



Keywords

Cloning,
Expression,
Purification,
Immobilization,
Characterization,
Glutathione S Transferase

Received: July 26, 2015

Revised: August 5, 2015

Accepted: August 7, 2015

Overexpression, Purification, Immobilization and Characterization of Thermophilic Lipase from *Burkholderia pseudomallei*

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Citation

Magdy M. Youssef. Overexpression, Purification, Immobilization and Characterization of Thermophilic Lipase from *Burkholderia pseudomallei*. *American Journal of Microbiology and Biotechnology*. Vol. 2, No. 6, 2015, pp. 82-91.

Abstract

Lipases (EC 3.1.1.3), triacylglycerol hydrolases, are a significant group of biotechnologically applicable enzymes and they find enormous applications in food, detergent and pharmaceutical industries. Lipases are largely produced from microbes, and they play a fundamental role in commercial ventures. A lipase from thermophilic *Burkholderia pseudomallei* bacterial strain was isolated from Saudi Arabian environment. Based on this strain, a lipase gene encoding 399 amino acids was cloned, and expressed in *E. coli* BL21 (DE3). The lipase protein fused with glutathione S-transferase was purified to homogeneity 128.2 fold. SDS- PAGE of the purified enzyme revealed it has *Mr* of 32 kDa. The recombinant lipase was efficiently immobilized in calcium alginate gelatin composites. The optimum temperature for free enzyme highest activity was recorded at 65°C however the immobilized enzyme exhibits the highest activity at 70°C. The immobilized enzyme retains most of its activity and shows high stability for 120 min at 70°C compared to 45 minutes for the native enzyme when incubated at 70°C. The free enzyme has an optimum pH at 7.5 but this optimum pH is shifted to 8.5 for the immobilized enzyme. The free and immobilized lipase catalytic function were enhanced in the presence of 1 mM of Ba⁺⁺, Ca⁺⁺ and Na⁺, but inhibited by 1mM of Ni⁺⁺, Hg⁺⁺, Cu⁺⁺ and Co⁺⁺. The free and the immobilized enzyme activities increased in the presence of 5 mM Fe⁺⁺, Co⁺⁺ or Li⁺.

1. Introduction

The natural production of fatty acids by the hydrolysis of natural oils and fats is a very important component in the economic utilization of these naturally produced renewable raw materials. A significant number of high value products require fatty acids in their manufactures. These include coatings, adhesives, specially lubricating oils, shampoos and other personal care products. Oils and fats are part of a group of compounds known as fatty esters or triglycerides, and their hydrolysis essentially involves reactions with water to produce valuable free fatty acids and glycerol [1]. Enzymatic hydrolysis of triglycerides may be carried out at ambient conditions. Microorganisms produce different classes of lipolytic enzymes, including lipases (EC 3.1.1.3), which specifically catalyze the hydrolysis of ester linkages of long chain triglycerides at an oil-water interface [2]. Lipases are currently attracting enormous attention because they constitute the most important group of biocatalysts for biotechnological applications [3-5]. Most of the

lipases used in industry are microbial enzymes, of both fungal and bacterial origin [6-7]. Bacterial lipases vary widely in enzymatic properties and substrate specificities. Consequently, they are currently receiving much attention because of their potential applications in various industrial processes and biotechnological applications [8]. The industrial demand for highly active preparations of lipolytic enzymes with appropriate specificity and stability to pH, temperature, ionic strength and organic solvents continues to stimulate the search for new enzyme sources [9].

Thermophilic bacteria are an important source of thermostable enzymes (in solvents and detergents), which give these enzymes considerable potential for many biotechnological and engineering applications [10]. Thermophilic lipases show higher thermostability, higher activity at elevated temperature and often show more resistance to chemical denaturation. This makes them ideal tools in industrial and chemical processes where relatively high reaction temperatures and/ or organic solvents are used. The industrial request search for the thermostable continues to stimulate the microorganism's produces of thermostable enzymes. A small number of thermophilic lipase producing bacteria have been described in the last decades [11]. It was reported that a few thermostable lipases have been isolated from thermophiles and hyperthermophiles [13]. The knowledge of thermostable lipolytic enzymes in industrial applications is increasing at a rapid and exciting rate [12].

