 2). The optimization of production medium is a very useful tool to produce the bulk amount of enzyme activity with economical preparation. Optimization of culture media for xylanase production with different nitrogen source using yeast extract, peptone and KNO_3 are very important for bulk production of xylanase (Nagar *et al.*, 2010).

Table 2: Effects of different concentration of various medium components to optimise enzyme production

Yeast Extract	Enzyme activity (IU/mL)	Peptone	Enzyme activity (IU/mL)	NaCl	Enzyme activity (IU/mL)	Xylan	Enzyme activity (IU/mL)
0%	1.04	0%	1.01	0.00%	1.33	0.00%	1.30
0.25%	1.60	0.50%	1.67	0.15%	2.42	0.25%	1.65
0.50%	2.31	1.00%	2.35	0.30%	2.95	0.50%	1.98
1.00%	1.35	2.00%	2.04	0.60%	1.98	1.00%	2.26

Evaluation of Crude Carbon Source as Substrate

Pure xylan is highly expensive to be used for large scale industrial production of xylanases therefore alternate substrates, especially crude agriculture based raw-material were explored as potential substrates for economic production of xylanase. The isolate showed higher xylanase production on wheat straw followed by sawdust and birchwood xylan after 72 hours of incubation. Production of xylanase using wheat straw was found double than birchwood xylan after 72hrs (Figure 4). Wheat straw has been reported to support the highest xylanase production amongst the crude substrates with *streptomyces sp.* SU9 (Bajaj *et al.*, 2010). Due to the structural complexity xylan served a as good inducers of xylanase, while simple carbon sources such as xylose and glucose don't induce xylanase secretion due to absence of structural heterogenity (Beg *et al.*, 2001).

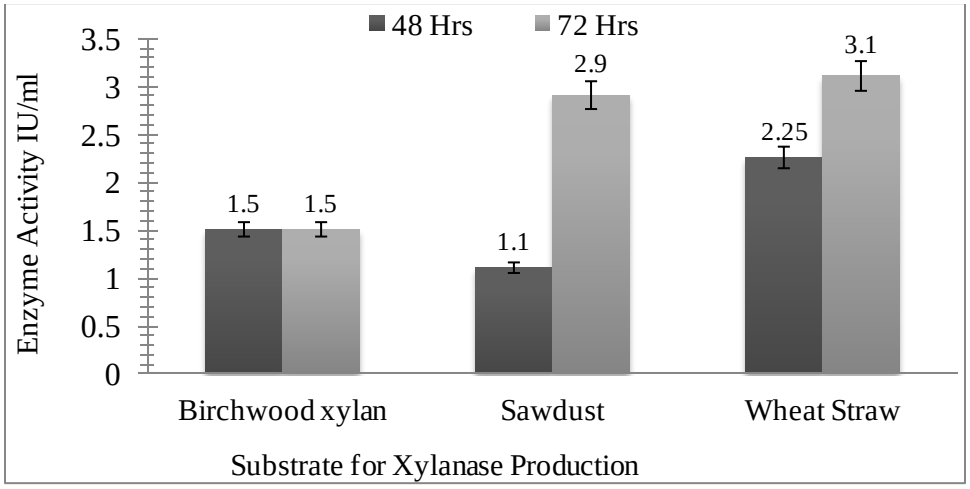

Figure 4: Evaluation of crude carbon sources for xylanase production

Characterization of Xylanase

Effect of pH on Enzyme Activity

Partially purified enzyme with enzyme activity (2.05 IU/ml) was used for characterization of enzyme to determine its robustness for industrial applications. The optimum pH for xylanase activity was found to be pH 9.0, and activity was 2.05 IU/mL (Figure 5). It was observed that the enzyme activity directly

