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Research Article

EXPLORATION OF THAR DESERT FOR THERMOPHILIC BACTERIAL STRAINS RELEASING INDUSTRIALLY VALUABLE ENZYMES

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ABSTRACT
Thar Desert of north western India was explored for thermo tolerant bacterial diversity. Ten randomly selected strains (BN1-BN10) were identified at genomic level by PCR amplification of 16S rDNA followed by sequencing of PCR products. Nucleotide sequences were analyzed with nucleotide-nucleotide basic local alignment search tool (BLASTn) available on National Center for Biotechnology Information (NCBI) website. At genomic level organisms were identified as four strains of *Bacillus licheniformis* (BN 1, 5, 6, 10), one strain of *Bacillus subtilis* (BN4), three strains of *Enterobacter cloacae* (BN 2, 7, 9), one strain of *Providencia stuartii* and *Pectobacterium carotovorum*. These 16S rDNA gene sequences were deposited to Genbank using BankIt submission tool and have been assigned with NCBI accession numbers. All of the thermophilic isolates were screened for extracellular enzyme production activity. Considerable extracellular enzyme producing activity has been shown by isolated strains viz; Casein hydrolytic activity (BN 2, 3, 4, 5, 6 and 7), cellulase (BN 2, 4, 5, 6 and 7), Gelatinase (BN 3), pectinase (BN2, 3, 4, 5 and 7) and urease (BN 1, 3 and 8), but amylase activity has not been shown by any of the isolates. While studying antibiotic sensitivity, all isolates have shown inhibition zones of different diameters (Figure 3 (i) to Figure 3 (ix)) against 12 antibiotics tested (cefpodexine, chloramphenol, vancomycin, streptomycin, rifampicin, levofloxacin, ceftriaxone, clindamycin, augmentin, amikacin, cefixime and tetracycline) except BN 1 which showed no inhibition zone by any of the antibiotics tested (Graph 1). Isolates were screened for their antibiotic potential also. None of the isolates have shown antimicrobial activity against any of the test organisms (*Escherichia coli, Staphylococcus aureus* and *Bacillus subtilus*).

Keywords: *Thermophilic Bacteria, 16S rDNA, PCR, Sequencing, Thar Desert*

INTRODUCTION
Thar Desert is considered as a unique ecosystem due to harsh environmental conditions for living beings. On the other hand bacteria have excellent ability of mutations and adaptations, hence, they develop biomolecules with specific features.

Some of these compounds, especially enzymes and proteins have proved to be beneficial for industries, being tolerable to harsh manual and mechanical processing. *Bacillus* and *Enterobacter* species are world wide spread (Brenner *et al.*, 2005).

Bacillus genus members are generally found in soil and species are Gram- positive, endospore forming, rod shaped bacteria (Waites *et al.*, 2008).

They represent a wide range of physiological abilities, allowing the organism to grow in every environment and compete desirably with other organisms within the environment due to their capability to form extremely resistant spores and metabolites that have antagonistic effects on other microorganisms (Kuta, 2008).

Enterobacteriaceae family has 44 genera and 176 species (approx) (Brenner *et al.*, 2005). Enterobacteriaceae may account for 80% of clinically significant isolates of Gram-negative bacilli and 50% of clinically significant bacteria in clinical microbiology (Murray *et al.*, 2003).

Enterobacteriaceae are Gram-negative rods with a length of 1 - 3 µm. They are facultative anaerobes, oxidase-negative, catalase positive, and grow on MacConkey agar, and their natural hosts are human and animal intestines (Forbes *et al.*, 2007).

MATERIALS AND METHODS

Collection of Sample

Soil samples were randomly collected from different areas of north western India. These samples were serially diluted in nutrient broth up to 10^{-7} dilution followed by plating on nutrient agar and ATCC 697 medium (NaCl 0.5%, peptone 0.5%, yeast 0.2%, beef extract 0.4%, agar 2% in 1L DW) and incubated at 50ºC for 24 hrs.

