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Deciphering Thermostable Xylanases from Hot Springs: the Heritage of Himachal Pradesh for Efficient Biomass Deconstruction

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Thermophilic bacteria isolated from hot springs of Himachal Pradesh were screened for xylanase activity and production. Of the 38 strains screened, 19 were xylanase positive and among them 5 promising strains with significant hydrolytic capacity were considered for further investigation. Under submerged conditions, xylanase production of these isolates ranged from 0.64 to 2.36 IU.ml^{-1} at 72 h using birch wood xylan as substrate. These isolates were further confirmed for the presence of xylanase gene (700bp) and protein profile showing high density activity bands between 75 to 90 KDa. The maximum level of xylanase production was noted with *Bacillus licheniformis* KBFB3, which also exhibited relatively more activity in a broad pH range between 4.5 and 8.5 and temperature up to 80°C. From the above results, it is vivid that the highly thermostable and alkali tolerant xylanase of *B.licheniformis* KBFB3 could find its application in efficient biomass valorization.

Key words: Hotsprings, ***Bacillus licheniformis,*** Xylanase, Biomass, Valorization

Lignocellulosic biomass contains 20–40 % of hemicellulose, which is a branched heteropolymer consisting of pentose (D-xylose and D-arabinose) and hexose (D-mannose, D-glucose, and D-galactose) sugars with xylose being the most abundant (Cano and Palet, 2007; Kumar *et al*., 2008). Hemicelluloses are classified according to the main sugar in the backbone of the polymer, e.g., xylan *(β-1*,4-linked xylose) or mannan *(*β-1,4-linked mannose*)* (Jorgensen *et al*., 2007). To obtain these linked sugars, there is a need to effectively break the locked polysaccharides from recalcitrant lignocellulose. Xylanase refers to a class of enzymes that specifically degrade xylan into oligosaccharides and xylose (Collins *et al*. 2005) and hydrolysis of xylan using enzymes such as xylanases provides a viable alternative to chemical hydrolysis as it is highly specific in nature apart from being an environment friendly process (Kuhad *et al*., 1997).

Extremophiles inhabiting hot springs are considered to be the closest living descendants of the earliest life forms on earth. Thermophilic microorganisms unequivocally represent a valuable source of highly thermostable robust extracellular hydrolytic enzymes, with numerous advantages towards biotechnological applications due to their overall inherent stability and high reaction rates at elevated temperatures (Vieille and Zeikus, 2001; Turner *et al*. 2007). One of the natural habitats of the thermophilic bacteria is the hot springs, where the temperature of the water is between 70 to 100 °C. Therefore, these springs provide deep insights into the origin and evolution of life, thereby serving as a potential reservoir for industrially important biomolecules. For example, thermophilic microbes of various genera, including *Bacillus*, *Geobacillus*, *Acidothermus*, *Cellulomonas*, *Paenibacillus*, *Thermoanaerobacterium*, *Actinomadura*, *Alicyclo bacillus*, *Anoxybacillus*, *Nesterenkonia* and *Enterobacter* have been reported to produce thermostable xylanases (Bhalla *et al*., 2013).

Thermostable enzymes have an obvious advantage as catalysts in the lignocellulose conversion processes due to better enzyme accessibility and cell-wall disorganization achieved at high-temperature reaction conditions (Paes and O'Donohue, 2006). Also, high temperature allows better solubility of reactants and products by lowering the viscosities, leading to faster hydrolysis (Viikari *et al*., 2007). Longer active life under high temperature conditions would make these enzymes favourable for enhanced and efficient biomass conversion. Therefore, to be an effective enzyme, thermostability and tolerance to acid / alkali are the most important attributes for the enzyme utilized under extreme bioprocessing conditions. The present work aimed to isolate thermophilic microbes from extreme environments *i.e.* hot springs of Himachal Pradesh for exploring potent thermostable and acid / alkali tolerant xylanases for biomass deconstruction.

