



RESEARCH ARTICLE

MOLECULAR BIOLOGY

ISOLATION AND IDENTIFICATION OF NEW LIPOLYTIC THERMOPHILIC BACTERIA FROM AN INDIAN HOT SPRING**BISHT, S.P.S* AND AMRITA KUMARI PANDA**Department of Biotechnology, Roland Institute of Pharmaceutical Sciences
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**BISHT, S.P.S**Department of Biotechnology, Roland Institute of Pharmaceutical Sciences
Berhampur-760010, Orissa, India**ABSTRACT**

Thermophilic lipase producing bacteria have been isolated and characterized from an Indian hot spring, isolates named as AK-P1, AK-P2 and AK-P3 respectively. The strain AK-P2 was first of its kind with reference to its lipase producing potential. The phylogenetic analysis of these strains using 16S rDNA sequence data revealed that the strain AK-P1 had highest homology (100%) with *Acinetobacter* sp. 01B0;, AK-P2 had highest homology (99.9%) with *Brevibacillus borstelensis*; AR9 and AK-P3 have shown 99.2 % similarities with *Porphyrobacter cryptus* (T); ALC-2. Maximal lipase production by AK-P2 was observed at the end of the stationary phase (36 h). The optimum temperature and pH for this crude enzyme activity was 60°C and 10.0 respectively. Enzyme extracted from AK-P2 exhibited a Km 0.052mM and Vmax 0.645 μM/min/ml with p-nitrophenyl laurate as a substrate.



KEYWORDS

Lipases, thermophiles, hot spring, 16S rDNA

INTRODUCTION

Lipases (triacylglycerol acylhydrolases) belong to the class of serine hydrolases and don't require any co-factor. The natural substrate of lipases are triacylglycerols, having very low solubility in water. Under natural conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved. Lipases catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol and fatty acids. Lipases have attracted much attention during the last decade due to the diversity of their applications. The enantioselective and regioselective nature of lipases have been utilized for the resolution of chiral drugs, fat modification, synthesis of cocoa butter constituents, bio-fuels, synthesis of personal care products and flavor enhancers (Gerhartz, W. 1990).

Lipases are ubiquitous enzymes (Brockerhoff 1974; Borgstrom 1984; Desnuelle 1986; Wooley 1994) which are found in animals, plants (Huang 1993; Mukherjee et al. 1994), fungi (Iwai et al. 1984) and bacteria (Brune & Goetz 1992; Jaeger et al. 1994; Jaeger & Reetz 1998).

Thermostable enzymes are of great demand and concern due to their high stability, which makes them attractive for several industrial processes and applications. It is not only their thermostability but also their greater stability under other extreme conditions such as high pH or low water concentration. The use of higher temperature in industrial processes reduces the risk of microbial contamination caused by mesophiles and simultaneously thermozymes are much more useful in the processing of lower viscosity fluids, as at higher temperature viscosity is usually reduced, that lowers shear

consequently, the costs of pumping, filtration, and centrifugation. Heat and mass transfer rates are also improved at high temperature, further at higher temperatures diffusion rates are higher and mass transfer is less limiting (Peek et al. 1992). In addition, more substrate will dissolve at higher temperatures, which can shift the equilibrium to a higher product yield in comparison to other enzymes of low temperature stability.

Hot springs are the emerged water bodies produced by geo-thermally heated groundwater (Kauze, T., et al., 2006). Hot springs are scattered all over the globe, on every continent and even under the sea and oceans. Taptapani is one of the hot springs in India which has been poorly explored from the microbiological point of view. The aim of this study was to isolate and identify new lipase producing bacteria from this Indian hot spring.

MATERIAL AND METHODS

Water samples were collected in sterile containers from the hot spring, Taptapani (19° 30' 0N latitude, 84° 24' 0E longitude and 1053 feet altitude) located in Ganjam District of the state of Orissa, India. The temperature of the water was between 50 °C to 55 °C and the pH of the water was around 7.5 at the site of release. The collected water samples were serially diluted up to 10⁻⁴ and spreaded on thermus agar plates and incubated at 50 °C.