Genomic Level Identification

The CTAB/NaCl method (Ausubel *et al.*, 1994) was used for isolation of genomic DNA. Then 16S rDNA was amplified by using 5'TACGGYTACCTTGTTACGACTT-3' reverse primer and 5'-AGAGTTTGATCMTGGCTCAG-3' forward primer. The PCR products were eluted from gel and DNA sequencing was performed with an automatic DNA sequencer (ABI prism) according to manufacturer's instructions.

Nucleotide sequences were analyzed with nucleotide-nucleotide basic local alignment search tool (BLASTn) program available on National Center for Biotechnology Information (NCBI) website. The 16S rDNA gene sequences of the species most closely related to our strains were retrieved from the database. Retrieved sequences were aligned by using the Clustal X program. A phylogenetic tree was constructed by the neighbor-joining method using the software package Clustalo 1.2.1. The 16S rRNA gene sequences have been deposited to Genbank using BankIt submission tool for getting Genbank accession numbers by NCBI.

Screening for Production of Extracellular Enzymes

Screening for Amylase Activity: After inoculation of the cultures on ATCC 697 and incubation for 1-2 days at 37^0C and 50^0C, iodine solution (I_2 =1 g, KI= 2 g/ 300 ml) was poured on the plates, white clear zones against a blue background around the colonies were taken as the indication of positive test for amylase activity.

Screening for Casein Hydrolysis Activity: After inoculation of the cultures on skim agar plate media (Skim milk powder 5.14% and agar 1.5% in 1L DW) and incubation for 3-4 days at 37^0C and 50^0C, clear zones around the colonies were taken as the evidence for the casein activity.

Screening for Cellulase Activity: After inoculation of the cultures on Czapek mineral salt agar media ($NaNo_3$ 0.02%, KH_2P0_4 0.01% $MgSo_4$ 0.048% Carboxymethyl cellulose 0.05% KCl 0.048% Peptone 0.02% Agar 2% in 1L DW) and incubation for 3-4 days at 37^0C and 50^0C, Congo red solution (1 %) was poured onto the plates. After 30 min incubation at room temperature, the plates were washed with 1 M NaCl solution. A clear zone around the colonies on a red background was taken as an evidence for the cellulase activity.

Screening for Gelatinase Activity: After inoculation of the cultures on the Gelatin based media (Peptone 0.4% Yeast 0.1% Gelatin 1.2% Agar 2% in 1L DW) and incubation at 37^0C and 50^0C for 24 hr. Clear zone occur around the colonies showed the presence of gelatinase.

Screening for Pectinase Activity: Cultures was inoculated in the pectin based media (Pectin powder 1.7% Yeast 1.5% Peptone 0.9% Agar 4% in 1L DW) and incubated for 2-3 days at 37^0C and 50^0C. Then, 1% cetyl-trimethyl-ammoniumbromide solution was poured onto the surface of the plates. After 10 min incubation at room temperature, colonies with clear zones indicated pectinase activity.

Screening for Urease Activity: Cultures were inoculated in Urea media (Urea base 2.1% Agar 2% Phenol 0.012% Urea 4% in 1L DW). As urea is unstable and gets easily break down at 15 psi, therefore, filter sterilized and added to the medium after autoclaving. Cultures were incubated for 2-3 days at 37^0C and 50^0C and examined the change in color.

Screening for Antibiotic Sensitivity

For antibiotic sensitivity testing, samples were inoculated on ATCC 697 medium plates by spread plate method and twelve antibiotic discs were placed on it, cultures were incubated overnight at 50^0C for 24 h. The antibiotics used were: cefpodexine, chloramphenol, vancomycin, streptomycin, rifampicin, levofloxacin, ceftriaxone, clindamycin, augmentin, amikacin, cefixime and tetracycline. Zones of Inhibition (ZOI) were measured in centimeters (cm) by scale.

RESULTS AND DISCUSSION

Bacterial Morphology and Culture Characterization

After 24 h incubation at 50^0C in nutrient broth, the cultures showed flocculating growth in aerobic condition. This indicated that these strains can be easily grown at high temperature. The strains showed smooth, moist translucent and milky colonies on nutrient agar and ATCC 697 medium.