Material and Methods

Isolation and screening of thermophilic bacterial isolates for xylanase activity

Isolation of thermophilic xylanase producing bacteria was done by dilution plate on basal medium

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(KH_2PO_4 - 2.5g, K_2HPO_4-2.5g, $(NH_4)_2HPO_4$ - 1.0g, $MgSO_4.7H_2O$-0.2g, $FeSO_4.7H_2O$-0.01g, $MnSO_4.7H_2O$ - 0.007g for 1L of medium) supplemented with 1% birch wood xylan for xylanolytic bacteria. The plates were incubated at 50°C till sufficient isolated colonies were observed. The distinct bacterial colonies were observed for morphological and colony characteristics. After incubation, the plates were flooded with 0.1% Congo red followed by detaining with 1M NaCl (Salem *et al*., 2008). Positive isolates showed a zone of clearance around the colony and the hydrolytic capacity of the bacterial isolates was calculated.

Identification of bacterial isolates by 16s rDNA sequencing and phylogeny

Genomic DNA extracted from the selected bacterial isolates was used for amplification of 16S rDNA gene sequences using universal primers 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'-ACGGCTACCTTGTTACGACTT-3') following the PCR conditions as follows: 95 °C for 5 min; 30 cycles of 94 °C for 1min, 55 °C for 1 min, and 72 °C for 90 s; and 72 °C for 10 min (Weisberg et al., 1991). PCR product of 1500 bp was resolved by electrophoresis in 1.2% agarose gel in 1 X TAE buffer. Gels were stained with ethidium bromide (10 mg. ml–1) and visualized on a gel documentation system and gel images were digitalized using a Bio-rad Gel DocXR+ system (Hercules,CA,USA). The PCR products were purified using GeneJET PCR Purification Kit (Thermoscientific, USA) and sequenced commercially at BioServe, India and the phylogenetic relationship was analysed using Mega 7.0.

PCR screening of the isolates for xylanase genes

The xylanolytic isolates thus, selected were screened for xylanse gene (*xln*B) using gene-specific primers as mentioned by Hussain *et al*. (2011) (Ba_xln F) 5'- ATGTTTAAGTTTAAAAAGAATTTC-3' and Ba_xln R 5'- TTACCACACTGTTACGTTAG- 3') and the amplicons were resolved in 1 % agarose gel.

Xylanase assay under submerged condition

Xylanolytic activity of the cell-free culture supernatant was determined according to Satyanarayana and Johri (1983) at 50 °C with 1 % birch wood xylan as substrate. Xylan was dissolved in 100 ml of sodium phosphate buffer (pH 6.5). The reaction mixture containing 0.5 ml of enzyme, 0.5 ml of buffer and 0.5 ml of substrate was incubated at 50 °C, for 30 min. The amount of reducing sugars liberated was estimated by following 3,5 -dinitro salicylic acid (DNS) method using xylose as standard. One unit of xylanase activity was expressed as µmole of reducing sugars (xylose equivalent) released in 1 min under the assay conditions.

SDS-polyacrylamide gel electrophoresis and activity of partially purified xylanase

The crude xylanase from the extracellular fraction was harvested and precipitated using 70% ammonium sulphate. The partially purified xylanase was separated by SDS-PAGE in 12% polyacrylamide gels according to Laemmli, (1970). Protein bands were detected by Coomasie-brlliant blue staining solution and destained with glacial acetic acid : methanol : water (5:45:50) and documented using Bio-rad Gel DocXR+ system (Hercules,CA,USA). Following this, the gel was placed on agarose plate containing 0.1% xylan and incubated for 1h, at 50°C. The plate was stained with 0.1% Congo red for 30 min and finally washed with 1M NaCl to detect enzyme activity (Zhang *et al*., 2009).

Optimization of conditions for xylanase activity from *B.licheniformis* KBFB3

Determination of optimum temperature and pH

For determination of optimal temperature, the assay was carried out using 0.5% (w/vol) birch wood xylan as substrate prepared using100 mM sodium phosphate buffer (pH 6.5). The assay mixture was incubated at 40, 50, 60, 70, 80 and 90°C for 30 min. and the reaction was stopped by boiling for 10 min. The free glucose released was measured as per Nelson (1944). The optimal pH for xylanase activity was determined in various buffers with different pH *viz.,* ammonium citrate (3.0), sodium acetate (4.5), sodium phosphate (7.0), tris (8.5) and ammonia buffer (9.5). Protein content was determined using Bradford reagent (Biorad) in a multimode microtitre plate reader (SpectraMax@i3x) with bovine serum albumin as standardat wavelength 595 nm. The specific activity of xylanase was calculated and expressed in terms of IU per mg of protein.

