Research Article

Biochemical Characterization and 16S rRNA Sequencing of Few Lipase-Producing Thermophilic Bacteria from Taptapani Hot Water Spring, Orissa, India

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Three lipase-producing thermophilic bacteria (AK-P1, AK-P2, and AK-P3) were isolated from the Taptapani hot water spring in Orissa, India. The crude extra cellular lipases from cell-free culture supernatant were reacted in an olive oil mixture, and their lipolytic activities were compared. Identification of the bacteria was carried out using biochemical tests, 16SrRNA sequencing and sequences submitted to NCBI GenBank. Strain AK-P3, exhibited the highest lipolytic activity of 5.5 U/mL was identified as *Porphyrobacter* sp. The lipolytic activities of strains AK-P1 and AK-P 2 were 4.5 U/mL and 3.5 U/mL, respectively. Strains AK-P1 and AK-P2 were identified as *Acinetobacter* sp. and *Brevibacillus* spp. The GenBank accession numbers of the 16S rRNA gene sequences determined in this study for the strains AK-P1, AK-P2, and AK-P3 are HM359120, HM359119, and HM359118, respectively.

1. Introduction

Thermophiles are a group of microbes adapted to thrive in ecological niches such as deep-sea hydrothermal vents, hot springs, and solfataric fields. Thermophilic microorganisms are found to be potential and good alternative source of thermostable enzymes [1, 2]. The investigation and application of thermostable enzymes from thermophilic microorganisms have received a lot of attention in industries ranging from food industry to waste management industries.

Thermophiles are of interest to enhance the thermostability of industrial enzymes. The modern concept of the nature of the thermostability of the enzymes from the thermophilic microorganisms can be reduced to two basic ideas. Firstly, the enhanced thermal stability of enzymes in thermophilic cells could be due to their molecular structure being different from that of their counter part mesophilic proteins. Secondly, the increased stability of the enzymes could be due to their supramolecular association with other components of the cell (lipids, polysaccharides, or proteins). In order to survive at high temperatures,

thermophilic prokaryotes (Archaea and Eubacteria) adopt different strategies like GC-rich codons, the ratio of charged amino acids compared to uncharged amino acids, ionic interactions, amino acid preferences and their distribution, posttranslational modifications, and solute accumulation [3]. Most of the industrial microbial lipases derived from fungi and mesophilic bacteria [4]. Bacteria produce different classes of lipolytic enzyme, including carboxylesterases (EC 3.1.1.1), which hydrolyse small ester containing molecules at least partly soluble in water, true lipases (EC 3.1.1.3), which display maximal activity towards water insoluble long-chain triglycerides, and various types of phospholipase [5].

More than 50 lipases have been identified, purified, and characterized to date which originate from natural sources such as animals, plants, and microorganisms (native or genetically engineered). A large number of beneficial thermophiles which produced lipases with good thermal stabilities have been found in diverse habitats [6, 7]. Their thermal stabilities particularly in the absence of sufficient amounts of water increase their popularity [8].

FIGURE 1: Lipolytic activities of lipase-producing bacteria isolated from Taptapani hot water spring.

TABLE 1: Colony morphology of isolates.

Strain	Colony size (mm)	Colony morphology	Gram reaction	Cellular morphology
AK-P1	1	Circular, entire edge, Smooth surface	–ve	Coccoid
AK-P2	2	Irregular and spreading Raised margin	+ve	Rods
AK-P3	1	Circular, entire edge, Smooth surface, Orange pigmented	–ve	Ovoid to short rods

The enormous potential of microbial lipases arises from the facts that they are (1) quite stable and active in organic solvents, (2) do not require cofactors, (3) exhibit a high degree of enantio- and regioselectivity, and (4) possess a wide range of substrate specificity for the conversion of various unnatural substrates [9, 10]. Therefore screening and identification of thermostable lipolytic bacteria will act as a meaningful addition to the database on bacterial lipase research.

2. Material and Methods

2.1. Conventional Identification Tests. All isolates were initially evaluated by conventional tests, that is, Gram stain, growth, and morphometric characteristics on thermus agar ATCC 697 medium, growth at 42°C, catalase, oxidase, motility, indole production, gelatin liquefaction, oxidative fermentative carbohydrate utilization, decarboxylation of lysine, and urease activity. Additional tests included phenylalanine deamination, nitrate reduction, citrate utilization, H₂S production, hydrolysis of starch, and growth in the presence of 6.5% sodium chloride.