16S rDNA Sequencing Analysis

Molecular characterization was performed by the multistep procedure including; extraction of genomic DNA, followed by amplification and sequencing of 16s rDNA, which was analyzed by bioinformatics tools available on NCBI website. The 16S rRNA gene sequence of strains showed 92% and 98% similarity with validly described strains of thermophilic bacteria (*Bacillus licheniformis, Enterobactor cloacae, Providencia stuartii, Pectobacterium carotovorum*). Phylogenetic study of the isolates has shown their close affiliation with thermophilic *Bacillus* species and *Enterobacter* species which are grouped within the clade of thermophiles. This demonstrates that isolates mainly belong to the thermophilic *Bacillus* and *Enterobacter* species (Table 1). Similar results with same methodology have been obtained by a number of researchers (LaMontagne *et al.*, 2002; Adetunji *et al.*, 2012; Kheyrodin and Ghazvinian, 2015). The sequences thus obtained have been submitted to Genbank with the help of sequence submission tool Bankit and accession number for each sequence have been obtained from NCBI, which gave further authentication to the work done (Table 1).

Table 1: NCBI Assigned Accession Numbers of Isolates

S. No.	Organism	Strain Similarity with Database	GenBank Accession Number
1.	Bacillus *licheniformis*	HT-Z14-B1 (97% similar)	KU051657
2.	Enterobactor *sp*.	HSL64 (98% similar)	KU253439
3	Providencia *stuartii*	5CMAC1 (92% similar)	KT989036
4.	Bacillus *subtilis*	CCMMBB1000 (96% similar)	KU051658
5.	Bacillus *licheniformis*	HT-Z14-B1 (95% similar)	KU284843
6.	Bacillus *licheniformis*	RPA (96% similar)	KU284842
7.	Enterobactor *cloacae*	MSSRFS8 (98% similar)	KU284841
8.	Pectobacterium *carotovorum*	Carotovorum 88% similar)	KU23910
9.	Enterobactor *cloacae*	PW 108 (97% similar)	KU199682
10.	Bacillus *licheniformis*	CY-012 (96% similar)	KU199683

Screening for Production of Extracellular Enzymes

Wide varieties of thermostable enzymes are produced by most of the thermophilic microorganisms. Thermostable enzymes are of great interest for industrial applications (Haki and Rakshit, 2003). Considering these facts, all of the thermophilic isolates were screened for extracellular enzyme production activity. Considerable extracellular enzyme producing activity has been shown by isolated strains viz; Casein hydrolytic activity (BN 2, 3, 4, 5, 6 and 7), cellulase (BN 2, 4, 5, 6 and 7), Gelatinase (BN 3), pectinase (BN2, 3, 4, 5 and 7) and urease (BN 1, 3 and 8), but amylase activity has not been shown by

any of the isolates (Table 2, Figure 2). Several workers have reported pectinase and cellulase activity from thermophilic *Bacillus* (Khatri *et al.*, 2015; Das *et al.*, 2010) and *Enterobacter* species (Ogbo and Okonkwo, 2012; El-Deeb *et al.*, 2013). Fira *et al.*, (2001) and Rath and Mohanta (2010) have also reported various extracellular enzymes from some thermophilic bacterial isolates.

