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Research Article

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Bioprocessing, biochemical characterisation and optimization of solid state fermentation of a new thermostable xylanase producing strain belonging to *Bacillus* genus

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ABSTRACT

The present study was aimed at isolation and characterization of a new xylan degrading strain of Bacillus from soil for production of xylanase. Different soil samples from Punjab state were used to isolate xylan degrading strains. We isolated a highly active xylanolytic strain and performed genetic characterisation using 16S r-RNA analysis. DNA sequencing and phylogenetic analysis using MEGA4 software showed our strain to be a novel strain (accession number JQ916900) which was named Bacillus lpuarvinder st. lpu002. Biochemical characterization of the enzyme showed optimum activity at 60 ^oC and pH 8.6. The enzyme was stable at 70 ^oC for up to 6 hours. The xylanase activity seems to be alleviated by divalent Ca^{2+}, Mn^{2+} and Mg^{2+} ions. The best enzyme activity was obtained when the cells were grown in Bushnell-Haas medium supplemented with 0.5% xylan and beef extract. The process optimization was done using solid state fermentation (SSF) using wheat straw as substrate. The enzyme isolated from SSF was analysed in a zymogram. The zymogram analysis showed prominent clear zone with congo red. The strain Bacillus lpuarvinder st. lpu002 produces a highly active xylanase, which may be taken to purification as it has immense biotechnological potential to be used in industries.

Keywords: Xylanase, *Bacillus*, Solid State Fermentation (SSF), Thermostable, MEGA4, Zymogram

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INTRODUCTION

Xylans are complex polysaccharides made up of β-D-xylose residues, linked together α-1,3- and/or β-1,4-glycosidic linkages. α-1,3 glycosidic linkages are found in the xylan component of softwoods, whereas β-1,4 linkages are ubiquitous in all xylans. Xylans are almost as predominantly found in the plant cell walls as cellulose and occurs in all the phyla of plant kingdom, be it *Bryophyta*, *Pteridophyta* or *Embryophyta* as well in fungi. Such posthumous occurrence of xylans makes them an economic carbon source for use in different fermentation processes. But the problem that the scientific community is facing currently is the deficiency of such highly active enzymes and/or strains possessing xylan metabolising activity. Xylanases are the class of enzymes capable of hydrolyzing complex xylans, major component of hemicellulose, to yield xylose and other xylooligosaccharides. Xylanase is the collective name given to the enzymes or β-1,4 endoxylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37). These enzymes find immense potential in the paper industry as it provides a significant by-pass to the eco-hazardous chlorine bleaching of pulp [1]. A well established application of xylanses comes from the food industry where it is an intrinsic component of fruit juice clearing along with pectinase and cellulase [2]. Having fundamental applications in industries, in recent years as well as currently, more and more research is being directed to the search for high activity xylanases for saccharification of complex lignocellulosic biomass. Shrivastava and coworkers have showed a thermophilic xylanase produced by *T. lanuginosus* SS-8 [3]. A similar work has been reported where a thermostable xylanase was ectopically expressed in *E. coli* and *Pichia pastoris* [4]. Hung and co-workers have also reported isolation of a thermophilic xylanase GH10 from *Thermoanaerobacterium saccharolyticum strain NTOU1* [5].Li et al isolated a thermostable xylanase from Paecilomyces thermophila strain J18, which showed stable activity up to 75 ^oC [6]. There also have been reports on organic solvent-tolerant xylanase from *Aspergillus awamori VTCC-*

F312, which retained 44-61% of its activity in organic solvent concentrations of as high as 80% (v/v) [7]. In the current study, we have isolated a novel xylanase producing strain of genus *Bacillus*, performed genetic and biochemical characterization of our strain and optimised xylanase production by non-sterile solid state and submerged fermentation.

Solid State Fermentation (SSF) is beneficial over submerged fermentation including lesser space requirement, lesser media requirement, ergonomy of the process, equipment and control systems, higher product yields, higher energy efficiency, highly economical process (lower capital and recurring expenditure in industry), more convenient scale-up of process, easier downstream processing and product recovery, improved yields, absence of foam build-up and easier maintenance of sterility due to the low moisture content of the system [8][9].

We report here a novel xylanse producing strain belonging to the genus *Bacillus* isolated from the soil collected from a unique niche of the state of Punjab, India. Optimisation of culture and nutritional parameters for our strain was performed including optimisation of carbon source, nitrogen source, optimum temperature, pH and inhibitor analysis. The enzyme shows high industrial potential owing to its rapid action and high catalytic efficiency.

EXPERIMENTAL SECTION

Isolation of Xylanase producing microbes: The soils samples were resuspended in sterile distilled water and plated in various serial dilutions on Mannitol Salt Agar (Himedia, India) plates. The plates were incubated at 37^{o}C for 24 hours. The resultant colonies were patched in duplicates onto fresh MSA plates containing 0.5% Xylan (SD Fine chemicals, India). The xylanase producers were screened by staining the xylan-MSA plates with 0.5% Congo red (Sigma Aldrich). Xylanase producing organisms displayed clear zones upon congo red staining.

Genetic identification of xylanase producing strains: A single colony from the pure culture of the isolated strain was used to inoculate Luria broth and incubated at 37^{o}C overnight. Next day, the cells were harvested by centrifugation and the genomic DNA was isolated from the cells using SDS/CTAB lysis followed by Phenol/Chloroform/Isoamyl alcohol extraction and isopropanol precipitation [10]. The genomic DNA was used in a PCR reaction to amplify 16S r-RNA genes using the following primers: E8F (AGAGTTTGATCCTGGCTCAG) and E1492R (GGTTACCTTGTTACGACTT). The expected product size using these primers was 1485 bp. The amplified PCR product was purified by gel extraction using Qiagen Gel extraction kit (Qiagen, U.S.A.) and sequenced at Bioserve Biotechnologies India private Limited. The obtained sequence was analysed using NCBI-BLAST and a phylogenetic analysis was carried out using Molecular Evolutionary Genetic Analysis (MEGA 4; http://www.megasoftware.net/) software with 1000 bootstrap analyses.