Screening for lipase producing bacteria

Screening of lipase producers was carried out using tributyrin agar plates according to Lawrence 1967. Each culture was streaked onto the tributyrin agar plate and incubated at



50°C for 2 days. The lipase producing bacteria were identified by the presence of clear hydrolytic zones.

DNA preparation and PCR amplification

Genomic DNA was extracted from the isolates using Chromous kit (RKT09). Each genomic DNA used as template was amplified by PCR with the aid of 16SrDNA primers (16S Forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3', 16S Reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3') and thermal cycler was programmed as denaturation at 94°C for 5 min followed by subsequent 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 2 min with the final extension at 72°C for 5 min. The presence of PCR products was determined by electrophoresis of 10 µl of the reaction product in 1% agarose gel.

16S rRNA sequencing and data analysis

Sequencing analysis was performed on a 1500 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The three 16SrRNA sequences were aligned and compared with other 16SrRNA genes in the GenBank by using the NCBI Basic Local alignment search tools BLAST n program (<http://www.ncbi.nlm.nih.gov/BLAST>). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees created using Weighbor (Weighted Neighbor Joining: A Likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000. The 16SrRNA gene sequences have been deposited to Genbank using BankIt submission tool and has been assigned with NCBI (National Centre for Biotechnology information) accession numbers (HM359118, HM359119, HM359120).

Enzyme Production

The identified bacterial lipase producers were cultivated in production medium (50 ml) in

triplicate (one control and two replicates/sample). After incubation at 50°C for 36h the cultures were centrifuged at 8000 rpm at 4°C for 15 min. The crude lipase solution was obtained by filtering through a 0.22µm pore-size membrane filter and was used as the source of crude lipase enzyme.

Enzyme Assay

Lipolytic activity was measured according to the method of Sigurgisladottir et al 1993 with slight modification using pNP-laurate as substrate. The enzyme activity was determined at 410 nm. Each reading was taken in triplicate and the activity was calculated as amount of enzyme required liberating one micromole equivalent fatty acid per mL/min.

Protein Determination:

Protein concentration was determined according to the Biuret method. Two ml of Biuret reagent was added to 50 µl of sample and the extinction was measured after 10 min at 560 nm. Different concentrations of bovine serum albumin (BSA) were used as a protein standard: 10, 20, 40, 60, 80, and 100 µg/ ml distilled water.

Effect of Temperature and pH on the Activity of Lipase:

The effect of temperature on catalytic activity of lipases was determined by measuring the enzyme activity at temperature range from 50 °C - 80 °C under the standard assay conditions. The effect of pH on enzyme activity was determined by measuring the enzyme activity at varying pH values ranging from 5 to 11 at 60 °C using suitable buffers, 50 mM sodium acetate (pH 5.0 and 6.0), 50 mM sodium phosphate (pH 7.0 and 8.0), 50 mM glycine-NaOH buffer (pH 9.0 and 10.0), sodium bicarbonate - NaOH buffer (pH 11.0) respectively.

Enzyme kinetic study

The effect of substrate pNP-laurate concentration (0.05–0.4 mM) on the reaction

rate was assayed by using standard enzyme assay. The Michaelis–Menten constant (Km) and maximum velocity for the reaction (Vmax) with pNP laurate as substrate were calculated by Lineweaver Burk plot.

RESULTS AND DISCUSSION

Thermophiles have molecular modifications at cellular and subcellular level to survive at high

temperature in the hot water bodies. Enzymes from thermophiles and hyperthermophiles are thermostable and display irreversible protein denaturation at high temperatures. Thermozyymes are of great interest for industrial applications (Cowan, 1996). Isolation of lipase producers from water samples of Taptapani hot water spring resulted in isolation of three different strains showing clear hydrolytic zone on Tributyrin agar (Fig. 1).

