LIPASE PRODUCING THERMOPHILIC BACTERIA ISOLATION AND CHARACTERIZATION FROM HOT SPRINGS OF CENTRAL INDIA

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ABSTRACT : Hot springs considered being a unique domain of novel extremophiles which are useful for production of industrially important enzymes, to understand the metabolic reactions and for producing other biotechnological products. The aim of the present research was isolation and characterization of thermophilic bacteria containing potential to produce extracellular lipase enzyme from the geothermal springs located in central India. Water is used as test sample in order to obtain thermophilic bacteria. 10 thermophilic bacteria were isolated from the water samples and subjected to screening for the potential of producing lipase enzyme of which 8 isolates are potential producer of extracellular lipase. Two isolates with higher zone of clearance around them were selected for the further investigation. Morphological, biochemical and molecular identification by 16S rRNA sequencing of these isolates (BAC23 and BAC26) could be identified as *Bacillus haynesii* and *Bacillus clausii*. The lipolytic activity of these isolates was recorded stable at optimum temperature range of 45°C-60°C with alkaline pH at 9.0-9.4 and at 0.5-1.0 M salinity. Capability to produce extracellular thermostable lipase at high temperature and pH, these strains of *Bacillus* spp. can be presented as promising contender for modern industrial applications.

Keywords : *Bacillus haynesii*, *Bacillus clausii*, Hot springs, Thermophilic bacteria, Thermostable lipase.

INTRODUCTION

A wide scope of organism's living spaces and be a basic aspect of all condition extending from moderate to brutal conditions to live like high temperature water springs, aqueous vents, salterns and other such situations with outrageous conditions, where common life conditions are not seen. Thermophiles are the life forms adjusted to develop ideally at high temperatures going from 55°C-121°C (Kumar *et al.*, 2013). Worldwide, concentrates on microbial communities in hot springs have essentially focused on natural surroundings at low rises like Yellowstone National Park (Mitchell, 2009), Kamchatka in Russia, Iceland (Reigstad *et al.*, 2010), Indonesia (Aditiawati *et al.*, 2009) and Tunisia (Sayeh *et al.*, 2010). India is the home to a few geothermal hot springs, particularly the Himalayan Geothermal Belt (HGB) which contains near 150 warm springs.

Microbial communities present in such environments comprise important hotspots for different biotechnological applications (Satyanarayana *et al.*, 2005 and Sayeh *et al.*, 2010). Thermophilic microorganisms have indicated colossal potential in biotechnology due to their capacity to deliver special thermostable enzymes and proteins with high stability and activity (Demirijan, 2001 and Tehei & Zaccai, 2005). One of commonly used enzyme in industries is the Taq DNA polymerase, which is a catalyst isolated from the hot springs bacterium *Thermus aquaticus* (Chien *et al.*, 1976 and Podar & Reysenbach, 2006). Thermostable enzymes from these microorganisms such as proteases, amylases, lipases cellulases, xylanases, pectinases, gelatinases, DNases (Verma *et al.*, 2014) are in extraordinary interest as they are not typically denatured at high temperature, however are somewhat more dynamic at raised temperature (Adams & Kelly, 1998 and Zeikus *et al.*, 1998).

Among them Lipases are a flexible group of catalysts and regularly express different activities like phospholipase, isophospholipase, cholesterol esterase, chitinase, amidase and other esterase kind of activity (Svendsen, 2000). Importantly they are used in paper, food, pharmaceutical, leather, textile, detergent and cosmetic industries. A few thermophilic lipases have been purged and portrayed from *Thermophilic Bacillus* sp. (Sharma *et al.*, 2002), *Bacillus thermoleovorans* (Lee *et al.*, 1999 and Markossian *et al.*, 2000), *Bacillus stearothermophilus* (Sinhaikul *et al.*, 2001), *Bacillus circulans* (Kademi *et al.*, 2000). Hence many more novel and useful genetic pool of thermophilic microbes is expected to be discovered from the unexplored hot springs of central India. These extremophiles are having biotechnological importance as they produce industrial enzymes which function in extreme environments (extremozymes). Therefore, the goal of this investigation is to isolate thermophilic bacteria from this region and screen them for the high potential of producing the industrial product such as enzyme that are able to tolerate high temperature.