Optimisation of Growth Conditions

Growth Media: Two media were used viz. M1 and M2 (Table 1). 100mL of each medium was inoculated with a 24 hour grown primary culture of *Bacillus st. arvin04.* The flasks were incubated for further 48 hours. The optimum media was chosen by estimating xylan saccharification using the standard DNSA method as described previously [11].

Optimization of carbon source: Enzyme production was carried out using the carbon sources viz. glucose, xylose, Birchwood Xylan, Oat spell Xylan and Carboxy methyl cellulose (CMC) at 0.5% (w/v) each and wheat straw, wheat bran, rice straw, rice husk and sugarcane bagasse, at concentration of 1.0% (w/v) each. The enzyme production was quantified using DNSA based estimation of reducing sugars.

Optimization of Nitrogen Source: Enzyme production was estimated in presence of various inorganic [sodium nitrate ($NaNO_3$), ammonium chloride (NH4Cl) and Ammonium nitrate (NH_4NO_3)] and organic (peptone, yeast extract, beef extract and tryptone) nitrogen sources at concentration of 1%(w/v) each and effect of Whey on Xylanase activity was also measured. Further, effect of the selected nitrogen source on enzyme production was investigated at its different concentrations.

Substrate Concentration: Xylan was added to the optimum medium at various concentrations as 0.25%, 0.5%, 0.75% and 1%. The saccharification of xylan was analysed after 48 hours of incubation.

Optimum Growth Temperature: Media M1 supplemented with 0.5% xylan was inoculated with a 24 hour grown culture of *Bacillus st. lpu02* in different Ehrlenmeyer flasks (Borosil, India). The flasks were incubated at 30^{o}C, 37^{o}C and 42^{o}C for 48 hours each. The optimum temperature was inferred by estimating xylan hydrolysis using DNSA method.

Optimum Growth pH: Media M1 containing 0.5% xylan was prepared at various pH *viz.* 6, 7, 8, 9 and 10 in Ehrlenmeyer flasks and inoculated with a day-grown culture of *Bacillus lpuarvinder strain002*. The cultures were incubated for 48 hours at 37^{o}C. The optimum pH was obtained by biochemical estimation of sugars present in the spent medium.

Optimum salt concentration for cell growth: NaCl concentration in media 2 was varied from 5-20% and supplemented with 0.5% xylan. The optimum salt concentration was denoted by quantifying xylan hydrolysis, taking salt-free media as control.

Biochemical characterisation of Xylanase

Determination of optimum growth temperature for Enzyme activity: 1% CMC was used as substrate in an enzymatic reaction containing 0.1M Tris-HCl, enzyme and substrate. The cells were cultured at different temperatures *viz.* 30^{o}C to 80^{o}C with 10^{o}C intervals. Xylanase activity was measured using DNSA assay.

Effect of temperature on Enzyme stability: The enzyme was pre-incubated for 120, 240 and 360 minutes at temperature interval of 10^{o}C from 30^{o}C to 80^{o}C for 30 minutes in 0.1M Tris-HCl. The residual enzyme activity was measured by incubating the enzyme and substrate at 60 ^{o}C for 30 minutes.

Effect of medium pH on Enzyme Activity: The cells were grown in BHB medium supplemented with 0.5% xylan at pH 6 to 10. The xylanase activity was determined using 1% CMC as substrate.

Effect of pH on Enzyme stability: The enzyme was pre-incubated in 0.1M HCl (pH 6, 7, 8, 9 and 10) for 120, 240 and 360 minutes and the enzyme activity was determined by incubation with CMC for 30 minutes.

Effect of Metal Ions on Xylanase activity: The enzyme was incubated with 1% CMC and supplemented with 1mM Ca^{2+}, Mg^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} and Hg^{2+}. The reaction was incubated at 60^{o}C for 60 minutes and xylanase activity was quantified.

*Enzymatic Saccharification***:** Enzymatic hydrolysis of wheat straw polysaccharides was investigated for the production of reducing sugars. Wheat straw was used for the enzyme production after thorough washing and air-drying in a sterilized tray for 2 hours at 60^{o}C. Straw was then sieved to 1mm size, pretreated with NaOH at 80^{o}C and re-sieved. Hydrolysis was initiated by adding 1ml (114 IU/g) of enzyme. An enzyme free parallel control was also maintained. Aliquots were drawn periodically and release of sugar content was measured.

Non-Sterile Submerged Fermentation (SmF)

Submerged fermentation employs fluidic substrates like whey, molasses and broths, which give readily usable nutrients for microbial metabolism. The main advantage of SmF is that downstream processing is easy and the recovery of product is more convenient than solid state fermentation systems. The enzyme production was done in media M1 supplemented with 1% (w/v) wheat straw alone or 1% (w/v) wheat straw and 1% whey (v/v). The media was inoculated and incubated at 37^{o}C for 24h, 48h, 72h and 96h. The enzyme activity was determined by estimating the reducing sugar content by DNS assay. The uninoculated media were used as the control sets for this experiment.

Non-Sterile Solid State fermentation (SSF)

Solid state fermentation makes use of solid substrates like straw, bran, bagasse etc, which gives a controlled-release nutrient flow. This is advantageous to the economy of the process because it allows recycling of agricultural wastes as substrates of SSF. SSF can be used only for those organisms which have low water activity[12]. Enzyme production was carried out in 250ml Erlenmeyer flasks containing 20% of media M1 ,wheat straw as a carbon source, 5% NaCl and 1% whey as a nitrogen source (non sterile). This set up was inoculated with *Bacillus lpuarvinder strain lpu002* and the xylanase activity was estimated after 48 hours, 96 hours and 120 hours. A similar parallel setup was made and not inoculated to be used as control.

Zymogram Analysis

Xylanase zymogram was prepared by copolymerizing 0.1% Xylan with 12% polyacrylamide in a native PAGE. The xylanase produced from SSF were loaded onto the native PAGE. For xylanase activity staining, the gel was incubated in 20 mM Tris–HCl buffer (pH 8.6, containing 5 % isopropanol) for 24 h. After several washings with 20 mM Tris–HCl buffer (pH 8.6), the xylanase band was visualized by staining the agarose gel with 0.5 % (w/v) Congo red and destaining with 1M NaCl.

