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# High level expression and characterization of a novel thermostable, organic solvent tolerant, 1,3-regioselective lipase from *Geobacillus* sp. strain ARM

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ABSTRACT

The mature ARM lipase gene was cloned into the pTrcHis expression vector and over-expressed in *Escherichia coli* TOP10 host. The optimum lipase expression was obtained after 18 h post induction incubation with 1.0 mM IPTG, where the lipase activity was approximately 1623-fold higher than wild type. A rapid, high efficient, one-step purification of the His-tagged recombinant lipase was achieved using immobilized metal affinity chromatography with 63.2% recovery and purification factor of 14.6. The purified lipase was characterized as a high active (7092 U $mg^{-1}$), serine-hydrolase, thermostable, organic solvent tolerant, 1,3-specific lipase with a molecular weight of about 44 kDa. The enzyme was a monomer with disulfide bond(s) in its structure, but was not a metalloenzyme. ARM lipase was active in a broad range of temperature and pH with optimum lipolytic activity at pH 8.0 and 65 °C. The enzyme retained 50% residual activity at pH 6.0–7.0, 50 °C for more than 150 min.



## 1. Introduction

Nowadays, lipases (EC 3.1.1.3) have developed into the most widely used class of enzymes in biotechnology and synthetic organic chemistry because of their ability to catalyze a broad range of novel and important reactions in aqueous and nonaqueous media. They are used to hydrolyze ester bonds of a variety of nonpolar substrates at high activity, regioselectivity, and stereoselectivity. Moreover, they are able to catalyze wide range of ester and amide bonds formation in nonpolar solvents. The reaction can be designed and optimized to produce a variety of novel products by changing substrate structure, solvents, additives, water activity, pressure, temperature, and the biocatalyst itself. The lipase used in each application is selected based on its activity, stability and selectivity (Hasan et al., 2006; Dizge et al., 2009).

Ironically, many of the industrial processes in which lipases would offer clear sustainable advantages do not operate under mild conditions. Finding enzymes that work optimally in harsh conditions and not losing their activities is a tall order. Thermostable enzymes in comparison to mesophilic enzymes display higher resistance to chemical denaturants and withstand higher substrate concentrations. They catalyze the reactions at higher process rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates at high temperature. The reactions result in higher process yield due to increased solubility of substrates and products and favorable equilibrium displacement in endothermic reactions (Vieille and Zeikus, 2001).

Furthermore, lipases that can function as biocatalysts in nearly anhydrous organic solvents offering new possibilities such as shifting of the thermodynamic equilibria in favor of synthesis, enabling the use of hydrophobic substrates, controlling or modifying enzyme selectivity by solvent engineering, suppressing undesirable water dependent side reactions, improving thermal stability of the enzymes and decreasing microbial contamination. Exploiting such advantages is often limited by the low stability and/or activity of biocatalysts in these systems. Since most lipases easily denature in organic solvents and therefore lose their catalytic activities, it is necessary to find lipases that are stable in nonaqueous systems (Ogino, 2008; Xu et al., 2010).

Substrate specificity of lipases is often very important to their applications for analytical and industrial purposes. Position-specific lipases (particularly 1,3-specifics) are the key point for specialty structured lipids production. Structured lipids are referred particular molecular species of triacylglycerols (TAGs) with defined molecular structure. Molecular structure of TAGs (i.e. composition and positions of the fatty acids in the molecule) influences their functionalities including metabolic fate in organisms (i.e. digestion and absorption) as well as their physical characteristics (e.g. melting points and crystallinity). Consequently, it is possible to control the behavior of TAGs by designing structured lipids with particular chemical structure, thereby improving the nutritional

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and pharmaceutical properties of TAGs (Öztürk et al., 2010; Akoh, 2002). In addition, 1,3-specific lipases are important catalyst in some pharmaceuticals synthesis, flavor making, biodiesel production, and cosmetics and perfumery industries (Hasan et al., 2006; Li et al., 2010).

Although many lipases have already been described, the search for new lipases with improved stability and selectivity is still an important field of research. Previously we isolated a thermophilic lipolytic bacterium from cooking oil contaminated soil in Selangor, Malaysia. It was identified as *Geobacillus* sp. strain ARM via 16S rDNA analysis (GenBank: EF025325) and deposited in DSMZ, Germany (DSM 21496) and NCIMB, UK (NCIMB 41583). In the present investigation, the thermostable lipase gene from strain ARM was cloned and overexpressed in *Escherichia coli* TOP10. Consequently, the purified recombinant lipase was characterized.

## 2. Methods

### 2.1. Strains, plasmids and culture conditions

*Geobacillus* sp. strain ARM, a thermophilic lipolytic bacterium, isolated from cooking oil contaminated soil was grown in nutrient broth at 60 °C. *E. coli* TOP10 was grown in Luria–Bertani (LB) medium at 37 °C and used as a transformation and expression host. Competent cells of *E. coli* TOP10 were prepared by using a conventional $CaCl_2$ method (Sambrook et al., 1989). pTrcHis TOPO vector (Invitrogen, USA) was used for cloning, sequencing and expression of the lipase gene.

### 2.2. DNA preparation

Genomic DNA from *Geobacillus* sp. strain ARM was extracted according to the method described by Sambrook et al. (1989). Plasmid DNA was isolated with a QIAprep Spin Miniprep Kit (QIAGEN, Germany) according to the manufacturer's instructions. The PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany) according to the manufacturer's instructions.