Results and Discussion

Isolation, screening and identification of xylanolytic thermophilic bacteria

Thermostable xylanases, optimally active at high temperatures and wide range of pH find wider application in harsh industrial processing (Turner *et al*., 2007). In this study, thermophilic bacteria were

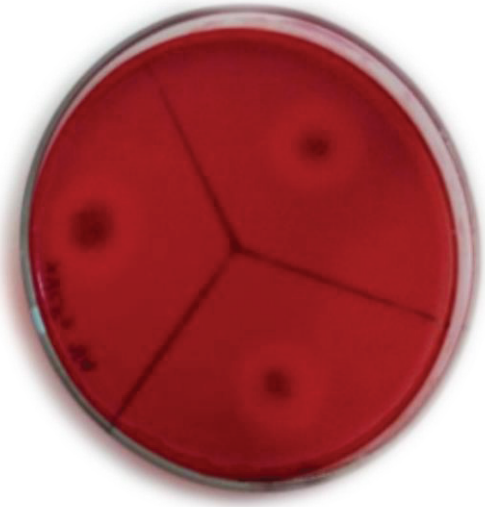

Fig. 1. Thermophilic bacterial isolates showing hydrolytic activity on xylan plates incubated at 50°C

isolated from differently enriched lignocellulosic substrates at hot springs *viz*., Manikaran (~95 °C), Kalath (~50°C) and Vasist (~65 °C) located at Himachal Pradesh, India. Among the 38 different morphotypes obtained, only 19 were positive for xylanase activity at temperature ≥50° C.The hydrolytic capacity index (HC) of five promising isolates VCB1, VCB2, VSDB4, KBFB3 and KBFB2 on birch wood xylan ranged between1.67 to 5.30, respectively (Fig. 1 and Table 1). The highest xylanase activity on Congo red plate assay was observed with KBFB3 (5.30) followed by KBFB2 (2.60) and VCB2 (2.18).

Table 1. Phylogenetic affiliation of thermophilic bacterial isolates by 16S rRNA gene sequence homology and their xylan hydrolyzing ability

Isolate	Sequence homology			Phylum	Xylan hydrolysing ability*
	Closest speciesa	Accession No.	Similarity (%) of sequence in BLAST analysis		
VCB1	*Bacillus tequilensis*	MGO98075	74	Firmicutes	2.14 (±0.13)b
VCB2	*Bacillus tequilensis*	MGO98076	99	Firmicutes	2.18 (±0.14)b
VSDB4	*Bacillus tequilensis*	MGO27696	99	Firmicutes	1.67 (±0.29)b
KBFB2	*Bacillus licheniformis*	MGO28595	99	Firmicutes	2.60 (±0.16)b
KBFB3	*Bacillus licheniformis*	MGO28594	99	Firmicutes	5.30 (±0.33)a

[a]Species identified based on 16S rRNA gene sequence similarity.
*Values represent mean (±standard error) (n=3) and the values representing same alphabets are not significant from each other as determined by DMRT at p=0.05

The potential thermophilic bacterial isolates were further confirmed for the presence of xylanase gene and the positive isolates generated amplicon of about 700bp (Fig. 2). This result is in accordance with the study of Hussain *et al.*,(2011) who isolated bacterial xylanase gene. The thermophilic bacterial isolates