In this work we describe the cloning and overexpression the lipase enzyme from thermophilic *Burkholderia pseudomallei* by using the molecular biology tools. The overexpressed protein is purified, immobilized and optimal pH; temperature, thermal stability and the effect of different cations are studied for both free and immobilized enzyme.

2. Materials and Methods

Chemicals: All the chemicals, restriction enzymes, DNA polymerase and DNA ligase used were AR or molecular biology grade, and obtained from Sigma, BDH chemicals LTD, Bio-Rad and stored as directed by supplier.

2.1. Bacterial Strains and Plasmid DNA

E. coli AB1157 strain (F⁻ thi-1 thr-1 araC14 leuB6 Δ(gpt proA2)62 lacY1 tsx-33 galK2 λ⁻Rac⁻ hisG4 rfbD1 rps131 str^R kdgK51 xylA5 mlt-1 argE3,[14]), DH5(supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1,[15]), BL21 (DE3) strain (hsd S gal (λc its857 indI Sma7 nin5 lac Uv5 T7geneI,[16]) and pGEX-2T DNA plasmid (GST gene fusion plasmid IPTG inducible ApR) were kindly provided by Dr Picklesley, S. M. (Biomedical Science Department, Bradford University, UK).

2.2. Media and Growth Conditions

Luria Bertani (LB) medium was made by dissolving 10g bacto-tryptone, 5g yeast extract, and 10g NaCl in 1 litre deionised water and sterilised by autoclaving. LB agar plates

were prepared by adding 20g agar to one litre of LB medium. The LB media was supplemented with 100μg/ml ampicillin (LBA).

2.3. Chromosomal and Plasmid DNA

Extraction and purification of both chromosomal and plasmid DNA were carried out using the procedure described by Sambrook, *et al.* [17]

2.4. Restriction Enzyme Digestion

The digestion of DNA by restriction enzymes was carried out according to the manufacturer's instructions. The reaction digestion was terminated by heating at 70°C for 15 min and adding 1/6 volume DNA loading dye.

2.5. Agarose Gels

DNA was analysed by using horizontal agarose gel electrophoresis. The DNA mixed with 1/6 volume of loading dye (10%w/v ficol 400, 0.06%w/v bromophenol blue and 0.5%w/v SDS) and loaded onto the 0.8% agarose gel in TAE buffer(0.04M Tris-HCl pH 7.9, 5mM sodium acetate, 1mM EDTA). The electrophoresis was performed in TAE buffer and stained with ethidium bromide (0.5μg/ml).

2.6. Polymerase Chain Reaction (PCR)

Oligonucleotides DNA primers (forward) (5'ACTCATTTCAGGGGATCCGCCATGGTTC3' *Bam* HI site is underline) and (reverse) (5'GGCCGAATTCTGCAATCGCTCATGCC3' *Eco* RI site is underline) were designed with defined restriction sites to facilitate the cloning process. DNA primers were designed in frame to amplify the *lipase* gene from the chromosomal DNA of thermophilic *B. pseudomallei*. The Polymerase Chain Reaction (PCR) was carried out in an overall volume of 50 μl containing 2.5 μl of each primer (50 ng/μl), 2.5 μl (2mM) deoxynucleoside triphosphate mix, 3 μl Mg⁺⁺ ion (25 mM), 5 μl buffer (10 X buffer delivered with the *Pfu* DNA polymerase), 1 μl template DNA (~0.1 ng), 1 μl of *Pfu* DNA polymerase, 5 μl dimethyl sulphoxide (DMSO), and the reaction was diluted to 50 μl with distilled water and mixed gently. The reaction mixture was incubated at 94°C for 4 min. The following PCR cycle was repeated 30 times: denaturation 94°C for 1 min, annealing of primers at 55°C for 1 min and DNA synthesis at 72°C for 2 min. This was followed by 4 minute incubation at 72°C before the mixture was stored at 4°C.

