Indian J Microbiol (October 2010) 50(Suppl 1):S2–S9
DOI: 10.1007/s12088-010-0071-4

ORIGINAL ARTICLE

Biochemical and molecular characterization of thermo-alkali tolerant xylanase producing bacteria from thermal springs of Manikaran

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Received: 30 July 2008 / Accepted: 26 November 2008
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Abstract One hundred ten alkalo-tolerant thermophilic bacteria were isolated from 17 samples (water and sediment) collected from Manikaran. Of 110 isolates, 70 showed the production of xylanases and were further screened for growth and production of xylanases at different temperature ranging from 40 to 75°C. Eleven isolates that showed growth and xylanase production at temperatures ≥50°C were selected for quantitative estimation in modified Reese mineral liquid medium containing wheat bran. Maximum xylanase activity was produced by isolate H-7 followed by H-9 and R-9 and was statistically superior to other isolates. The microscopic observation showed that the isolates possessed the typical rod with endospore, characteristic of genus *Bacillus*. The isolates were found to be oxidase and catalase positive. Using BIOLOG Microlog 3 software, the isolates H7, H9 and R9 were identified as *Paenibacillus ehemensis, Bacillus cereus*/*B. thuringiensis* and *B. subtilis* respectively, based on utilization of 95 carbon sources. PCR-RFLP analysis of 16S rDNA indicated that the isolates were genetically different from each other. DNA sequencing of the three isolates and phylogenetic analysis revealed that all the isolates obtained from Manikaran thermal springs showed 97 to 100% similarity with the sequences within the GenBank. The closest phylogenetic neighbours according to the 16S rRNA gene sequence data for the three isolates H-7, H-9, and R-9 were *Paenibacillus ehemensis, Bacillus cereus* and *Bacillus subtilis*, respectively.

Keywords Xylanase · Thermo-alkali tolerant · Manikaran · 16S rDNA PCR-RFLP · DNA sequencing

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Introduction

Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which catalyse the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan. They are a widespread group of enzymes, involved in the production of xylose. Xylan, the substrate of xylanases, is a major structural polysaccharide in plant cells, and is the second most abundant polysaccharide in nature, accounting for approximately one-third of all renewable organic carbon on earth [24]. Hydrolysis of xylan is undoubtedly an important step toward proper utilization of abundantly available lignocellulosic material in nature. Xylan hydrolysis 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 [3, 20]. Thermostable xylanases active at alkaline pH are of great importance for application in pulp and paper industry to decrease the consumption of chlorine chemicals [17]. There are reports of isolation of xylan degrading alkali tolerant thermophiles from the hot springs in Bulgaria [12] and Portugal [5]. Bacteria belonging to the genus *Bacillus* have been largely reported to produce cellulase free xylanases [6, 30].

The aim of the present study was to isolate alkali tolerant thermophilic bacteria from the hot water springs of Manikaran, India, screen for xylanase production and to

identify the isolates efficient for xylanases activity based on carbon utilization pattern and sequencing of 16S rDNA.

Material and method

Isolation of thermo-tolerant bacteria

The water and sediment samples were collected from thermal springs of Manikaran. The temperature and pH of thermal spring at sampling site was 90°C and 8.0–8.2 respectively. For enrichment of alkalophilic thermotolernat bacteria, the water and sediment samples were inoculated to modified Thermus broth containing (% w/v) Tryptone 0.8, yeast extract 0.4, NaCl 0.2 and glucose 1.0. The broth was incubated at 75°C for 4d at 180rpm. Following incubation, the samples were serially diluted and spread on Thermus media (pH 9.0) and incubated at 45°C for 24 hours. Plates were constantly observed for the appearance of bacterial colonies. Single colonies with distinct morphology were selected from each of the plates and examined by Gram staining. A total of 110 isolates were retained for further studies. Spore staining was also done for selected cultures and examined under phase contrast microscope. The cultural and morphological characters were also recorded for the selected isolates.