2.2. Culture Maintenance. The cultures AK-P1, AK-P2, and AK-P3 isolated from Taptapani hot water spring (19° 30' 0N lat and 84° 24' 0E long) of Ganjam District, Orissa, India were maintained on thermus agar and stored at 4°C.

2.3. Screening for Lipase-Producing Bacteria. Screening of lipase producers was carried out using tributyrin agar plate according to Lawrence 1967. Each culture was streaked onto the tributyrin agar plate and incubated at 48°C for 2 days.

The lipase producing bacteria were identified by the presence of surrounding clear hydrolytic zones.

2.4. Lipolytic Assay of Lipase Producers. The identified bacterial lipase producers were cultivated in production medium (50 mL) in triplicate (one control and two replicates/sample). After incubation at 48°C for 36 h the cultures were centrifuged at 8000 rpm at 4°C for 15 min. The crude lipase solution was obtained by filtering through a millipore 0.22 µm filter membrane and lipolytic activity was measured by universal titrimetric method [11, 12]. 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.

2.5. DNA Preparation and PCR Amplification. Genomic DNA was extracted from the isolates using Chromous Genomic DNA isolation kit (RKT09). Each genomic DNA used as template was amplified by PCR with the aid of 16S rDNA primers (16S Forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3' 16S Reverse Primer: 5'-CGYTAMCTTWTACGRCT-3') with the programme consisted of denaturation at 94°C for 5 min and subsequent 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min followed by 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 a 1% agarose gel.

2.6. 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

FIGURE 2: Thermal stability of crude lipase produced by AK-P3.

FIGURE 3: Phylogenetic position based on 16S rRNA gene sequence analysis of strain AK-P1.

FIGURE 4: Phylogenetic position based on 16S rRNA gene sequence analysis of strain AK-P2.

FIGURE 5: Phylogenetic position based on 16S rRNA gene sequence analysis of strain AK-P3.

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 accession numbers.

3. Results and Discussion

3.1. Comparative Lipolytic Activity of Lipase Producers. Lipolytic activities of three lipase-producing bacteria are shown in Figure 1. All the three cultures showed good lipolytic activities. Strain AK-P3 exhibited the highest lipolytic activity of 5.5 U/mL followed by strains AK-P1 and AK-P2 with an activity of 4.5 U/mL and 3.5 U/mL, respectively, (Figure 1).

3.2. Thermostability of Crude Lipase of AK-P3. The enzyme has shown very good stability up to 65°C for 20 min. Further it decreased considerably at 70°C followed by a sharp decline in enzyme activity after 70°C. The exposure of the enzyme at 75°C for 20 min decreases the enzyme activity to 26% of the total activity (Figure 2).

3.3. Colony Morphology, Gram Reaction. See Tables 1 and 2.

3.4. 16S rRNA Sequence Analysis. More than 1400 bp of the 16S rRNA genes of strains AK-P1, AK-P2, and AK-P3 were sequenced. Analysis of the 16S rRNA sequences confirmed

TABLE 2: Results of biochemical tests.

Biochemical tests	Results		
	AK-P1	AK-P2	AK-P3
Carbohydrate fermentation			
Glucose	+ve	–ve	–ve
Adonitol	–ve	–ve	+ve, weak
Lactose	–ve	–ve	–ve
Arabinose	–ve	–ve	+ve, weak
Sorbitol	–ve	–ve	–ve
Citrate utilization	–ve	–ve	–ve
Indole	–ve	–ve	+ve
Motility	–ve	–ve	+ve
Nitrate reduction	+ve	+ve	–ve
Lysine utilization	–ve	–ve	–ve
Ornithine utilization	–ve	–ve	–ve
Phenylalanine deamination	–ve	–ve	–ve
Urease production	–ve	–ve	–ve
H ₂ S production	–ve	–ve	+ve
Catalase	+ve	+ve	+ve, weak
Oxidase	–ve	–ve	–ve
Methyl Red	–ve	–ve	–ve
Voges-Proskauer	–ve	+ve	–ve
Starch hydrolysis	–ve	+ve	+ve
Gelatin liquefaction	–ve	–ve	–ve

+ Positive result; – Negative result.

the strain AK-P1 was most similar to *Acinetobacter* sp. 01B0 (Figure 3). The strain AK-P2 was found to be most similar to *Brevibacillus* sp. B2 (Figure 4) and the strain AK-P3 shares closest homology with *Porphyrobacter cryptus* (T) ALC-2 (Figure 5).

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