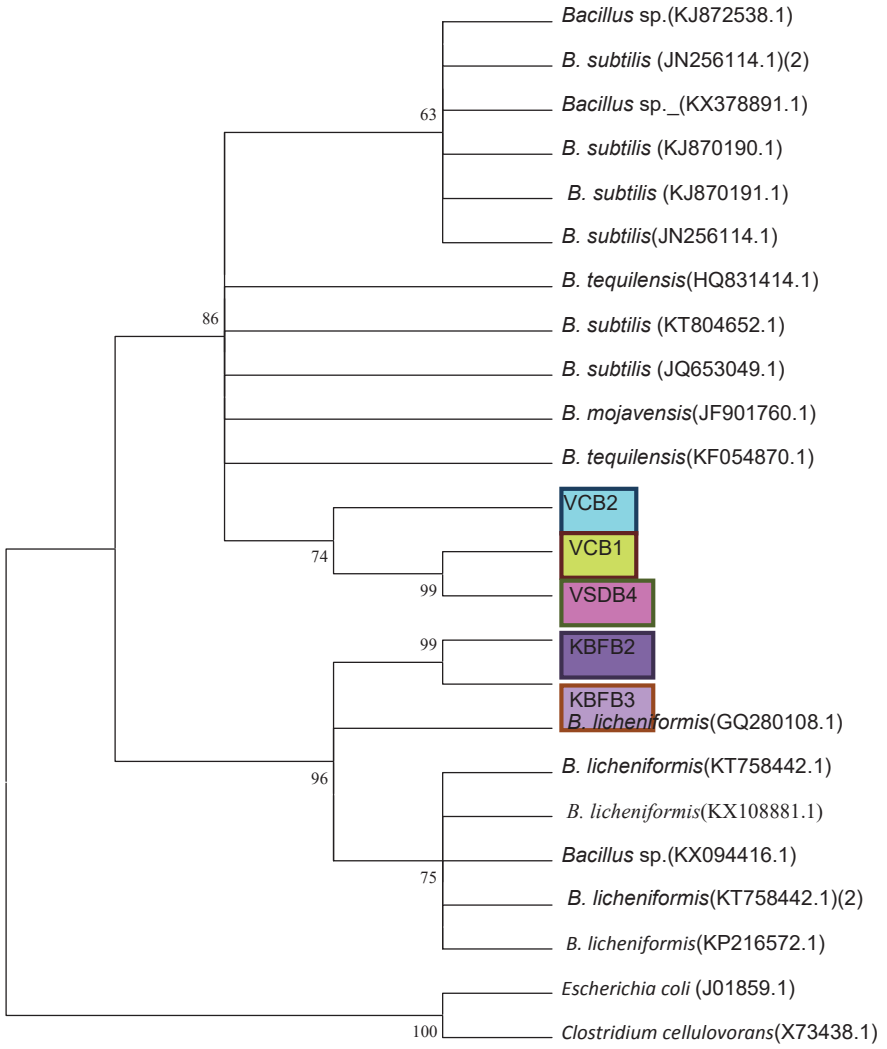

Fig. 3. Phylogentic tree construction

were further subjected to molecular identification by 16S rDNAgene sequence analysis. The 16S rDNA amplified by PCR was sequenced and further analysed by NCBI BLAST nucleotide search and the phylogenetic tree was constructed on the aligned data

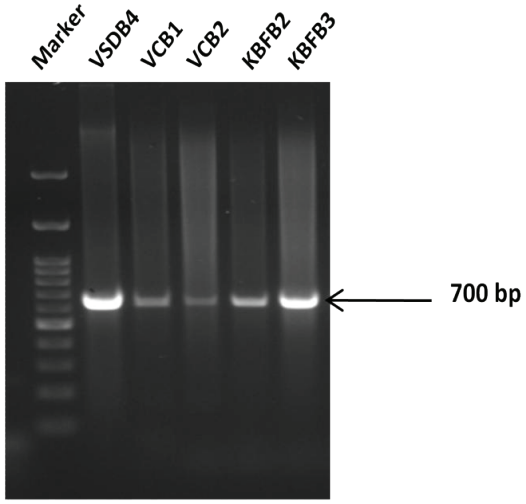

Fig. 2. PCR amplification of xylanase gene (xln B) confirmed the presence of xylanolytic activity

sets using neighbour joining (NJ) method using MEGA 7.0 (Fig.3). The results revealed that all the isolates obtained from thermal springs were gram positive and belong to the phylum Firmicutes. The closest phylogenetic neighbours for the isolates VCB1, VCB2, and VSDB4 were *Bacillus tequilensis* with 74 and 99 % homology, respectively. The isolates, KBFB2 and KBFB3 have close identity with *Bacillus licheniformis* (99 per cent). The sequences were submitted in NCBI database and the accession numbers along with their phylogenetic affiliation is given in Table1.