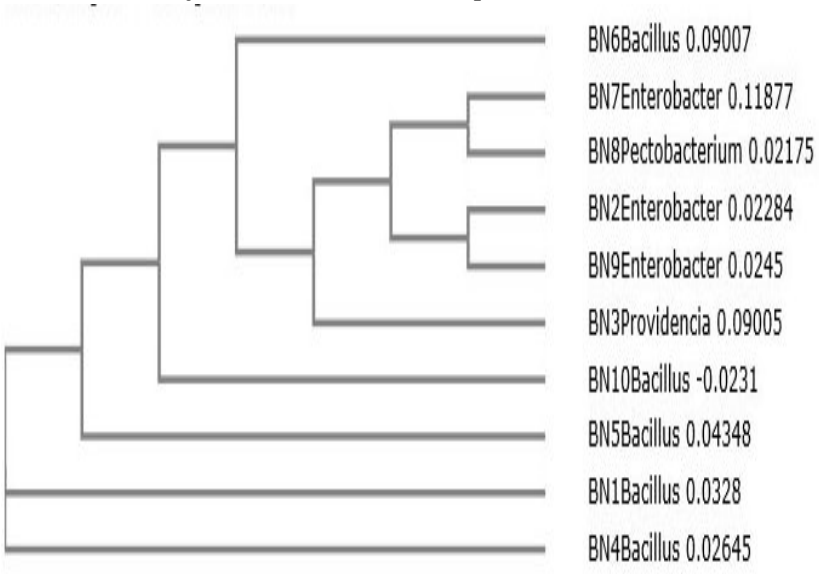


Figure 1: Phylogenetic Tree of the Strain BN1 to BN10 and Closest NCBI (BLASTn) Strains Based on the 16S rRNA Gene Sequences (Neighbor Joining Tree Method)

Table 2: Extracellular Enzymes Profile

S. No	Isolates	Amylase	Casein Hydrolyase	Cellulase	Gelatinase	Pectinase	Urease
1.	BN1	-	-	-	-	-	+
2.	BN2	-	+	+	-	+	-
3.	BN3	-	+	-	+	+	+
4.	BN4	-	+	+	-	+	-
5.	BN5	-	+	+	-	+	-
6.	BN6	-	+	+	-	-	-
7.	BN7	-	+	+	-	+	-
8.	BN8	-	-	-	-	-	+
9.	BN9	-	-	-	-	-	-
10.	BN10	-	-	-	-	-	-

(i) (ii) (iii)

Figure 2 (i) Pectin Hydrolysis Test, (ii) Casein Hydrolysis Test, (iii) Urease Test

Screening for Antibiotic Sensitivity and Antimicrobial Potential

Thermophilic microorganisms were screened for antibiotic resistance by disc diffusion method. It is mandatory for the clinical microbiology to screen isolates for determining resistance/sensitivity to the therapeutic drugs of choice (Alfredson *et al.*, 2003). Disc diffusion tests for *Bacillus* and *Enterobacter* sp. have been proposed previously (Mezzatesta *et al.*, 2012; Regli and Pages, 2015). The new interpretive criteria for disc susceptibility testing of thermophilic Campylobacter sp. were proposed by Huysmans and Turnidge (1997). The antibiotics used were; cefpodexine, chloramphenol, vancomycin, streptomycin, rifampicin, levofloxacin, ceftriaxone, clindamycin, augmentin, amikacin, cefixime and tetracycline. All isolates have shown inhibition zones of different diameters (Figure 3 (i) to Figure 3 (ix)) which were measured in centimeters (cm), except BN 1 which showed no inhibition zone by any of the antibiotics tested (Graph 1). Isolates were screened for their antibiotic potential also. None of the isolates showed antimicrobial activity against any of the test organisms (*Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilus*). Bacterial isolates were screened for their inhibitory effect on test bacteria by agar well diffusion method as reported by Yilmaz *et al.*, (2006).