2.7. Cloning of *Lipase* Gene Into pGEX-2T DNA Plasmid

The PCR products of the amplified *lipase* gene was treated with *Bam*HI and *Eco* RI restriction enzymes and purified by low melting point agarose [17]. A plasmid pGEX-2T DNA vector was purified and linearized with *Bam* H I and *Eco*R I restriction enzymes. The *lipase* gene digested with *Bam* H I

and *Eco* R I restriction enzymes was ligated into the plasmid previously treated with both *Bam* H I and *Eco* RI restriction enzymes. The ligation mixture was transformed into competent cells of *E. coli* DH5 and plated onto LBA plates and incubated at 37°C overnight. Individual colonies were examined by plasmid mini prep with restriction enzyme digestion to identify the recombinant plasmids. The recombinant plasmid designated pMMY103 (pGEX-2T-*lipaseBps*IPTG- inducible Ap^R) contains the whole *lipase* gene in-frame with the Glutathione S transferase (GST) fusion protein. *E. coli* BL21 (DE3) cells were transformed with pMMY103DNA plasmid to express the GST-lipase fusion protein. The generated *E. coli* strain designated *E.coli* Lipase.

2.8. Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli, [18].

2.9. Time Course of Overexpression of GST-Lipase Fusion Protein

E. coli Lipase was streaked onto LB ampicillin plates and incubated overnight at 37°C. A single colony was used to inoculate 10 mL of LB broth supplemented with 100 µg/mL ampicillin, and grown overnight at 37°C and 200 rpm in a shaking incubator. The overnight cultures were used to inoculate 100 ml LBA media. The cultures were incubated at 37°C and 200 rpm, until they reached to the mid-logarithmic growth phase OD_{650nm} of 0.4 - 0.6, then 1mM of isopropyl-1-thio-B-galacto-pyranoside (IPTG) was added. At various times 1ml samples were removed and the cells were pelleted by centrifugation at 6,000 rpm for 5min. Cells were then resuspended in 100µl of 1X SDS gel loading buffer [100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM dithiothreitol (DTT)], boiled for 4min, sonicated three times for 5sec and analysed by SDS-PAGE.

2.10. Purification of GST-Lipase Fusion Protein

The bacterial strain BL21(DE3) carrying plasmid pGEX-2T lipase gene that overexpresses GST-Lipase fusion protein was prepared from 500 mL culture. The cell pellet was resuspended in buffer A (50mM Tris-HCl pH 8.0, 50mM NaCl, 5mM dithiothreitol [DTT], 1.0mM phenyl methyl sulphonyl fluoride [PMSF], 0.1mM Benzamidine, 10% (v/v) glycerol and Proteinase Inhibitor Cocktail Tablet, Boehringer Mannheim) and then placed in an ice-water bath, and finally sonicated five times for 30 s (with a 5 mm diameter tip at maximum output using a MSE 150 Watt Ultrasonic Fisher). The lysate was cleaned by centrifugation for 30 min at 4°C with 15,000 rpm. The resulting supernatant was applied to a 12 ml DEAE-Sepharose fast flow (Amersham Pharmacia biotech) column at 30 mL/h, previously equilibrated with at least ten bed volumes of buffer A. The unbound proteins were removed from the column by passing 100 ml buffer A

through the column and . The bound proteins were eluted from the column by using 100 ml gradient of 50-500 mM NaCl in buffer A. Fractions of 5 ml were collected and assayed for the GST-Lipase fusion protein by SDS-PAGE. The best fractions containing the GST-Lipase fusion protein, judged by SDS- PAGE were collected and dialysed overnight against 2 liters of buffer B (25 mM Phosphate, 125 mM NaCl, 1 mM PMSF, 0.1 mM Benzamidine and 10% (v/v) glycerol). The dialysed proteins were applied on to a 2 ml Glutathione S sepharose 4B column (Amersham Pharmacia biotech) previously equilibrated with 50 ml buffer B with flow rate 30 ml/h. The unbound proteins were washed away from the column with 50 ml buffer B. The bound protein was eluted from the column with a 10 ml buffer B containing 10 mM reduced glutathione ~30 ml/h and 2 ml fractions were collected for GST-Lipase protein and then assayed by 10% SDS-PAGE.