### 2.3. Amplification, sequencing and analysis of the thermostable lipase gene

A pair of degenerate oligonucleotide primers was employed based on significant gene sequences sharing and structural similarities of *Bacillus* spp. thermostable lipases (Leow et al., 2004) as follows: CJH-F1: 5′-AGS RTG ATG AAA KGC TGY GGG CTK ATG K-3′ and CJH-R1: 5′-KYW TTA AGG CYG CAA RCT CGC CA-3′. The following thermal cycle was used to amplify the thermostable lipase gene by *Taq* DNA polymerase using genomic DNA as template. One cycle of pre-denaturation: 94 °C for 4 min; 30 cycles of: 94 °C for 1 min (denaturation), 60 °C for 2 min (annealing) and 72 °C for 1.5 min (extension); and one cycle final primer extension: 72 °C for 7 min.

The purified PCR products were sequenced by First BASE Laboratories Sdn. Bhd. (Shah Alam, Selangor, Malaysia). According to the sequencing results, new sets of primers were designed in order to perform primer walking. The homology search was performed with the NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast). The open reading frame was predicted by NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The putative signal peptide and its cleavage sites were predicted by the SignalP V3.0 (http://www.cbs.dtu.dk/services/SignalP).

### 2.4. Cloning of the thermostable lipase gene

The mature lipase gene was amplified with mature ARM lipase primers, forward: 5′-GCGGCTTCGCGAGCCAACGAT-3′ and reverse: 5′-TTAAGGTTGCAAGCTCGCCAACTGC-3′, using genomic DNA as template. Thirty rounds of amplification were performed with the following cycles: One cycle of pre-denaturation: 94 °C for 4 min; 30 cycles of: 94 °C for 1 min (denaturation), 65 °C for 1 min (annealing) and 72 °C for 1.5 min (extension); and one cycle final primer extension: 72 °C for 7 min. The purified PCR product was cloned into the pTrcHis TOPO vector (Invitrogen, USA), according to the manufacturer's instructions and transformed into the *E. coli* TOP10 competent cells using heat shock method (Sambrook et al., 1989). The transformed cells were plated on LB tributryrin agar (1% tributryin in LB agar) containing 50 μg/ml ampicillin. The positive transformed colonies (with clear surrounding zone on ampicillin tributyrin agar plate) were subcultured. The presence of the lipase gene (insert) was confirmed by lipase gene amplification (PCR) and sequencing after the plasmid extraction.

### 2.5. Expression of the thermostable ARM lipase gene

The transformed colony harboring the pTrcHis/ARM recombinant plasmid was cultured in 5 ml LB medium containing 50 μg/ml ampicillin and incubated overnight at 37 °C with 150 rpm shaking. Blue cap bottle (1000 ml) containing 200 ml LB broth medium supplemented with 50 μg/ml ampicillin was inoculated with 1% (v/v) of precultured cells. The culture was grown at 37 °C with 150 rpm shaking and induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at $OD_{600}$ = 0.5. Aliquots (10 ml) were harvested every 3 h for 30 h to assess gene expression. The aliquots were centrifuged (10,000*g*, 10 min, 4 °C) and pellets were resuspended in 10 ml sodium phosphate buffer (50 mM, pH 7.0) prior to sonication (Branson 250 sonifier: 20 cycles of intermittent sonication at 25 W for 30 s). The cell extracts were obtained by centrifugation (12,000*g*, 10 min, 4 °C). The lipase activities of super natants were assayed according to the method described by Kwon and Rhee (1986) using olive oil as substrate. One unit of lipase activity was defined as 1.0 μmol of free fatty acid liberated $min^{-1}$ and reported as U $ml^{-1}$. The expression level was tested as a factor of post induction incubation time as well as inducer (IPTG) concentration (0.1–1.5 mM).

### 2.6. Purification of recombinant ARM lipase

The fusion His-tagged recombinant ARM lipase was purified using the immobilized metal ion affinity chromatography (ProBond™ Purification System (Invitrogen)) following the manufacturer's purification protocol for native conditions. The harvested transformed cells (*E. coli* TOP10/pTrcHis/ARM), after 18 h post induction incubation with 1.0 mM IPTG, were resuspended in 50 ml native binding buffer (50 mM $NaH_2PO_4$, pH 8 containing 0.5 M NaCl and 10 mM imidazole). The cells were lysed by intermittent sonication. The disrupted cells were removed by centrifugation (10,000*g*, 15 min, 4 °C) and the supernatant was filtered through a membrane filter with pore size of 0.22 μm.

The purification was performed at 4 °C using 10 ml purification column filled with 2 ml of ProBond™ Nickel-Chelating Resin. The resin was washed with 10 volumes of distilled water followed by equilibration with 10 volumes of native binding buffer (50 mM $NaH_2PO_4$, pH 8 containing 0.5 M NaCl and 10 mM imidazole). Five ml of the crude enzyme preparation was loaded in the column. The column was washed with 24 volumes of native wash buffer (50 mM $NaH_2PO_4$, pH 8 containing 0.5 M NaCl and 20 mM imidazole). The binding protein was eluted off the column in 1 ml fractions with native elution buffer (50 mM $NaH_2PO_4$, pH 8 containing 0.5 M NaCl and 250 mM imidazole). The homogeneity of the purified enzyme and its unfolded protein size was monitored by SDS–PAGE on a 12% (w/v) polyacrylamide gel (Laemmli, 1970). The marker proteins were protein molecular weight standards in