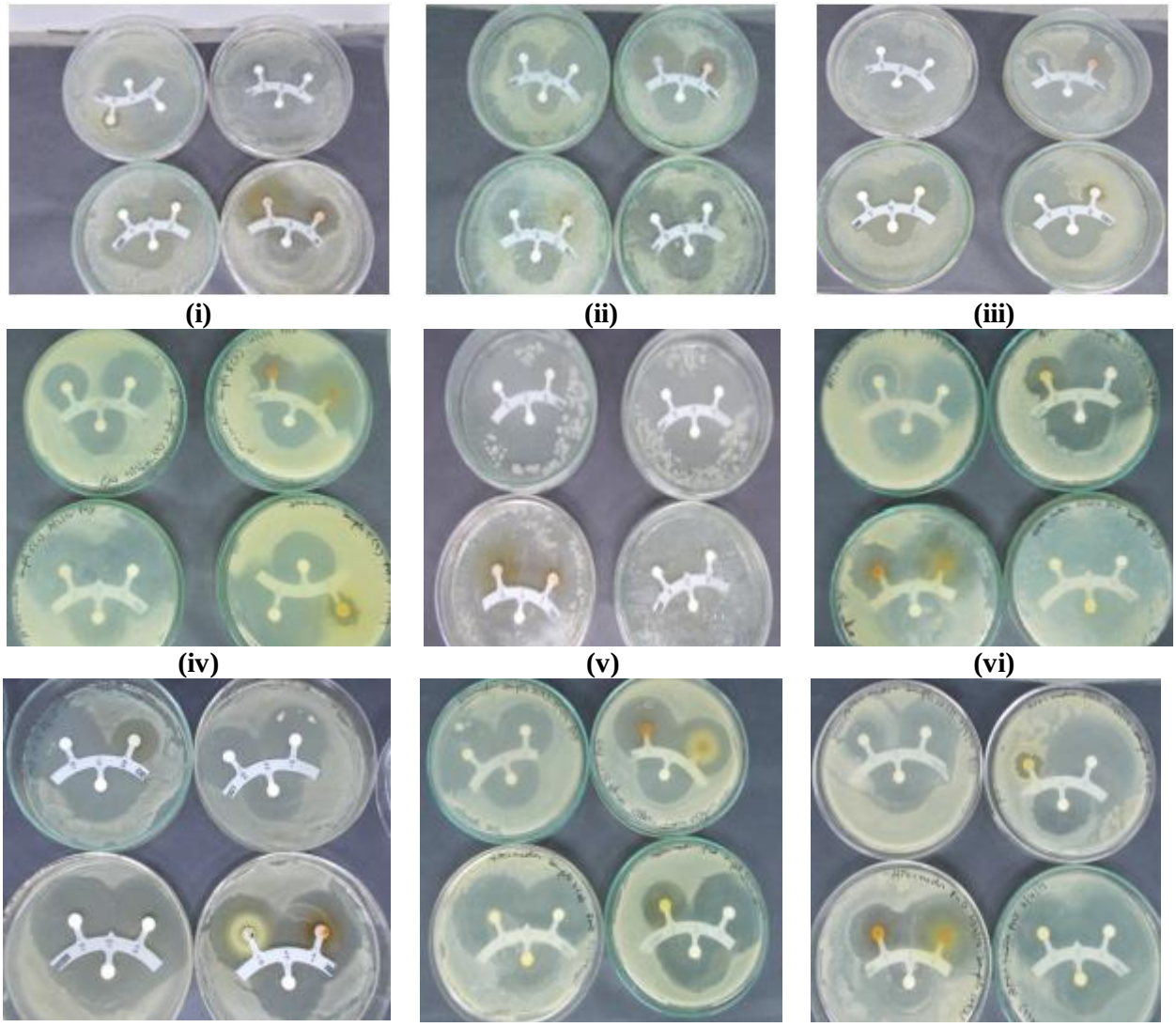
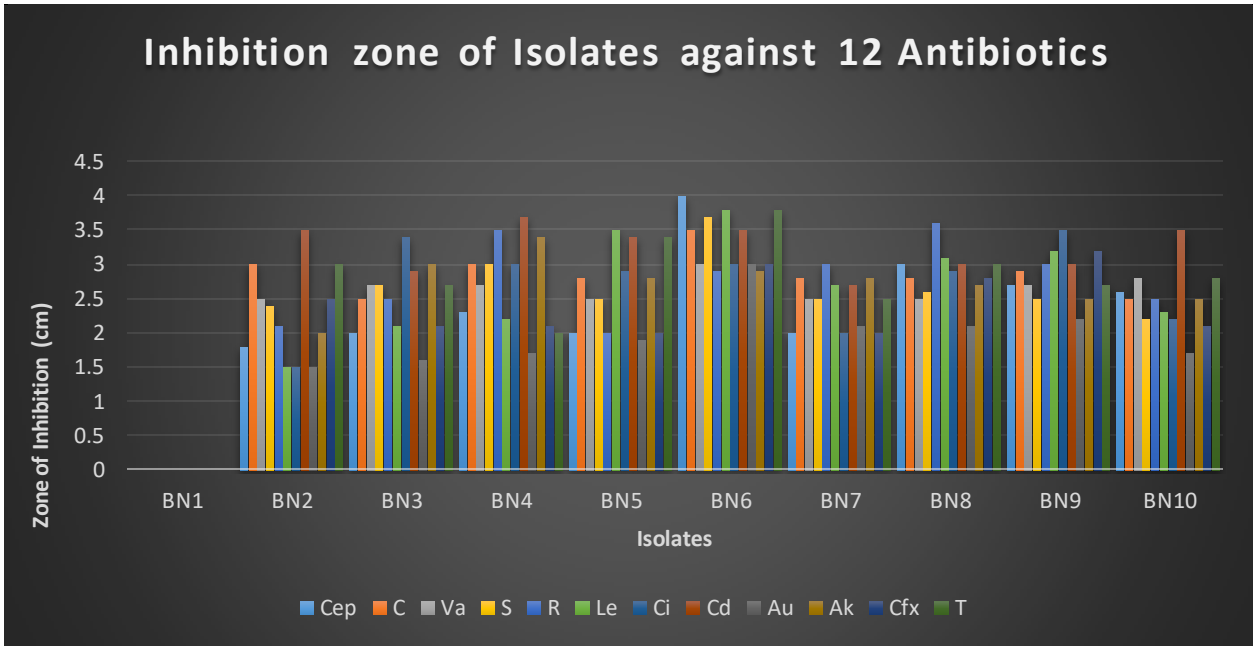