MATERIAL AND METHODS

Sample collection :

Water samples in different season were aseptically collected from Choti Anthoni Pachmari (CAP) Hoshangabad and Badi anthoni in Chindwada district (BAC), the two selected hot spring areas of Madhya Pradesh for this research. The sample is withdrawn from the source of hot water in an air tight glass vials and these vials placed in a thermo flask containing a temperature more than that of source, to maintain the temperature as well as avoidance of damage of samples during transportation and used for the investigation of physical parameters (Anderson *et al.*, 2006).

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Isolation of Bacteria and conventional methodology for identification : The sample were serially diluted up to 10⁻⁹ and grown in Erlenmeyer flask containing Luria Bertani (Himedia, Mumbai) broth of different pH, and incubated at 50°C for 24 hours. After growth 100 µl from each flask is than spreaded on NAM (Himedia, Mumbai) plates and incubated again at 50°C for 24 hours. Various bacterial colonies with different morphologies are selected and subjected to subculturing in order to obtain pure culture in NA plates. Morphological characterization of each pure culture was carried out by examining colour, size, colony morphology gram staining as described by (Kristjansson *et al.*,1992). Biochemical characterization test such as catalase, oxidase, citrate, sugar utilization, motility, and urease was applied by using the method described previously by (Aneza,2003). Temperature effects, pH and salt concentration effects on all the isolates were carried out by growing at different temperature, pH and NaCl concentration at 0.5-2.0 M for 24-48 hours.

Screening for Enzyme producing potential and Molecular identification

Lipase activity : Lipolytic activity was detected by using tributyrin agar (peptone 5 grams, yeast extract 3 grams, agar 15 grams and tributyrin 10 ml) by streaking single line of the culture followed by the incubation of 24 hrs at 55°C (Rollof *et al.*,1987). The pH of the media was adjusted to alkaline at 8.0. For the detection of lipase activity presence of halo clear zone around the inocula indicate lipase activity.

16s rDNA Amplification : The DNA was isolated from the bacteria by using the method described by Sambrook and Russel in 2001. In the present study PCR amplification of 16s rDNA was achieved by using PCR reaction mixture with universal primers 27F and 1492R (eurofins Genomics Luxembourg). The final reaction mixture is 50 µl for 16S rRNA gene amplification with universal primer 1492R (5'CGGTTACCTTGTTACGACTT3') and 27F

(5'AGAGTTTGATCMTGGCTCAG3'). The composition of the reaction mixture used is 5 µl of 10x *Taq* Buffer (pH 9.1 at 20°C), 2 µl - 50 mM MgCl₂, 5 µl of 2 mM dNTPs, 1 µl of *Taq* polymerase 3 µl of 27F primer and 5 µl of 1492R primer is added along with the 3 µl of template DNA at the end and the final volume is brought up to 50 µl (Heuer *et al.*,1997). The PCR reaction were performed by using Applied Biosystems Veriti™ 96-Well Thermal Cyclers under the following thermocycling condition set as initial denaturation at 95°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds and elongation at 72°C for 2 minutes followed by extension at 72°C for 8 minutes. The band patterns were visualized on automated transilluminator Gel doc instrument (Azure Biosystems, Inc.).

Sequencing of 16s rDNA amplicons and phylogenetic analysis : The amplified product obtained by 16S rDNA amplification was subjected for purification by PCR cleanup Kit and the method is followed as described by the manufacturer (Gentix Biotech). 16S rDNA PCR amplicon is purified, diluted for sequencing as 100 ng/2 µl and sequenced for both DNA strands using the same primer as used in 16s rDNA amplification *i.e.* 27F and 1492R (Genombio Technologies Pvt. Ltd., Pune). The sequence obtained after sequencing were used for the analysis using similarity search by online web server blastn (nucleotide blast) for 16S ribosomal RNA available at NCBI. The sequences are then used for multiple sequence alignment by another web server ClustalW (Thompson *et al.*,1994). Finally, phylogenetic tree was constructed using MEGAX 10.1 computer programme by using Maximum Parsimony method as mentioned by Tamura *et al.* (2001).

RESULTS AND DISCUSSION

In the present study the presence of thermophilic bacteria belongs to genus *Bacillus* were investigated from the samples isolated from the hot springs of Madhya Pradesh. The

Table. 1 Physiological parameters of water samples.