Screening of xylanase-producing thermophilic bacteria

Qualitative assay: The alkali-tolerant bacterial isolates were initially screened for their ability to produce xylanase by growing them on xylan-agar medium (nutrient agar added with 2.5 g/l xylan) at 37°C for 24 hours. The plates were then stained with Congo red solution composed of 0.5% (w/v) Congo red and 5% (v/v) ethanol in distilled water for 15 minutes and destained with 1 M NaCl for 30 min. The presence of yellow zones around the colonies against the red background indicated the presence of xylanase producing bacteria [35]. The xylanase producers were further screened qualitatively for xylanase activity at temperatures ranging from 40 to 75°C. The isolates that showed the production of xylanase at temperature ≥50°C were selected for quantitative assay.

Quantitative assay: Selected isolates were quantitatively assayed by growing them in modified Reese mineral liquid medium [28] devoid of glucose and supplemented with 0.7% (w/v) wheat bran (pH 9.0) at temperatures 50 to 65°C for 24 hours under shaking conditions (200 rpm). Xylanase activity in the culture broth was assayed at regular intervals of 24 hours by measuring reducing sugars using Dinitro Salicylic Acid (DNSA) according to the method of Miller [22].

Estimation of enzyme activity: Xylanase activities were estimated by quantifying the release of reducing sugar [22] and expressed in terms IU ml^{-1}. One international unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol reducing sugar ml^{-1} min^{-1} under the assay conditions. All of the experiments were performed in triplicate.

Carbon utilization pattern through BIOLOG™

Carbon utilization pattern of three alkalophilic thermo tolerant isolates that showed higher production of xylanases was studied. The bacterial isolates were identified using BIOLOG™ microplate test (Biolog, Incorporated, Hayward, CA) based on the utilization of 95 carbon sources [36] as per manufactures instructions.

Extraction of total genomic DNA from bacterial isolates for PCR analysis

Pelleted cells from 1.5 ml broth were resuspended in 0.5 ml SET buffer (75 Mm NaCl, 25 mM EDTA and 20 Mm Tris) with 10 µl of lysozyme (10 mg $m^{-1}l$). The subsequent genomic DNA extraction method was as described by Pospiech and Neumann [23]. Finally the washed DNA pellet was incubated at 37°C for 25 to 30 min to completely remove ethanol, and then resuspended in 50 µl of TE buffer.

PCR amplification of 16S rDNA

PCR reactions were performed on a Biorad thermal cycler. Primers pA (5'AGAGTTTGATCCTGGCTCAG3') and pH (5'AAGGAGGTGATCCAGCCGCA3') were used for the amplification of 16S rDNA [14]. The amplification was carried out in a 100 µL volume by mixing 50–90 ng template DNA with the polymerase reaction buffer (10x); 100 µM (each) dATP, dCTP, dTTP and dGTP; primers pA and pH (100 ng each) and 1.0 U *Taq* polymerase. The amplification conditions were as follows: initial denaturation of 5 min at 94 °C, followed by 40 cycles of 40 s at 94°C, 40s at 50°C and 1 min 30s at 72°C and a final extension period of 7 min at 72°C. After amplification the PCR product 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 (Alpha-Imager) and gel images were digitalized.

Restriction fragment length polymorphism

Aliquots of purified 16S rDNA PCR products were digested with 3U of restriction endonuclease in 25 µL reaction volume by using the manufacturer's recommended buffer

(2.5 µL of 10x) and temperature. The 16S PCR product was digested with restriction endonucleases *Alu*I and *Hae*III. Restricted DNA was analysed by horizontal electrophoresis in 2% agarose gels. Electrophoresis was carried out at 70V for 2h and 30 min with standard gels (11 × 14 cm). Ethidium bromide was added in the gel and a pre run was always given before loading the samples. The gels were visualized under UV on a gel documentation system (Alpha-Imager) and gel images were digitalized.

16S rDNA sequencing

The PCR amplified 16S rDNA were purified with a Quaquick purification kit (Qiagen). The DNA sequence was double checked by sequencing both strands using primers pA and pH for forward and reverse reaction respectively. The nucleotide sequences were di-deoxy cycle sequenced with fluorescent terminators (Big Dye, Applied Biosystems) and run in 3130xl Applied Biosystems ABI prism automated DNA sequencer.