RESULTS AND DISCUSSION

India is an agriculture-dominated sub-continent that generates 700 million tonnes of agricultural waste every year [13]. This waste is mainly rich in hemi-cellulose, lignins and xylans. These polysaccharides form a substantial percentage of the waste biomass and may readily be used as cheap carbon sources for production of fermentable sugars for bio ethanol fermentation. Realising such a potential of agricultural waste requires highly active cellulolytic and xylanolytic strains that can readily saccharify the polysaccharides to yield significant amounts of fermentable sugars. In the present study, we have isolated a new strain of bacillus, which possesses high xylanolytic activity. The 16S r-RNA gene of this high activity strain was sequenced and analysed to determine the phylogeny of the strain.

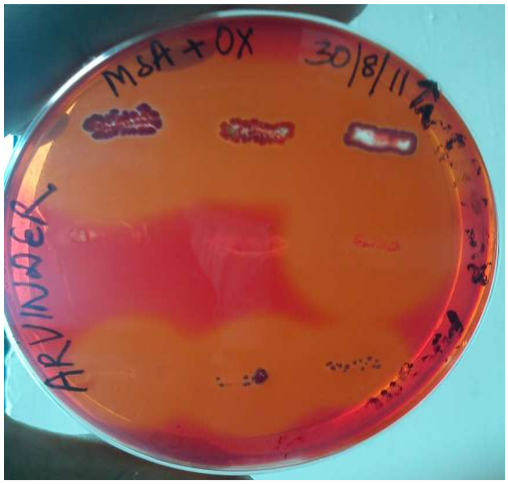

Fig. 1: Photograph of congo red stained MSA-Xylan plate showing clear zones indicating xylanase activity

Isolation of Xylanase producing microbes: Xylanase producers were isolated on MSA plates supplemented with 0.5% Xylan and were re-patched onto fresh MSA plates and stained with congo red. Congo red binds to xylan which contains β-1,4 glycosidic linkages. The xylanase activity was confirmed with a clear zone owing to hydrolysis of xylan by Xylanase secreted by the cells. (Fig. 1)

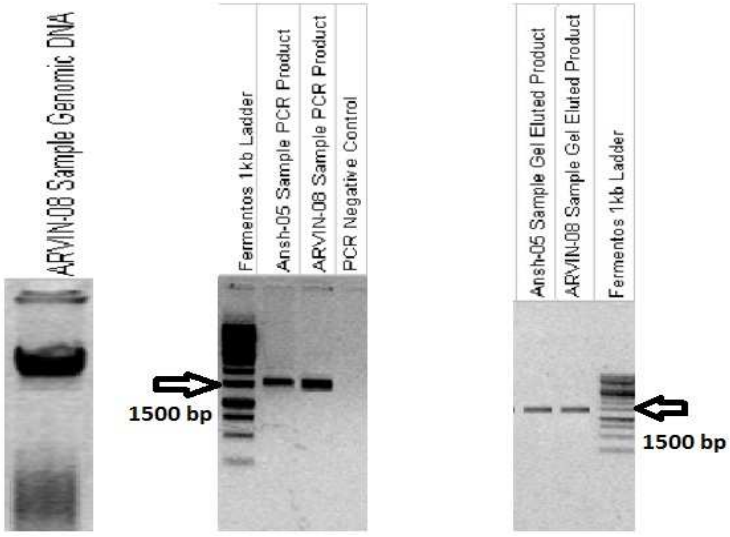

Fig. 2a: Agarose Gel Electrophoresis showing PCR amplified 16S r-RNA gene fragments from the positive xylanase producing strains

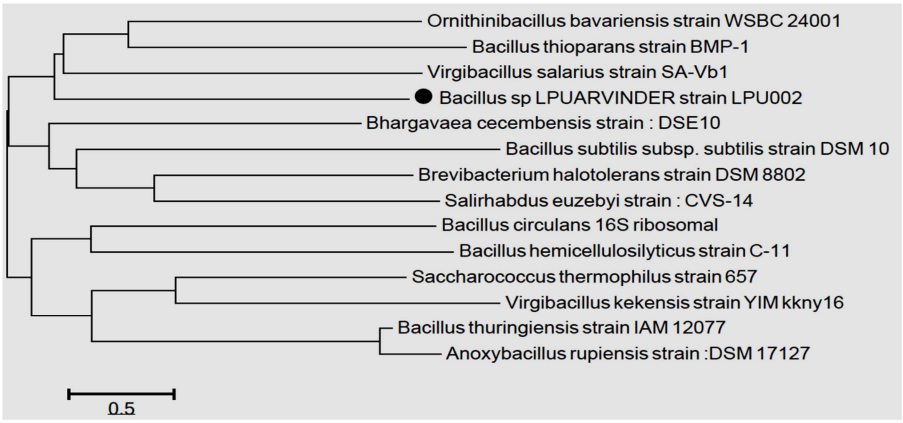

Fig. 2b Phylogenetic tree showing the position of novel strain *Bacillus lpuarvinder strain lpu002*