2.11. Immobilization of Lipase in Calcium Alginate-Gelatin Composites

Combinations of Gelatin alginate mixtures were prepared by adding gelatin (3%) to sodium alginate solution (5%) in water and then cross-linking with glutaraldehyde. Typically, sodium alginate (500 mg) and gelatin (300 mg) were added to distilled water (8 ml) in a conical flask, and autoclaved for 15 min at 120°C. The hot solution was allowed to cool to room temperature with constant stirring with a magnetic bead. The purified lipase protein (3 ml) was then added, and the mixture was stirred for 15 min. Glutaraldehyde (0.3 ml of 25% solution in water) was added, and the contents were stirred for an additional 15 min. This slurry was then transferred to a dropping funnel with a plastic tip and allowed to fall dropwise into cold CaCl₂ solution (4°C). The beads were left in the CaCl₂ solution for 30 min to harden. The supernatant was decanted, and the beads were then washed with distilled water and stored in a refrigerator.

2.12. Enzyme Assay

Lipase activity was determined by spectrophotometer using *p*-nitrophenylpalmitate (*p*-NPP) as a substrate. One milliliter of 99% ethanol containing 50 mg of *p*-NPP was mixed with 9 mL of 50 mM sodium phosphate buffer (at optimum pH) containing gum Arabic (0.11%) and triton X-100 (0.44%). The diluted enzyme solution (0.1 mL) was added to 1 mL sodium phosphate buffer (50 mM, optimum pH) and the reaction mixture was pre-warmed to 30°C and then mixed with 1 mL of freshly prepared substrate solution. The reaction mixture was incubated at optimum temperature for 30 min, and subjected to colorimetric assay at 410 nm.

One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µM *p*-nitrophenol per minute under the assay conditions. Under the conditions described, the extinction coefficient of *p*-nitrophenol is 1.46 x 10⁵ Cm² M⁻¹.

2.13. Protein Determination

Protein concentration was determined as described by

Bradford [19] using Bovine Serum Albumin (BSA) as standard.

2.14. Determination the Optimum pH, Temperature of Lipase Activity

The optimum pH of lipase activity was determined by estimating the enzyme activity at various pH values (4.0–12.0) of buffer under standard assay conditions. The buffer systems were utilized at a concentration of 50 mM: glycine–HCl buffer (pH 4.0–5.0), sodium hydrogen phosphate–NaOH buffer (pH 5.0–7.0), Tris–HCl buffer (pH 7.0–9.0), disodium hydrogen orthophosphate NaOH buffer (pH 9.0–11.0), and glycine–NaOH buffer (pH 10.0–12.0). The enzyme activity in the range of pH 4.0–12.0 was examined by incubating the enzyme solution for 40 min at the optimum temperature at different pH values, and then the residual activity of lipase was performed according to the standard assay protocol described above. Estimation of the lipase activity at different temperatures (20–80°C) was carried out at standard assay conditions.

All data about determination the optimum pH, temperature of lipase activity are the average of triplicate analyses.

2.15. Effect of Metal Ions on the Enzymatic Activity

The effects of various metal ions (1 mM and 5 mM) including Ni^{++} , Cr^{++} , Co^{+2} , Mg^{2+} , Cu^{2+} , Fe^{2+} , Ca^{2+} , Zn^{2+} , Mn^{2+} , Ba^{2+} , Na^+ , K^+ , Cs^+ and Li^+ as chloride salts were investigated. In these experiments, the enzyme was pre-incubated with metal ions for 40 min at the optimum temperature and pH and then the residual lipase activity was tested on *p*-NPP substrate. Lipase activity in the absence of metal ion was taken as 100%.

All data about the effect of metal ions on the lipase activity are the average of triplicate.

3. Results and Discussion

3.1. Chromosomal DNA Isolation and PCR Amplification of the Lipase Gene

Thermophilic *B. pseudomallei* strain was isolated from Saudi Arabian environment. The strain has displayed the highest lipolytic activity among 93 identified microorganisms (Author unpublished data). The nucleotide sequence of the lipase gene in the *B. pseudomallei* strain K96243 has been reported and the nucleotide sequence was identified as a part of the *B. pseudomallei* genome sequence [20]. The sequence of lipase gene of *B. pseudomallei* strain contain 1197 nucleotide base pair (bp) distributed in the following number and percentage 462G (38.60%), 431C (36.01%), 127A (10.61%) and 177T (14.79%). The lipase gene of *B. pseudomallei* has a high GC contents ratio.