Accession number

The sequences were submitted to GenBank and the following accession numbers were assigned for isolates H-7 (EU661710), H-9 (EU661711), and R-9 (EU661712).

Blast search and phylogenetic analysis

The partial 16S r DNA sequences of the isolated strains were compared with those available in the databases. Identification to the species level was determined as a 16S rDNA sequence similarity of ≥97% with that of a prototype strain sequence in the GenBank. Sequence alignment and comparison was performed using the multiple sequence alignment program CLUSTAL W2 [21], with default parameters and the data converted to PHYLIP format. Minor modifications were done manually on the basis of conserved domains and columns containing more than 50% gaps were removed. The phylogenetic tree was constructed on the aligned datasets using neighbor joining (NJ) method using the program MEGA 4.0.2 [34]. Bootstrap analysis was performed as described by Felsentein [15]. On 1000 random samples taken from the multiple alignments.

Results

Isolation, screening, and selection of xylanase-producing thermophilic bacteria

One hundred ten alkali-tolerant thermophilic bacteria were isolated from 17 samples (water and sediment) collected from Manikaran. Of 110 isolates, 70 showed the production of xylanases as indicated by the appearance of yellow clearing zone around the colonies after Congo red staining at 37°C. These isolates were further screened for growth and production of xylanases at different temperatures ranging from 40 to 75°C (data not shown). Eleven isolates that showed growth and xylanase production at temperatures ≥ 50°C were selected for quantitative estimation. Of the 11 isolates, only three isolates (H-7, H-9 and H-11) showed xylanase activity at 65°C while 5 isolates showed growth and xylanase activity only upto 50°C (Table 1). All the isolates were found to produce >75 IU ml^{-1} xylanases (Table 1). Maximum xylanase activity was produced by isolate H-7 (at 50°C) followed by H-9 and R-9 (both at 65°C) and was statistically superior to other isolates. These three isolates were further selected for cultural, morphological and molecular characterization. Further they were identified based on carbon utilization pattern and DNA sequencing of 16S rDNA.

Cultural and morphological characteristics

All the cultures showed large-sized spreading type colonies characterized with peculiar brownish or off –white colony colour. The microscopic observation showed that the isolates possessed the typical rod with endospore, characteristic of the genus *Bacillus*. The isolates were found to be oxidase and catalase positive (Table 2).

Identification of selected isolates based on carbon utilization pattern

The data on utilization of 95 carbon sources by three bacterial isolates is given in table 3. The isolates H7, H9 and R9 were identified as *Paenibacillus sp, Bacillus cereus/B.*

Table 1 Xylanase production by thermo-alkali tolerant bacterial isolates

Isolate	Xylanase Activity (IU ml^{-1}) at different temperatures		
	50 °C	60 °C	65 °C
R-9	185.0	ND[1]	ND
H-9	183.2	179.9	173.0
H-7	161.4	149.6	145.0
H-11	123.8	118.4	112.0
11	110.1	102.0	ND
37	102.8	95.5	ND
37A	94.3	93.8	ND
R-1	91.0	ND	ND
R-1	87.0	ND	ND
R-10	81.0	ND	ND
R-11	75.5	ND	ND

ND: Not Detected

Table 2 Morphological, cultural and biochemical characteristics of isolates from Manikaran thermal springs

Isolate No	Colony colour	Shape, size and pigmentation	Margin	Elevation	Gram reaction and Shape	Oxidase	Catalase	Nearest Phylogenetic neighbour and % similarity	Family
H-7	Brownish	Big, irregular, no	Wavy	Concave	Gram +, rod	+	+	*Paenibacillus ehemencis* (95 %)	Paenibacillaceae
H-9	Off -white	Medium, round, no	Smooth	Flat	Gram +, rod	+	+	*Bacillus cereus* (99%)	Bacillaceae
R-9	Brownish	Big irregular, no	Rough	Convex	Gram +, rod	+	+	*Bacillus subtilis* (99%)	Bacillaceae

Table 3 Characterization of isolates R-9, H-7 and H-9 using BIOLOG identification system