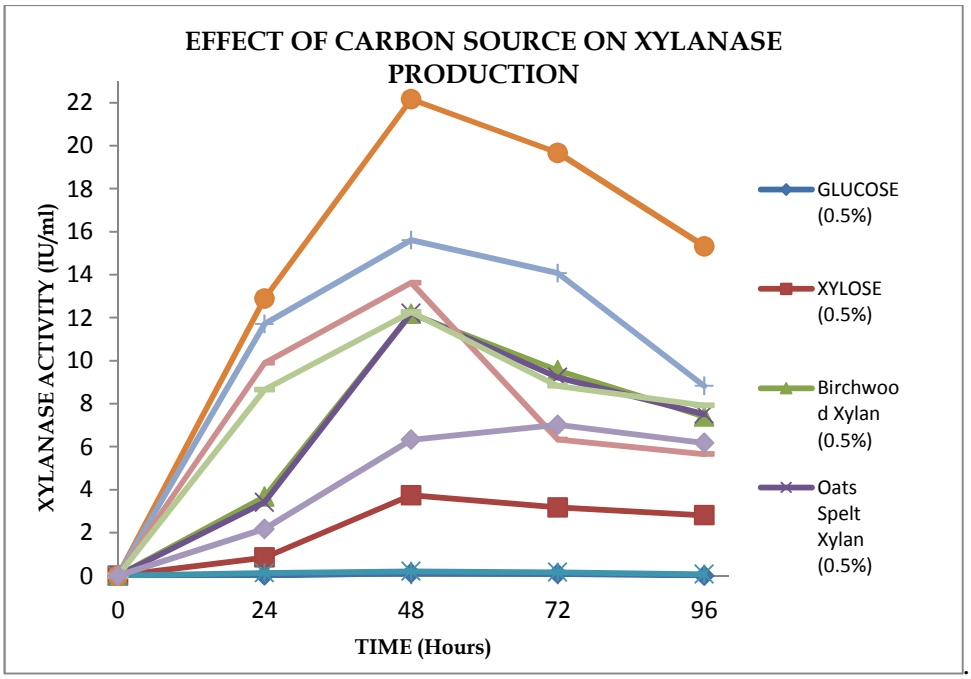

Fig. 3. Effect of Carbon Source on Xylanase production

Genetic identification of Xylanase producing strains: The xylanase producing strains were cultured in Mannitol Salt broth for 24 hours and total genomic DNA was isolated. The genomic DNA was used in a PCR reaction to amplify the 16S r-DNA gene. The 16S r-DNA analysis is the most convenient one for classification and identification of microbes and moreover, the sequences for 16S r-DNA genes for most microbes are available on public-access databases (Fig. 2a).

The DNA fragments were eluted from the gel, purified and sequenced. The sequencing results were analysed using the software MEGA 4 [14] and the phylogenetic tree was developed (Fig. 2b). The sequence has been submitted to NCBI with accession number JQ916900. Phylogenetic analysis showed clear branching out of our strain *Bacillus lpuarvinder st. lpu002*, indicating that it is a novel strain.

Optimization of growth conditions

Growth media: M1 and M2 media (Table 1) were used for media optimisation with varying concentrations of xylan as indicated. The optimum media was determined by measuring the xylanase enzyme activity using the standard DNSA method. The results were depicted in a tabulated manner (Table 2). Growth was better supported in media M1, i.e., Bushnell Haas Broth supplemented with 0.5% xylan.

Best Carbon Source: A cheap and readily metabolizable carbon source is one of the key parameters of the economy of the process. So it is imperative that the best carbon source be optimized. Enzyme production was carried out using glucose, xylose, Birchwood Xylan, Oat spell Xylan and Carboxy methyl cellulose (CMC) at 0.5%(w/v) each and wheat straw, wheat bran, rice straw, rice husk and sugarcane bagasse, at concentration of 1.0% (w/v) each as

carbon sources. Among different carbon sources, wheat straw yielded highest xylanase (22.16 IU/ml) production of 22.16 IU/mL and 19.67 IU/mL at 48 hours and 72 hours respectively, followed by wheat bran that gave 15.6 IU/mL at 48 hours. Birch-wood xylan resulted in production of 12.18 IU/ml xylanase (Fig. 3). The enzyme titer in presence of sugars was much lower compared with wheat bran or xylan. Although hemicellulosic substrates such as wheat bran, rice straw, wheat straw, rice husk and sugarcane bagasse were found to increase xylanase production, the highest enzyme titer was obtained with wheat straw, which may be attributed to high xylan content in wheat.