Sample	Electrical conductivity ms	Turbidity NTU	DO mg/l	B OD µg/ml	COD mg/l	TDS (ppm)	NaCl conc.
Site 1 Water	7.91	7.12	9	187	389	412	1.6%
Site 2 Water	827	5.96	17.1	120	1144	376	2.4%

Table. 2 Morphological characteristics of isolated bacterial samples of hot springs.

Strain	Morphological characteristics						
	Size (mm)	Color	Gram	Shape (cells)	Spore	Motility	Shape
CAP1	2	Creamy white	+ve	Rod	+ve	Motile	circular
CAP2	2.8	Yellowish	-ve	Rod	+ve	Motile	Irregular
CAP3	2	Creamy yellow	-ve	Rod	+ve	Motile	Irregular
CAP4	1.6		-ve	Cocci	+ve	Motile	Irregular
BAC22		Yellowish	-ve		+ve	Non motile	
BAC23	0.9	Creamy white	+ve	Rod	+ve	Motile	circular
BAC24	2	Creamy yellow	+ve	Cocci	+ve	Non Motile	rhizoid
BAC25	1.7	Yellowish		Cocci	+ve	Motile	rhizoid
BAC26	3-4	Creamy white, circular	+ve	Rod	+ve	Motile	circular
BAC27	2	Yellowish	+ve	Cocci	+ve	Motile	Circular

temperature of surface water is different and it gradually increases when the thermometer is dipped deeper in the kund (Source of hot spring). Temperature and pH of both the source was 55°C pH 8.0-8.5 for CAP and 55-68°C with pH 9.0 for BAC. Physio-chemical parameter was measured by HANNA instruments for measuring the listed parameters Table.1. Among the two water sample collected from both the sites 10 pure isolates were obtained and assigned the codes viz. CAP1, CAP2, CAP3, CAP4, BAC22, BAC23, BAC24, BAC25, BAC26, BAC27. Morphological characterization i.e colony shape, size, margin, form, elevation, surface and gram staining of the all isolates were listed in (Table.2). All the strain shown positive growth against different range of temperature from 45-65°C except few which shows negative results and growth beyond 65°C is not observed in any strains (Table.3). Likewise, maximum growth was observed at pH ranges from 8-10 for all the strains (Table.4). Isolates CAP1, CAP2, CAP3, CAP4, BAC22, BAC23, BAC24, BAC25, BAC26, BAC27 are positive for catalase, oxidase, citrate, sugar utilization and citrate except few which shows negative results (Table.5).

Nearly all the strains are able to produce halo zone of clearance around the inocula except CAP4, among which two strain i.e. BAC23 and BAC 26 shows the highest zone of clearance around the single line streaked inocula, indicates the high potential of producing thermostable lipase and these two strains are used for sequencing. These strains can able to grow at optimum temperature of about 55-65°C and can toler-

ate high pH of about 9-10. Both these strains are capable of fermenting different sugars such as maltose, dextrose, raffinose, arabinose, ribose, glucose, sucrose and fructose while producing the acids and gas as the end products. Genotypic and phenotypic identification of the thermophilic strains was carried out previously in many geothermal areas including India (Sharma *et al.*,2008), Greece (Sievert *et al.*,2000), Italy (Maugeri *et al.*,2001), Turkey (Gul-Guven *et al.*,2008), Bulgaria (Derekova *et al.*,2008) and China (Lau *et al.*,2009).

16S rDNA amplification by using the universal forward and reverse primer i.e. 1492R and 27F of these two isolates produce amplified products as PCR bands and these were subjected to electrophoresis which reveals the size of the product is between 1500 bp by using reference ladder DNA of 500bp. Sequencing of these two isolates produce 781 bp for BAC 23 and 784 bp for BAC26 with 50-60% G+C content. Both strains are used for *Insilico* study in order to obtain highest similarity using online web server nucleotide blastn based on the BLAST alignment and set as rRNA/ITS database for 16S ribosomal RNA sequences. These isolates were found belong to genus *Bacillus clausii* (DSM 8716) and *Bacillus haynesii* (NRRL B-41327) for BAC 23 and BAC26 with 98% and 97% similarity respectively and were subjected to multiple sequence alignment by using online server clustal Khalil (2011) identified *Bacillus* sp., *Brevibacillus borstelenesis* by using molecular characterization from the hot springs in Saudi Arabia and reported that these species are potential producers of thermostable lipases and can be active at temperature range of 55°C-65°C with alkaline pH of about 8.5-9.5.