Carbon source	Growth			Carbon source	Growth		
	R-9	H-7	H-9		R-9	H-7	H-9
Water (control)	-	-	-	D-Tagatose	-	-	-
α-Cyclodextrin	+	+	+	Trehalose	+	+	+
β- Cyclodextrin	+	+	-	Turanose	+	+	+
Dextrin	+	+	+	Xylitol	-	-	-
Glycogen	+	+	+	D-xylose	-	-	-
Inulin	-	-	-	Acetic Acid	-	+	-
Mannon	-	+	-	α-Hydroxybutyric acid	-	-	+
Tween 40	-	-	+	β- Hydroxybutyric acid	-	-	+
Tween 80	-	-	+	γ- Hydroxybutyric acid	-	-	-
N-Acetyl-D Glucosamide	+	-	+	p-Hydroxyphenyl-acetic acid	-	-	-
N-Acetyl- β -D Mannosamide	-	-	+	α-Keto Glutaric Acid	-	-	+
α-mygdolin	+	+	-	α-Keto Valeric Acid	+	-	+
L-Arabinose	-	-	-	Lactamide	-	-	+
D-Arabitol	-	-	-	D-Lactic acid Methyl ester	-	-	+
Arbutin	+	+	-	L-Lactic acid	+	-	+
D-Cellobiose	+	+	+	D-Malic acid	-	-	+
D-Fructose	+	+	+	L-Malic acid	+	-	+
L-Fucose	-	-	-	Pyruvic acid Methyl ester	+	-	+
D-Galactose	+	+	-	Succinic acid Mono-Methyl ester	+	-	-
D-Galacturonic Acid	-	-	-	Propionic acid	-	-	-
Gentiobiose	+	+	-	Pyruvic acid	+	+	+
D-Gluconic Acid	+	-	-	Succinamic acid	-	+	-
α-D-Glucose	+	+	+	Succinic acid	+	-	-
m-Inositol	+	-	-	N-Acetyl-L-Glutamic	-	+	+
α-D-Lactose	-	+	-	L-Alaninamide	-	-	+
Lactulose	-	+	-	D-Alanine	+	-	+
Maltose	+	+	+	L-Alanine	+	-	+
Maltotriose	+	+	+	L-Alanyl-glycine	-	-	+
D-Mannitol	+	-	-	L-Asparagine	+	-	+
D-Mannose	+	+	+	L-Glutamic acid	+	-	+
D-Melezitose	-	-	-	Glycyl-L-Glutamic acid	-	-	+
D-Melibiose	-	+	-	L-Pyroglutamic acid	-	-	+

Table 3 *Continued*

α-Methyl- D-Galactoside	-	+	-	L-Serine	+	-	+
β-Methyl- D- Galactoside	-	+	-	Putrescine	-	+	-
3-Methyl-D-Glucose	+	+	+	2,3-Butanediol	+	+	-
α-Methyl-D-Glucoside	+	-	+	Glycerol	+	+	+
β -Methyl-D-Glucoside	+	+	+	Adenosine	+	+	+
α-Methyl-D-Mannoside	-	-	-	2’-Deoxy Adenosine	+	-	+
Palatinose	+	+	+	Inosine	+	+	+
D-Psicose	+	+	+	Thymidine	+	-	+
D-Raffinose	-	+	-	Uridine	+	+	+
L-Rhamnose	-	-	-	Adenosine-5’-Monophosphate	-	-	-
D-Ribose	-	-	-	Thymidine-5’- Monophosphate	+	-	+
Salicin	+	+	+	Uridine-5’- Monophosphate	-	-	+
Sedoheptulosan	-	-	-	D-Fructose-6-phosphate	-	-	-
D-Sorbitol	+	-	-	α-D-Glucose-1-phosphate	-	-	-
Stachyose	-	+	-	D-Glucose-6-phosphate	-	-	+
Sucrose	+	+	+	D,L- α-Glycerol phosphate	+	-	+

thuringiensis, and *Bacillus subtilis* respectively by using the BIOLOG Microlog 3 software (Table 3).