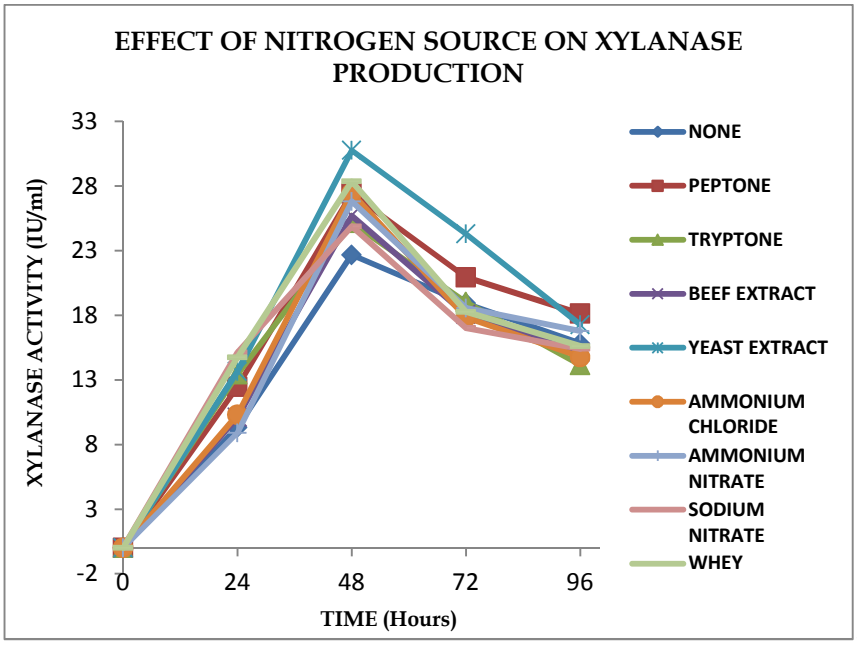

Fig. 4. Graph showing the effect of Nitrogen source on Xylanase production

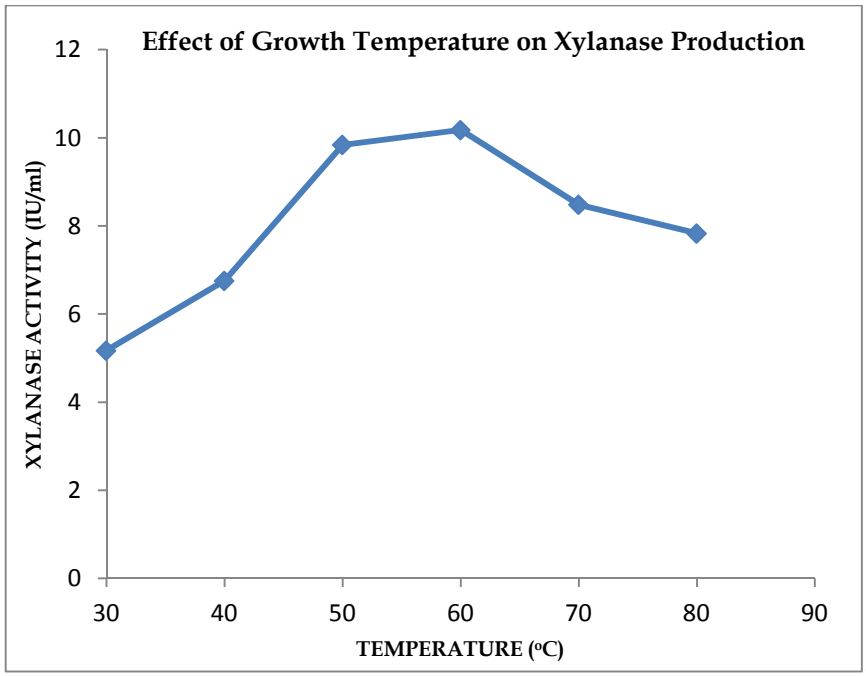

Fig.5: Graph showing the effect of growth temperature on xylanase production

Optimum Nitrogen source: Various inorganic [sodium nitrate ($NaNO_3$), ammonium chloride (NH4Cl) and Ammonium nitrate (NH_4NO_3)] and organic (peptone, yeast extract, beef extract and tryptone) nitrogen sources at concentration of 1%(w/v) each and Whey 1%(v/v) were the chosen candidates for optimizing the best Nitrogen source. The medium M1, supplemented with 0.5% xylan and the indicated concentrations of various nitrogen sources, was inoculated with *Bacillus lpuarvinder st. lpu002* cells and cultured for 24h, 48h, 72h and 96h. The enzyme activity was measured using the standard DNS assay. Organic nitrogen sources such as yeast extract (30.76

IU/ml), peptone (27.42 IU/ml), tryptone (25.14 IU/ml) and beef extract (25.71 IU/ml) resulted in higher enzyme titer compared with inorganic compounds such as NH_4NO_3 (24.81 IU/ml), NH_4Cl (27.45 IU/ml), and $NaNO_3$ (24.88 IU/ml). Xylanase production was also measured in the presence of whey, which gave an activity of 28.36 IU/mL (Fig. 4).