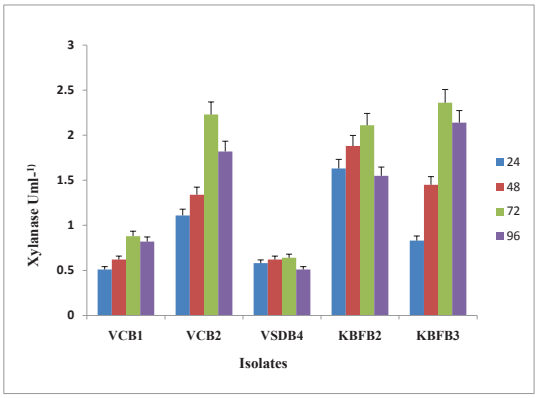

Fig 4. Xylanase activity profile by the thermophilic bacterial isolates with xylan under submerged conditions incubated at 50°C and agitated at 200 rpm.

Xylanase assay under submerged condition

In general, the increase in enzyme activity is associated with an increase in total extracellular protein and cell biomass. The maximum xylanase activity by the isolates was observed at 72 h (day 3) of inoculation ranging from 0.64 to 2.36 IU.ml^{-1} and showed a gradual decline thereafter. *B. licheniformis* KBFB3 (2.36 IU.ml^{-1}) and *B. tequilensis* VCB2(2.23 IU.ml^{-1}) have registered the maximum activity whereas, VSDB4 (0.64 IU.ml^{-1}) and VCB1 (0.88 IU. ml^{-1}) have recorded the least (Fig. 4). The isolate KBFB2 recorded the maximum activity (2.11) on 72 h which was comparable with KBFB3 and VCB2. The data on crude xylanase activities from thermophilic *Bacillus* sp. were generated from culture supernatants under unoptimized medium conditions; therefore, further experiments were performed to optimize the xylanase activity. This finding is in preponderance with the investigation of Bhalla *et al.*(2015) in which a thermophilic *Geobacillus* sp. WSUCF1 produced the maximum crude xylanase activity on 4th day, when the culture had nearly reached to plateau phase. Further decrease in extracellular crude xylanase activity might be due to the cessation of growth and depletion of substrate level in the growth medium.

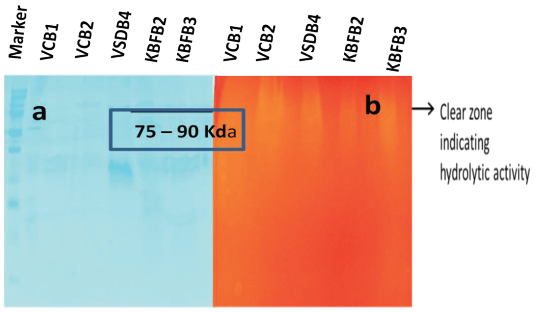

Fig 5. SDS PAGE and zymogram analysis showing xylanolytic activity. a) SDS profile of xylanase by the promising thermophilic isolates grown on culture media with xylanse b) xylanase activity in polyacrylamide gel with xylan and stained with congored

Characterization of xylanase from thermophilic bacterial isolates

Crude xylanases were expressed at high levels in the production medium containing xylan as substrate. SDS-PAGE analysis performed using the partially purified xylanases (Fig. 5a and Fig. 5b) revealed the high-density activity band smear between 75 and 90 kDa region for endoxylanase activity with Birchwood xylan as substrate. This is in contrary with the trend noticed for Praire Cord grass (PCG), where four activity bands (45, 38, 34 and 17 kDa) and two activity bands (34 and 17 kDa) were observed with untreated and and Pretreated PCG using xylanase of *Geobacillus* sp.WSUCF1 (Bhalla *et al*., 2015), respectively. The observed trend very well explains the difference in expression of enzymes by a microbe when grown on differently treated complex substrates.

