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

SHREYANSH PARSAI, KAMLESH CHOURE, ARPIT SRIVASTAVA, PIYUSH KANT RAI, VIVEK AGNIHOTRI and SOURABH SINGH GOUR

Department of Biotechnology, A.K.S. University, Satna (M.P.)

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, 2000), Kamchatka in Russia, Iceland (Reigstad et al., 2010), Indonesia (Aditawati 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 (Sutyanarayana 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 (Demirjian, 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 de- natured 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, amidas 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 thermocovorans (Lee et al., 1999 and Markossian et al., 2000), Bacillus stearothemophilus (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 Anhoni Pachmari (CAP) Hoshangabad and Badi anhoni in Chhindwada 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 flasks containing a temperature more than that of source, to maintain the temperature as well as avoidance of damage of samples during transporta- tion and used for the investigation of physical parameters (Anderson et al., 2006).

Keywords:
- Bacillus haynesii
- Bacillus clausii
- Hot springs
- Thermophilic bacteria
- Thermostable lipase

*Corresponding author (email: kamlesh.chaure@gmail.com)
Received 28.03.2020
Accepted 27.04.2020
Isolation of Bacteria and conventional methodology for identification: The sample were serially diluted up to 10−5 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 temperatures, 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., 1997). 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 et al. (1989). The pH of the media was adjusted to alkaline at 8.0. The final reaction mixture is 50 µl for 16S PCR amplification i.e. 27F and 1492R (Genombio Technologies Pvt. Ltd., Pune). The sequence obtained after sequencing were submitted for purification by PCR cleanup Kit and the method is followed as described by the manufacturer (Eurofins Genomics Technologies Pvt. Ltd., Pune). The sequence is than 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
temperature of surface water is different and it gradually in-
creases 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 stain-
ing 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 utili-
zation 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 fer-
menting different sugars such as maltose, dextrose, raffinose,
arabinose, ribose, glucose, sucrose and fructose while pro-
ducing the acids and gas as the end products. Genotypic and
phenotypic identification of the thermophilic strains was car-
ried 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 (Dereckova et al.,2008) and China (Lau et al.,2009).

16S rDNA amplification by using the universal for-
ward and reverse primer i.e. 1492R and 27F of these two iso-
lates 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 \textit{Insitilo} study in order to obtain high-
est 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 be-
long to genus \textit{Bacillus clausii} (DSM 8716) and \textit{Bacillus
haynesii} (NRRL B-41327) for BAC 23 and BAC26 with 98%
and 97% similarity respectively and were subjected to multi-
ple sequence alignment by using online server clustal Khalil
(2011) identified \textit{Bacillus sp., Brevibacillus horstelenses} by
using molecular characterization from the hot springs in
Saudi Arabia and reported that these species are potential pro-
ducers of thermostable lipases and can be active at tempera-
ture range of 55°C-65°C with alkaline pH of about 8.5-9.5.

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

\begin{table}
\centering
\caption{Bacterial isolates growth at various temperature range.}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{Temperature in °C} & \textbf{45} & \textbf{50} & \textbf{55} & \textbf{60} & \textbf{65} & \textbf{70} \\
\hline
\textbf{CAP1} & + & - & + & + & - & - & - \\
\textbf{CAP2} & + & - & + & + & + & + & + \\
\textbf{CAP3} & + & + & + & + & + & + & + \\
\textbf{CAP4} & + & + & + & + & + & + & + \\
\textbf{BAC22} & - & + & + & + & + & + & + \\
\textbf{BAC23} & + & + & + & + & + & + & + \\
\textbf{BAC24} & + & + & + & + & + & + & + \\
\textbf{BAC25} & + & + & + & + & + & + & + \\
\textbf{BAC26} & + & + & + & + & + & + & + \\
\textbf{BAC27} & + & + & + & + & + & + & + \\
\hline
\end{tabular}
\begin{flushright}
+ = Presence of activity, - = Absence of activity.
\end{flushright}
\end{table}

\begin{table}
\centering
\caption{Bacterial isolates growth at various pH range.}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{pH} & \textbf{6} & \textbf{8} & \textbf{9} & \textbf{10} & \textbf{12} & \textbf{14} \\
\hline
\textbf{CAP1} & - & - & + & + & + & + & + \\
\textbf{CAP2} & - & - & + & + & + & + & + \\
\textbf{CAP3} & + & + & + & + & + & + & + \\
\textbf{CAP4} & + & + & + & + & + & + & + \\
\textbf{BAC22} & - & + & + & + & + & + & + \\
\textbf{BAC23} & + & + & + & + & + & + & + \\
\textbf{BAC24} & + & + & + & + & + & + & + \\
\textbf{BAC25} & + & + & + & + & + & + & + \\
\textbf{BAC26} & + & + & + & + & + & + & + \\
\textbf{BAC27} & + & + & + & + & + & + & + \\
\hline
\end{tabular}
\begin{flushright}
+ = Presence of activity, - = Absence of activity.
\end{flushright}
\end{table}

\begin{table}
\centering
\caption{Biochemical characterization and lipase acivity of
bacterial isolates.}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{Biochemical test} & \textbf{Catalse} & \textbf{Oxidase} & \textbf{Citrate} & \textbf{Urease} & \textbf{Sugar} & \textbf{Lipase} \\
\hline
\textbf{CAP1} & + & + & + & + & + & + & + \\
\textbf{CAP2} & + & + & + & + & + & + & + \\
\textbf{CAP3} & + & + & + & + & + & + & + \\
\textbf{CAP4} & + & + & + & + & + & + & + \\
\textbf{BAC22} & - & + & - & + & + & + & + \\
\textbf{BAC23} & + & + & + & + & + & + & + \\
\textbf{BAC24} & + & + & + & + & + & + & + \\
\textbf{BAC25} & + & + & + & + & + & + & + \\
\textbf{BAC26} & + & + & + & + & + & + & + \\
\textbf{BAC27} & + & + & + & + & + & + & + \\
\hline
\end{tabular}
\begin{flushright}
+ = Presence of activity, - = Absence of activity.
\end{flushright}
\end{table}
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 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 thermostability, alkaliophilicity and potential of producing thermostable lipase could be used as a promising candidate in industries which uses lipases.

REFERENCES

THERMOPHILIC BACTERIA ISOLATION AND CHARACTERIZATION