Determination of the molecular community by phylogenetic analysis of 16S rRNA gene sequence indicate that all the phylotypes are associated with *Firmicutes* retrieved from enrichment culture. Phylogenetic tree was constructed using maximum likelihood parsimony (Felstein,1996) with computer programme MEGAX 10.1. The phylogenetic analysis of the 16S rRNA gene sequences of these two thermophilic isolates reveals the 98% homology *Bacillus clausii* and *Bacillus haynesii* (Figs.1&2). It was revealed that *Bacillus* isolates BAC23 be put under the species *Calusii* with 98% homology and about 97% homologues of rhizospharae. While *Bacillus* isolate BAC26 reveals 96% homology with

Table. 3 Bacterial isolates growth at various temperature range.

Strain	Temperature in °C					
	45	50	55	60	65	70
CAP1	+	-	+	+	-	-
CAP2	+	-	+	+	-	-
CAP3	-	+	+	+	+	+
CAP4	+	+	+	+	+	+
BAC22	-	+	+	+	-	-
BAC23	+	+	+	+	+	+
BAC24	-	+	+	+	+	+
BAC25	+	+	+	+	+	-
BAC26	-	+	+	+	+	+
BAC27	+	+	+	+	+	-

+= Presence of activity, -= Absence of activity.

Table. 4 Bacterial isolates growth at various pH range.

Strain	pH					
	6	8	9	10	12	14
CAP1	-	-	+	+	-	-
CAP2	-	-	+	+	-	-
CAP3	-	+	+	+	+	-
CAP4	-	+	+	+	-	-
BAC22	-	+	+	+	-	-
BAC23	+	+	+	+	+	-
BAC24	-	+	+	+	-	-
BAC25	+	+	+	+	-	-
BAC26	-	+	+	+	-	-
BAC27	+	+	+	+	-	-

+= Presence of activity, -= Absence of activity.

Table. 5 Biochemical characterization and lipase activity of bacterial isolates.

Strain	Biochemical test					
	Catalase	Oxidase	Citrate	Urease	Sugar	Lipase
CAP1	+	+	+	+	+	+
CAP2	+	+	+	+	+	+
CAP3	+	+	+	+	+	+
CAP4	+	-	+	-	+	-
BAC22	+	-	+	-	+	+
BAC23	+	+	+	+	+	+
BAC24	+	-	-	+	+	+
BAC25	+	+	-	-	+	+
BAC26	+	+	+	+	+	+
BAC27	+	+	+	+	+	+

+= Presence of activity, -= Absence of activity.

Fig. 1 Phylogenetic relationship of isolate BAC23 showing with the analyzed sequences by maximum likelihood method based on 16S rRNA gene sequence

Fig. 2 Phylogenetic relationship of isolate BAC26 showing with the analysed sequences by maximum likelihood method based on 16S rRNA gene sequence

Haynesii and 94-95% homology with other species of *Bacillus sonorensis*, *B.aerius*, *B.licheniformis* of different strain. Ugras (2017) in his study on the hot spring area of Hayran thermal springs in Giresun identified *Bacillus Licheniformis* and *Bacillus subtilis* on the basis of biochemical and molecular characterization by 16S rDNA sequencing and shows the maximum activity of the lipases enzyme is at temperature of 90°C at 9 pH.

The aim of this study was to isolate thermophilic bacteria from the hot springs of Madhya Pradesh and has potential to produce thermostable enzyme lipase. We have isolated

10 thermophilic bacteria and screen for the potential of extracellular lipase production. Further isolates were used for the biochemical and morphological characterization. Two isolates having the highest turbid halo zone of clearance around the inocula is used for the molecular characterization. Analysis of molecular characterization reveals the isolates belong to *Bacillus clausii* and *Bacillus haynesii*, that can be able to grow at the temperature of 60°C and can tolerate high pH of about 9.0. Characteristics of both the strains such as thermo stability, alkalophilicity and potential of producing thermostable lipase could be used as a promising candidate in industries which uses lipases.

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