PCR-RFLP analysis of 16S rDNA

To look for the species variation among the morphotypes selected, PCR amplification of 16S rDNA followed by RFLP analysis with two restriction endonucleases was carried out. When 16S rDNA amplicons were digested with restriction enzymes, different profiles containing between 4 and 7 fragments ranging in size from 86 to 700 base pairs were seen with three isolates. The variations in restriction profile (size and number of the bands) of the PCR products for three isolates by *Alu*I and *Hae*III (Fig. 1) indicated that the isolates were genetically different from each other.

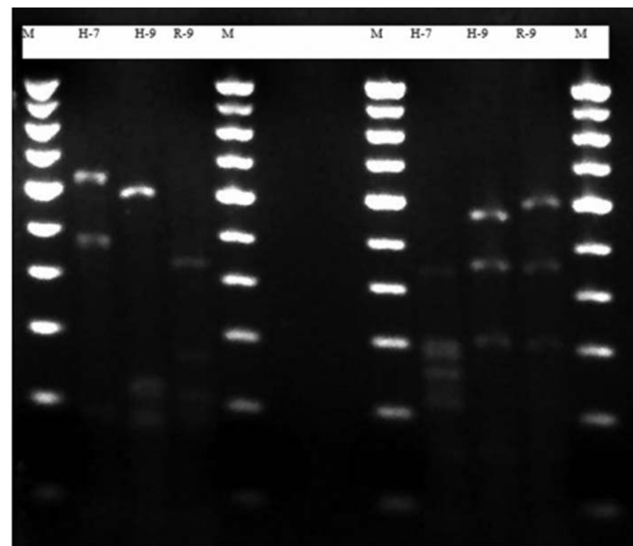

Fig. 1 RFLP of 16S rRNA of xylanases producing isolates using restriction endonucleases *Alu* I and *Hae* III. Lane 1, 100-bpDNA marker; lane 2, isolate H-7; lane 3, isolate H-9; lane 4, isolate R-9.

Analysis of 16S rRNA gene sequences of three bacterial isolates

Three selected isolates were sequenced and the sequence data was analysed by BLAST and the nearest match from GenBank data was reported. Sequences were deposited in the GenBank. DNA sequencing and phylogenetic analysis revealed that all the isolates obtained from Manikaran thermal springs showed 95 to 100% similarity with the sequences within the GenBank. The closest Phylogenetic neighbours according to the 16S rRNA gene sequence data for the three isolates H-7, H-9, and R-9 were *Paenibacillus ehimensis, Bacillus cereus* and *Bacillus subtilis*, respectively (Fig. 2).

Discussion

Microbial xylanases have important applications in the biodegradation of xylan. Thermostable xylanases active at alkaline pH are of significant importance in paper and pulp industry [16]. Thermal springs represent extreme niches that have maintained some degree of pristine quality and their biotechnological potential has remained unrealized. In the last one-decade, several attempts have been made for phylogenetic characterization of microflora from thermal