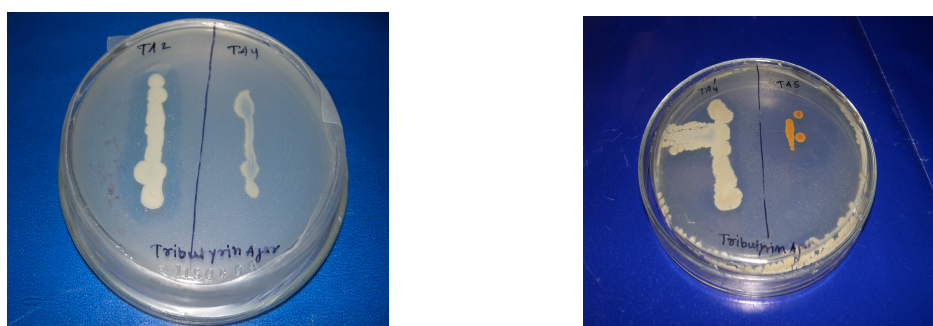


Fig. 1

Isolation of lipase producing thermophilic bacteria. Clear zone indicates the hydrolysis of Tributyrin as a result of lipase production.

Identification of the lipase Producing Thermophilic bacteria:

Three strains showing clear hydrolytic zone designated as AK-P1, AK-P2 and AK-P3. The phylogenetic analysis of these strains using its 16S rDNA sequence shows that strain AK-P1 had highest homology (100%) with *Acinetobacter* sp. 01B0; AK-P2 had highest homology (99.9%) with *Brevibacillus borstelensis*; AR9 and AK-P3 showed 99.2 % similarities with *Porphyrobacter cryptus* (T); ALC-2. To best of the knowledge reports are scanty about the production of lipase enzymes by *Brevibacillus borstelensis*. Though there are reports on production of lipases from thermophilic bacteria, *Bacillus thermoleoverans* (Lee *et al.*, 1999; Lee *et al.*, 2001; Markossian *et al.*, 2000), *Bacillus stearotherophilus* (Sinchaikul *et al.*, 2001), thermoacidophilic bacteria, *Bacillus acidocaldarius* (D'Auria *et al.*, 2000), and alkaliphilic bacteria, *Bacillus* sp.

strain A 30-1 (Wang *et al.*, 1995), *Bacillus* sp. J33 (Nawani and Kaur 2000).

Production of Lipases by *Brevibacillus borstelensis* AK-P2

The production of enzyme was carried out in the production medium as per Kumar & Valsa 2007. The production of lipase was detected after 12 h of cultivation and increased during growth and maximum level (40.0 U/ml) at the end of stationary phase, 36h of growth. The production remained constant during prolonged cultivation up to 72h. The lipase was completely secreted into the culture medium. The specific activity of the enzyme was found to be 150 U/mg Protein in cell-free supernatant.

Effect of Temperature on the enzyme activity



The effect of temperature on the activity of crude lipase was determined at various temperature ranging from 50 °C to 80 °C at pH 7.0. The enzyme has shown a good activity between 60°C to 65°C with a maximum at 60°C (Fig. 2). The optimum temperature for lipase activity produced by other thermophilic *Bacillus stearothermophilus*, *Bacillus thermocatenletus* and *Bacillus thermoleovorans* ID-1 were 68°C,

60°C-70°C and 70°C-75°C respectively (Haki and Rakshit, 2003). There are very few reports of thermostable lipases from archaeal origin. Phospholipase A2 which was secreted from the archaea *Pyrococcus horikoshii* (Yan et al., 2000) was involved in crude oil refining. This enzyme was found to optimally react at 95 °C and pH of 7.0.