Figure 3: Antibiotic Sensitivity Tests (i) BN2 (ii) BN3 (iii) BN4 (iv) BN5 (v) BN6 (vi) BN7 (vii) BN8 (viii) BN9 (ix) BN10



Graph 1: Cep- Cefpodexine, C- Chloramphenol, Va- Vancomycin, S- Streptomycin, R- Rifampicin, Le- Levofloxacin, Ci- Ceftriaxone, Cd- Clindamycin, Au- Augmentin, Ak- Amikacin, Cfx- Cefixime, T- Tetracycline

Conclusion

Thar Desert is nowadays being explored as rich source of novel genes due to harsh climatic conditions, hence, bacterial strains were isolated from sand dunes of Thar Desert of India. Ten randomly selected strains (BN1 to BN10) were identified as species of *Bacillus* (BN 1, 4, 5, 6 and 10) and *Enterobacter* (BN 2, 3, 7, 8, and 9) on the basis of 16 s rDNA sequencing. All of these strains were found to be tolerant to 50^0C temperature and 2 % salt concentration.

In this study ten different thermophilic bacterial species have been isolated and characterized molecularly. These isolated organisms mainly fall within the two genus of thermophiles; Bacillus and Enterobacter. The isolated organisms showed significant extracellular enzyme producing ability such as casein hydrolyase, cellulase, gelatinase, pectinase and urease. This ability makes them an important biotechnological tool for thermostable enzymes production. The investigation clearly indicates that the north western India, especially, sand dunes are a rich source of many thermophilic bacteria. The enzymes isolated from some thermophiles have been proven to be of great use in the modern fields of biological sciences e.g. heat stable DNA polymerase for polymerase chain reaction, surfactants and in medicine as they are able to do work under such conditions that would denature enzymes taken from mesophilic organisms. These isolates as well as their proteins and enzymes can be exploited in various useful industrial processes. The present study opens a way for utilizing microbial diversity present in Bikaner zone for extraction of thermostable enzymes and cloning of their genes in search of a cost effective production technology.

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