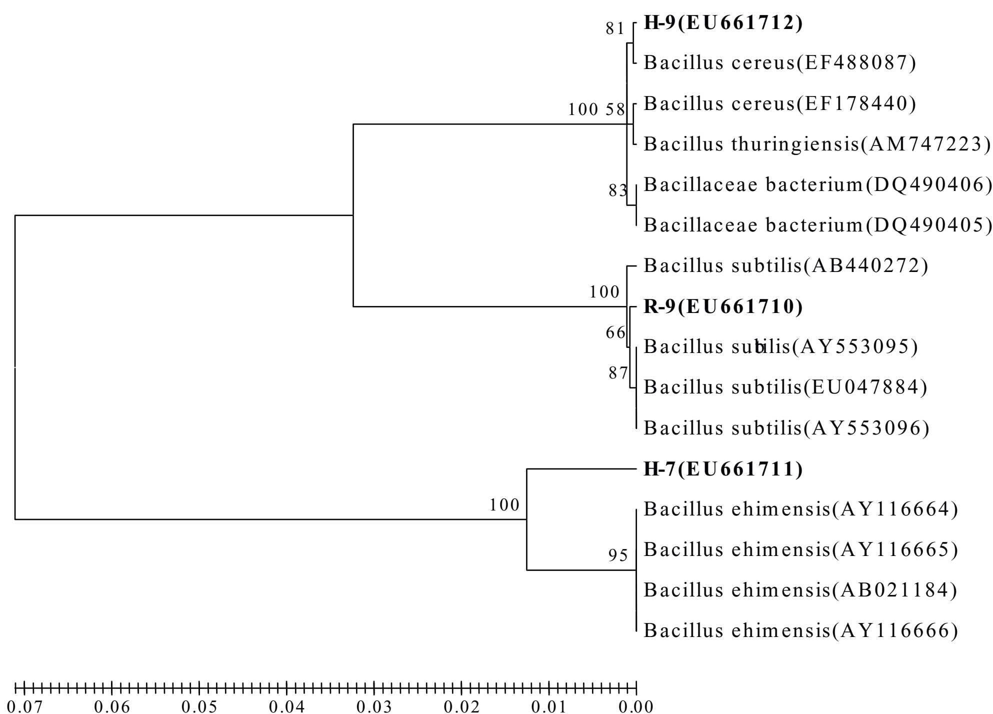

Fig. 2 Unrooted phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of xylanase producing isolates and some of their closest phylogenetic relatives. The phylogenetic tree was constructed on the aligned datasets using Neighbour Joining (NJ) method using the program MEGA 4.0.2. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1000 random samples. Isolates characterized in the present study are indicated in bold.

springs in different parts of the world [10, 28, 31]. There are reports of isolation of xylan degrading alkali tolerant thermophiles from the hot springs in Bulgaria [12] and Portugal [5]. Manikaran thermal springs located in Himachal Pradesh, India is famous for its hot water springs where the temperature is near boiling. It was pertinent to isolate thermotolernat xylanolytic bacteria from these springs as they have earlier been reported to be a good source of thermotolernat amylolytic bacteria [32]. Recently the xylanases from the strains of *Cellulomonas uda*, *Microbacterium ulmi*, *M. xylanilyticum*, *Bacillus amyloliquefaciens*, *B. firmus*, *B. thermoleovorans*, *B. pumilus*, *Streptomyces* sp., *Microbacterium barkeri*, *Bacillus niabensis*, *B. funiculus*, *B. megaterium*, *Pseudoxanthomonas suwonensis*, *Cupriavidus gilardi* and *Rhodococcus* have been reported [1, 7–9, 13, 18, 19, 25, 26, 29, 33]. The importance of xylanases in industry has motivated the workers to constantly search for new isolates with higher efficiency. It requires careful and planned screening for specific trait. In the present study, from a diverse pool of 110 isolates we narrowed down to three that could produce significantly high amount of xylanases at a temperature >50°C. These three isolates R9, H9 and H7 produced 185, 173 and 145 IU ml^{-1} of xylanases. There are reports suggesting variability in xylanase production, *Bacillus circulans* AB 16 (50 IU/ml) [11], *Bacillus subtilis* C 01 (135 IU gds/l) [2], Bacillus sp. (180 U/ml) [4] and *Bacillus subtilis* ASH (8,964 U of xylanase/g dry wheat bran) [30]. The optimisation of various growth and nutritional parameters could further enhance the efficiency of the three isolates selected in the present study. After optimization of various production parameters, an increase of nearly 13-fold in xylanase production (5407 IU/ml) has been reported [6].

The morphological, cultural, biochemical (C-utilization) and molecular characterization revealed the identity of three cultures to be *Bacillus* sp. However the sequencing of the 16S rDNA could identify the three cultures H-7, H-9, and R-9 as *Paenibacillus ehimensis, B. cereus, B. subtilis*, respectively. The C-utilization pattern and identification through BIOLOG has certain limitations, as it is difficult to distinguish closely related species like *Bacillus cereus* and *B. thuringiensis*. DNA sequencing is more reliable and could help in the identification of isolates up to species level. Of the three species identified in the present study *B. subtilis* and *B. cereus* have been reported to be producer of xylanse whereas no reports are available for *Paenibacillus ehimensis*. Optimization of conditions for enhanced production of xylanases from *Paenibacillus ehimensis* and biochemical characterization of the enzyme system is in progress at present.

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