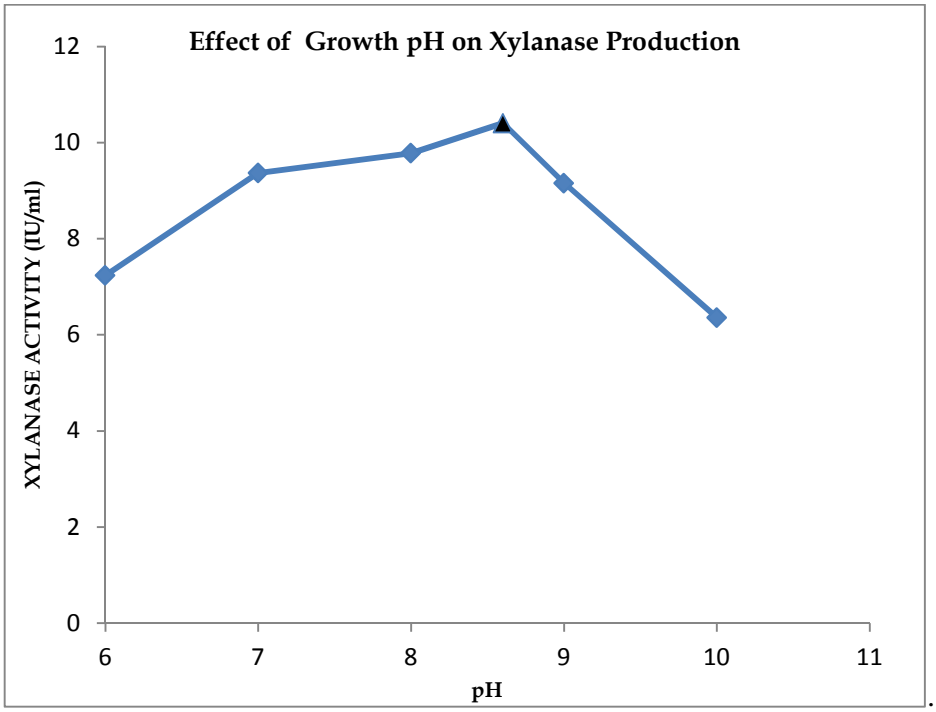

Fig. 6: Graph showing the effect of growth pH on Xylanase production

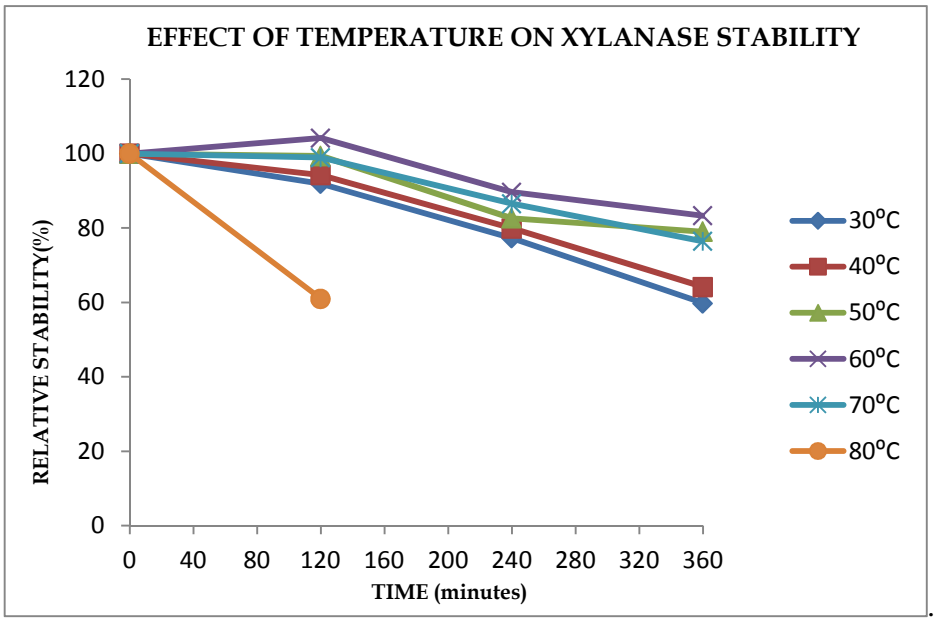

Fig. 7: Graph showing the effect of temperature on xylanase stability

Optimum Growth Temperature: *Bacillus lpuarvinder strain lpu002* cells were grown in media M1 and incubated at various temperatures for 48 hours. The enzyme production was estimated using the standard DNSA method and the data could be depicted as shown in Fig. 5. The maximum enzyme activity of 10 IU/mL was observed at a growth temperature of 60^{o}C, which indicated that the strain is thermo-tolerant.

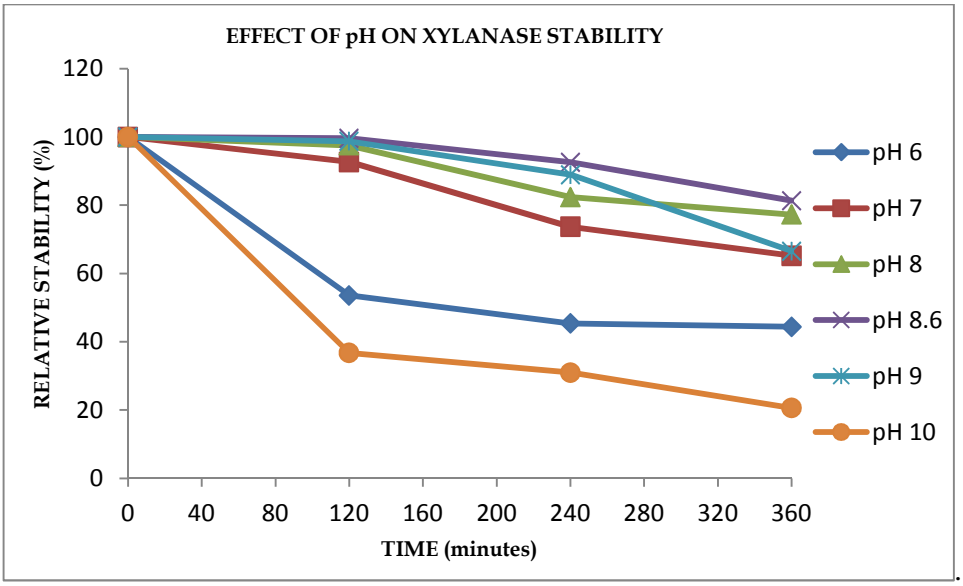

Fig. 8: Graph showing the effect of pH on xylanase stability

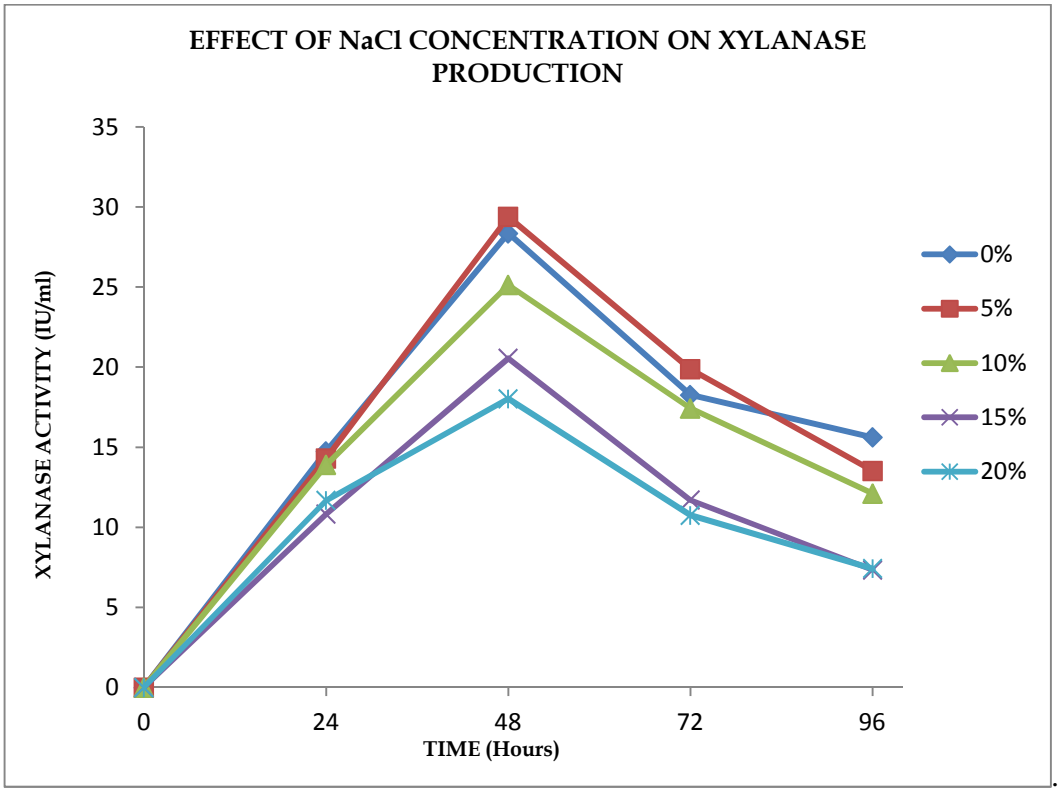

Fig. 9: Graph showing the effect of NaCl on xylanase production

Optimum Growth pH: The xylanase producing strain *lpu002* was grown in media M1 at various pH values ranging from 6 to 10. The enzyme production was quantified using the standard DNSA method of glucose estimation. The results were depicted as in Fig. 6. The optimum enzyme activity of 10.2 IU/mL was observed at pH 8.5.