correlates with growth of isolate. Activity reduction after 60hrs of fermentation may be due to production of certain proteases in the fermentation broth as the organism was protease producer. The culture conditions were found to have profound influence on xylanase production. The pH optima of the xylanases isolated from many bacteria were reported near the neutral pH. *Bacillus sp*. C-125 exhibited an optimum pH of 6.0 and xylanase of *B. stearothermophilus* had an optimum pH of 6.0 to 7.0. Optimum pH for xylanase from *Bacillus pumilus* SV-85S and *Paenibacillus macquariensis* RC 1819 were reported to be 6.0 and 8.6 respectively (Nagar *et al.*, 2010; Sharma *et al.*, 2013). Recently, few xylanases of bacteria such as *Enterobacter sp*. MTCC 5112 (Khanderkar and Bhosle, 2006) and *Staphylococus sp*. (Gupta *et al.*, 2000) had reported an optimum pH of 9.0. which is in agreement with our result.

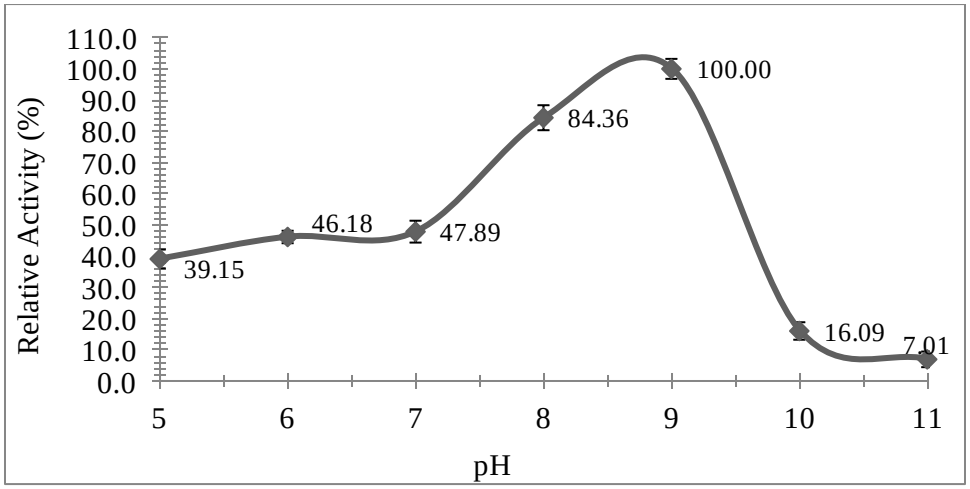

Figure 5: Effect of pH on xylanase activity

Effects of Temperature Enzyme Activity and Tharmostability

The optimum temperature for the xylanase activity was found to be 70°C, with maximum enzyme activity of 2.05 IU/mL (Figure 6). Thermostability profiles of the xylanase showed that, it was fairly stable at 70°C with residual activity 96% for 30min. Also at higher temperatures of 80°C and 90°C, it retained considerable relative activity after 30 min (Figure 7). Our results of thermostability of enzymes at elevated temperature appears superior than the thermostability of xylanase from *Bacillus circulans* (Dhillon *et al.*, 2000).