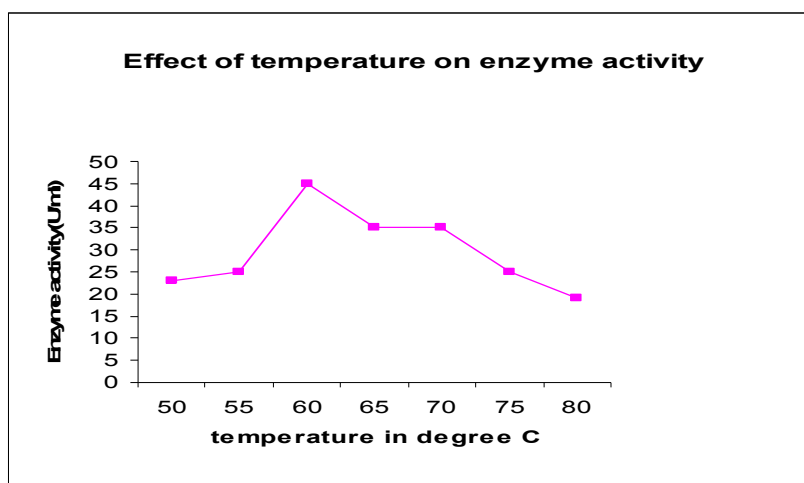


Fig. 2

Effect of pH on the lipase activity

The effect of the pH on the crude lipase activity of *Brevibacillus* AK-P2 was examined at various pHs ranging from 5.0 to 11.0 as shown in (Fig.3). The enzyme has a broad range of enzyme activity (pH 8-11) with optimal pH at 10

which is close to the optimum pH value of most *Bacillus* lipases (Haki and Rakshit, 2003) and almost same to extracellular lipase of *Bacillus subtilis* 168 (Lesuisse E et al., 1993). The enzyme had retained about 66% activity even at pH11.

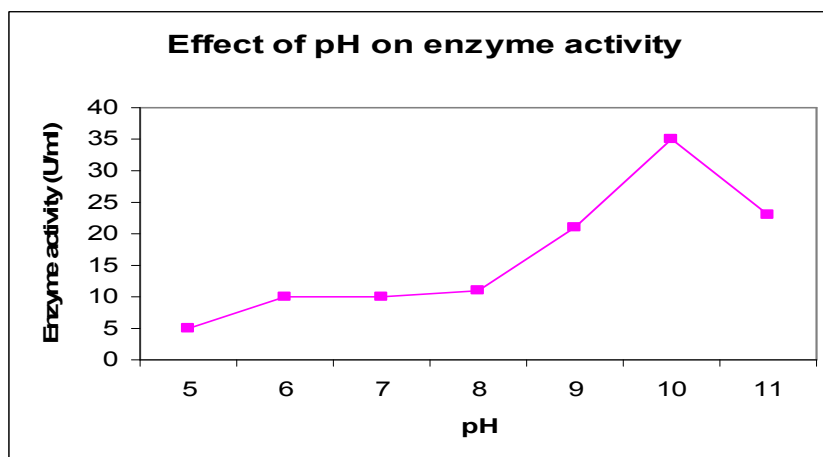


Fig. 3



Effect of substrate concentration on the lipase activity

The Michaelis-Menten constant and V_{max} was calculated to be 0.052mM and 0.645 μ M/min/ml for lipase secreted by the strain AK-P2.

CONCLUSION

There are reports that *Brevibacillus borstelensis* strain MH301 produces hydantoinase and carbamoylase which are key biocatalysts for the production of optically pure amino acids from dl-5-substituted hydantoins (Yanzhen Me, 2009). In the present study the *Brevibacillus* AK-P2 have shown promising results for the production of

lipase and this indicates the commercial utility of this strain is of diverse in nature. The investigations clearly indicate that the Taptapani hot spring of South-eastern India, is a rich source of many thermophilic bacteria and need to be explored for the industrially important enzymes by further studies on the microbiological aspects and meta-genomics to explore the uncultivated organisms.

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