Effect of temperature on Enzyme stability: The enzyme was pre-incubated at different temperatures ranging from 30^{o}C to 80^{o}C at intervals of 10^{o}C for 120 minutes, 240 minutes and 360 minutes. The stability was estimated by measuring residual enzyme activity using DNSA method. The data was depicted as follows. The enzyme showed maximum stability at 30^{o}C where it retained 84% of the activity ever after 360 minutes of incubation. The enzyme also retained >70% activity at 70^{o}C even after 360 minutes of incubation (Fig. 7)

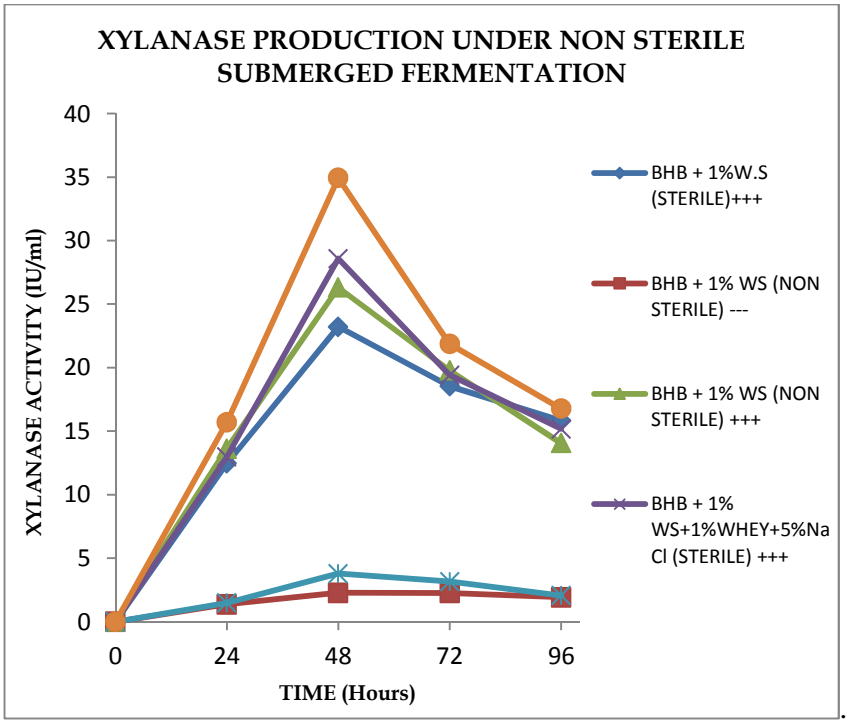

Fig. 10:Production of xylanase in non-sterile submerged fermentation

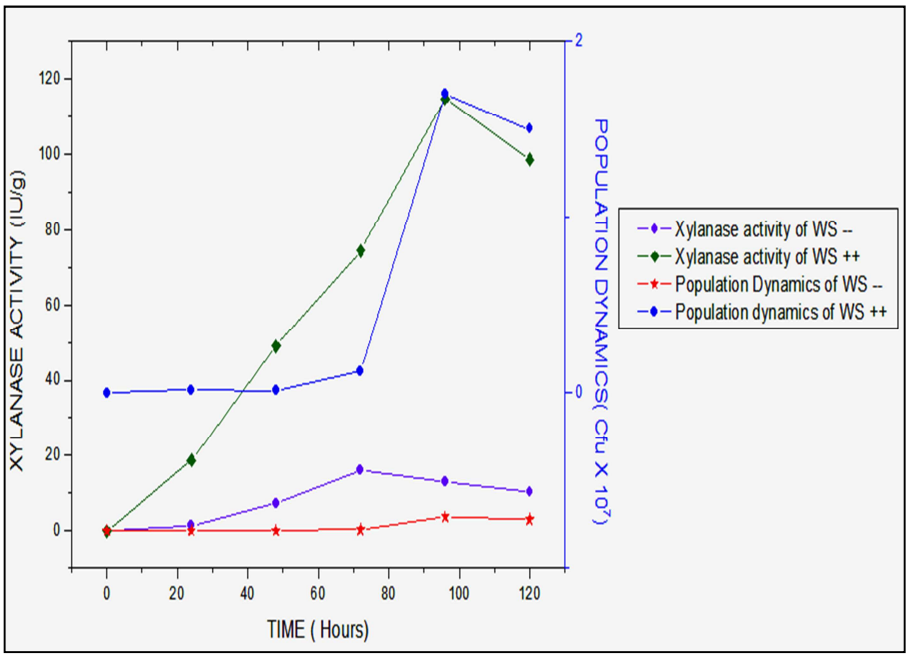

Fig. 11: Graph showing the correlation between solid state fermentation of *Bacillus lpuarvinder strain lpu002* and xylanase activity

Effect of pH on enzyme stability: The xylanse enzyme was incubated in 100mM Tris-HCl at various pH ranging from 6 to 10 for 120 minutes, 240 minutes and 360 minutes. The enzyme stability was quantified by measuring the residual enzyme activity using the DNSA method. The enzyme showed maximum stability at buffer pH 8.6 where 95% of the activity was retained even after 360 minutes of incubation. The enzyme was significantly unstable at pH 6 and 10 and rapidly lost activity at these pH values (Fig. 8).

Optimum salt concentration for cell growth: The cells were cultured in media M1 supplemented with 0.5% xylan and varying concentrations of NaCl from 0% (control) to 20%. The Xylanase activity was measured by quantifying reducing sugars by DNSA method. Optimum xylanase activity was observed at a salt concentration of 5%, which declined upon increasing the salt concentration further (Fig. 9). This might be due to the osmotic stress caused by NaCl.