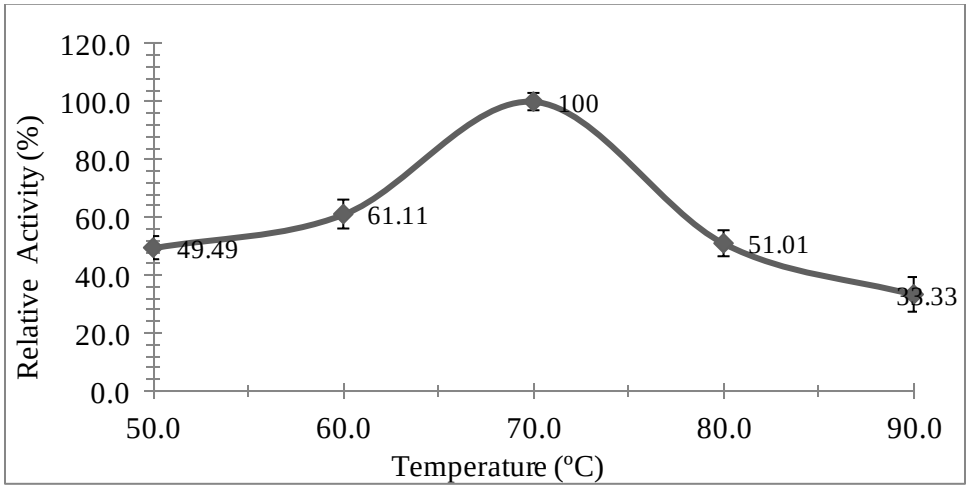

Figure 6: Effect of temperature on xylanase activity

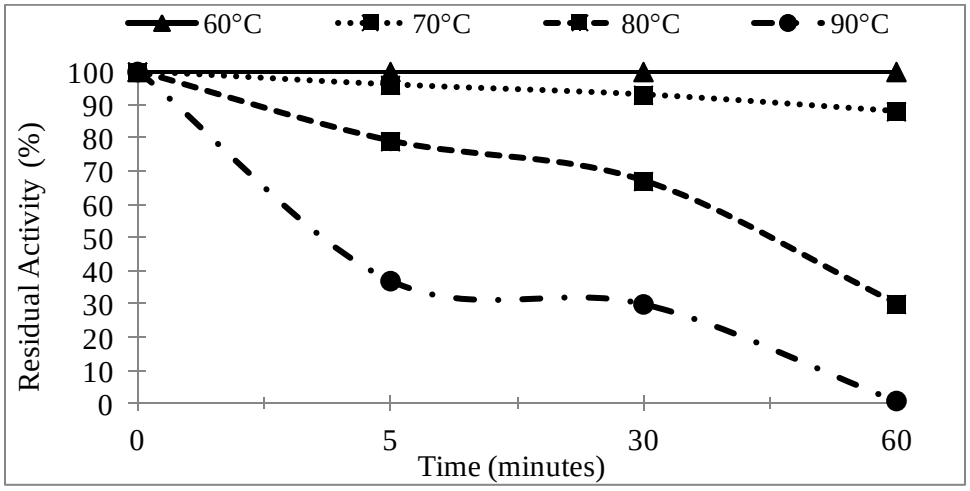

Figure 7: Thermostability of the enzyme at different time interval

Analysis of Hydrolysis Product of Birchwood Xylan

A hydrolysis experiment on birchwood xylan was carried out at 37°C at different time intervals using partially purified xylanase as the enzymatic source (Figure 8). In the early stage of the reaction, a large number of products were obtained but there was no trace of xylose until 11hrs of reaction time. After 12hrs of incubation, xylose was detected as one of the hydrolysis products. These results indicated that initially xylanase cleaved the substrate to liberate xylo-oligosaccharides and then release xylose.

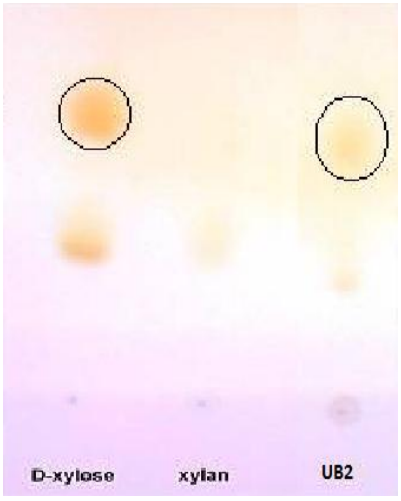

Figure 9: Chromatogram of the hydrolysis products of birchwood xylan by xylanase enzyme from isolate

Black circle indicate the D-xylose is end product of the xylan hydrolaysis by enzyme and its *Rf* value is concur with standard D-xylose.

Conclusion

This is the first report on the production of xylanase by *Moellerella species* to the best of our knowledge. The xylanase showed considerable activity at alkaline pH 9.0 and temperature 70ºC indicating its impending application in biobleaching. Additionally the bacterium showed sufficient enzyme production on cheap carbon source wheat straw indicating the potential of this isolate for cost effective enzyme production.

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