To further optimize the xylanase activity, the highest xylanase producer *B.licheniformis* KBFB3 was considered. The impact of different temperatures on the xylanase activity is shown in Fig. 6a. Maximum xylanase activity was observed at 80°C which showed a linear increase with the increase in temperature up

to 80°C and thereafter, it declined; however, at 90°C about 89% of its maximum activity was still retained. While at lower temperatures of 40, 50 and 60°C, 61, 82 and 80 % of xylanase activities, respectively were observed. Xylanases from *Geobacillus thermodenitrificans* TSAA1 (Verma*et al*., 2013), *Bacillus* sp. JB 99 (Shrinivas *et al*., 2010), *Bacillus licheniformis* 77-2 (Damiano *et al*., 2006), and *B. flavothermus* strain LB3A (Sunna *et al*., 1997) showed

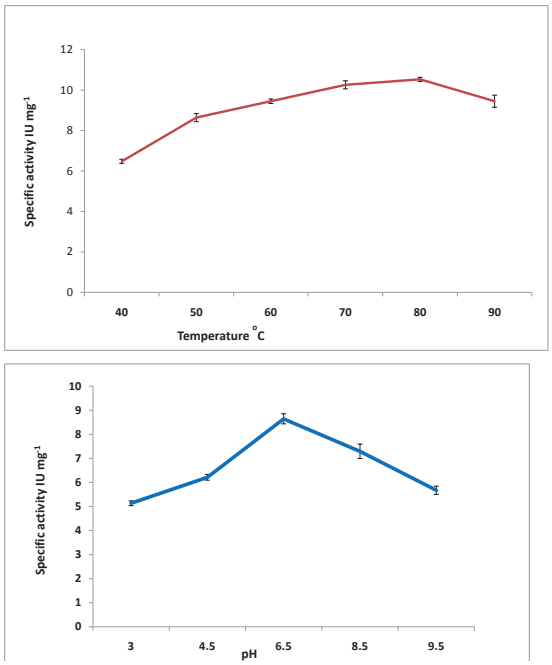

Fig 6. Impact of (a) pH and (b) temperature on the xylanase activity of B. licheniformisKBFB3. The points are the averages of triplicates, and error bars indicate ±SDs of the means (n = 3).

their optimum temperature at 70°C. But surprisingly, the xylanase of *B. licheniformis KBFB3* in the present study could be able to withstand temperature up to 90°C. Hence, the results demonstrated that *B.licheniformis* KBFB3 xylanase activity was resistant to change in temperature and hence, it is well suited for harsh process conditions entailed in bioprocessing of biomass.

The effect of pH on *B. licheniformis* KBFB3 crude xylanase activity was examined from pH 3.0 to 9.5 (Fig. 6b). *B.licheniformis* KBFB3 produced the maximum xylanase activity in sodium phosphate buffer at pH 6.5 and exhibited activity in a broader pH range of 4.5–8.5, retaining more than 60% relative activity at pH 9.5 and 59 % relative activity at pH 3. Xylanases from *Thermo anaerobacterium saccharolyticum* NTOU1 (Hung *et al*., 2011), *Clostridium* sp. TCW1 (Lo *et al*., 2011), *Actinomadura* sp. S14 (Sriyapai *et al*., 2011), *Bacillus* sp. (Sapre *et al*., 2005), *Bacillus flavothermus* strain LB3A (Sunna *et al.,* 1997), *B. stearothermophilus* T-6 (Khasin *et al*., 1993) and *Geobacillus* sp. (Bhalla *et al*., 2015) also showed their pH optima at 6.0–8.5. Therefore, the highly thermostable and alkali tolerant *B.licheniformis* KBFB3 xylanase warrants its application in biomass valorization processes, which employs high temperature and can be explored further for industrial applications especially biomass based biorefineries and paper industries.

Conclusion

Thermophilic microbes are repertoires for highly thermostable and alkalinetolerant biocatalysts, which can be explored to develop feasible low-cost technologies for biomass conversion. In this perspective, our investigation has resulted in an efficient strain *B.licheniformis* KBFB3 with an inherent potential of high temperature (80 °C) and alkaline tolerance (pH 8.5), which can be effectively used to develop a cocktail along with cellulases and can be employed in process optimization for consolidated bio-processing.

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