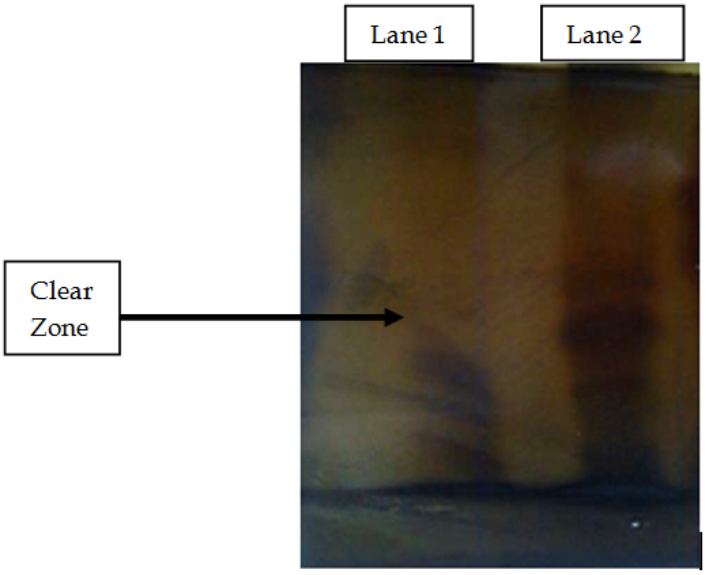

Fig. 12: Zymogram analysis of xylanase produced by solid state fermentation in wheat straw
Lane 1: Xylanase activity in SSF xylanase and Lane 2: Control

Table 1: Compositions of different media (M1 and M2) used in the study

	Component	Concentration (g/L)
Bushnell Haas Medium (M1)	Magnesium sulphate	0.200
	Calcium chloride	0.020
	Potassium dihydrogen phosphate	1.000
	Dipotassium phosphate	1.000
	Ammonium nitrate	1.000
	Ferric chloride	0.050
	Xylan	0.5% (w/v)
	Final pH	**7.0 ± 0.2**
Mineral Salts Solution (M2)	Potassium dihydrogen Phosphate	0.070
	Dipotassium hydrogen Phosphate	0.070
	Magnesium Sulphate Heptahydrate	0.070
	Ammonium Nitrate	0.100
	Sodium Chloride	0.005
	Ferrous Sulphate	0.002
	Zinc sulphate	0.001
	Manganous Sulphate	0.001
	Final pH	**7.0 ± 0.2**

Table 2: showing the enzyme activity using different substrate concentrations in media M1

Substrate Conc.	Enzyme Activity (48 Hours)
M1 + 0.25%	9.33
M1 + 0.5%	10.76
M1 + 0.75%	10.01
M1 + 1%	9.37
M2 + 0.5%	4.15

Table 3. Effect of metal ions on xylanase activity; * statistically significant (data not shown)

Metal ion	Residual Activity* %
Control (No metal ion)	100 %
Ca^{+2}	124.8%
Mg^{+2}	136.38%
Hg^{+2}	82.24%
Fe^{+2}	117.55%
Cu^{+2}	126.74%
Mn^{+2}	103.26%
Co^{+2}	116.54%
Zn^{+2}	104.61%
EDTA	66%

*Effect of Metal ions on xylanase activity:*Enzyme was equilibrated with 1 mM solution of either Ca^{+2}, Mg^{+2}, Hg^{+2} , Fe^{+2}, Cu^{+2} , Mn^{+2}, CO^{+2} and Zn^{+2} for 1 hour at room temperature. Residual activity was measured at 60 ^{o}C using enzyme assay described above. When incubated with Mg^{2+}, Residual activity was 136.38% followed by 126.74% when Cu^{2+} was used. 124.8 % of residual activity was observed when Ca^{2+} was used (Table 3).

Production of Xylanase using non-sterile submerged fermentation: In non-sterile submerged fermentation (SmF), xylanase production was 26.39 IU/ml using 1% wheat straw (inoculated) as substrate after 48 h, while xylanase production after 72hr and 96 hr was 19.77 and 14.05 IU/ml when bacterial strain was grown on BHB supplemented with 1% wheat straw (Inoculated). Xylanase production was 34.93 IU/ml after 48 hours in BHB supplemented with 1% wheat straw, 1% whey.

The activity was reduced to 21.87 IU/ml after 72 hours and to 16.78 IU/ml after 96 hours respectively under non sterile conditions. When media was un-inoculated the activity was 3.79 IU/ml after 48 hours under same conditions (Fig. 10).

Production of Xylanase using non-sterile Solid State fermentation: Enzyme production was carried out in 250ml Erlenmeyer flasks containing media M1 , 1% (w/v) wheat straw as a carbon source, 5% NaCl and 1% whey as a nitrogen source (non sterile). This set up was inoculated and after 96hr, 114.72 IU/gram of activity was investigated followed by 98.65 IU/gm at 120 hours and 74.48 IU/gm after 48 hours. The control setup containing M1, non sterile wheat straw, 1% whey as a nitrogen source and 5% NaCl was left un-inoculated. Activity in this case was 16.02 IU/gm after 72 hr followed by 12.88 IU/gm after 96 hr and 10.24 IU/gm after 120 hrs (Fig. 11).

Zymogram Analysis: Native PAGE was run using 0.5% xylan copolymerised with 12% acrylamide/bis-acrylamide. The gel was incubated with Tris-HCl and stained with congo-red. Destaining was performed using 1M NaCl for 4 hours. The zymogram stain showed diffused clear zone showing enzyme production (Fig. 12). The clear zone is due to hydrolysis of xylan by Xylanase enzyme present in the gel.

CONCLUSION

It is absolutely evident from the present study that our strain *Bacillus lpuarvinder strain lpu002* produces a highly active xylanase enzyme, which may be taken to the purification stage. The enzyme encoded by our strain is highly capable of hydrolysing complex polysaccharides present in agricultural wastes, to yield industrially acceptable amounts of fermentable sugars. These fermentable sugars can be immediately used in fermentation bioprocesses for production of bio ethanol, making this strain industrially valuable.

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