The lipolytic enzymes isolated from bacteria were classified into eight different families according to their amino acid sequence [21]. The largest family (family I), consists of six subfamilies, including the best studied “true

lipases,” such as *Pseudomonas* lipases [22] and *Bacillus* lipases. The family I.2 includes lipases from *Chromobacterium viscosum* [23], *Burkholderia glumae* [24], and *Burkholderia cepacia* [25–26]. Although a number of lipase producing bacterial sources are accessible, only a few are commercially exploited as wild or recombinant strains [27–28]. The PCR oligonucleotide forward and reverse primers were utilized to amplify the entire lipase gene of bacterial strain *B. pseudomallei* 21bp upstream of the lipase gene to 14bp downstream of the lipase gene with *EcoR* I and *BamH* I restriction sites. The 1.2 kbp DNA PCR product of the lipase gene of bacterial strain *B. pseudomallei* was amplified, digested with both *EcoR* I and *BamH* I restriction enzymes and purified from low melting point agarose gel.

3.2. Cloning the Lipase Gene Into the pGEX-2T DNA Vector

The pGEX-2T is a suitable vector for the cloning process because its complete sequence is known. The pGEX-2T contains a useful and unique *EcoR* I and *BamH* I restriction sites, and expresses GST protein tagged of the target protein of interest Lipase gene of *B. pseudomallei*. The pGEX-2T DNA was digested with *EcoR* I and *BamH* I to linearize the circular DNA vector. The 1.2 *EcoR* I and *BamH* I DNA fragments containing the entire lipase gene were inserted downstream the GST gene in the pGEX-2T DNA, as represented in Figure 1. The *EcoR* I and *BamH* I DNA fragment of pGEX-2T and the 1.2 kbp *EcoR* I and *BamH* I DNA fragment of *B. pseudomallei* lipase PCR product were ligated using T4 DNA ligase. The ligation mixture was transformed into competent cells of the *E. coli* strain DH5 and plated onto LB agar plates supplemented with 100 µg/ml ampicillin. The recombinant plasmid DNA was designated pMMY103.

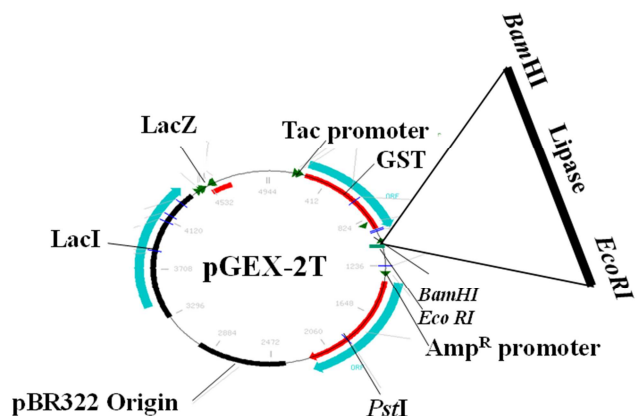


Figure 1. Schematic diagram of the construct used for overexpression of recombinant lipase. The lipase gene (*lip Bps*) fused to GST sequence and was cloned downstream of the Tac promoter in pGEX-2T DNA expression vector, which also contained the genes for *lacI* and *lacZ* repressors, pBR322 origin and ampicillin resistance.

The recombinant plasmid containing the lipase gene of bacterial strain *B. pseudomallei* was transformed into competent cells of *E. coli* strain BL21 (DE3). The *E. coli*

BL21 (DE3) protease deficient host strain containing the recombinant lipase gene of *B. pseudomallei* with pGEX-2T DNA (PMMY103) was designated *E. coli lipBps*.

3.3. Overexpression of GST-Lipase *B. pseudomallei* Fusion Protein

A time course of the overproduction of GST-Lipase *B. pseudomallei* fusion protein was studied by adding IPTG to a final concentration 1 mM at time 0, 1, 2... h, and a 1.0 mL sample was withdrawn for SDS-PAGE analysis at each time interval. The results of over production of the GST- Lipase *B. pseudomallei* fusion protein (~ 58kDa) are shown in Figure 2. The overproduction of GST-Lipase *B. pseudomallei* is evident after 1 hour of IPTG induction (Figure 2 Lane 3). The GST-Lipase *B. pseudomallei* fusion protein is optimally expressed after 6 hours of IPTG induction. The best overexpression of GST- lipase *B. pseudomallei* fusion protein from bacterial strain *E. coli lip Bps* appears after 6 hours as represented in Figure 2 Lane 8.

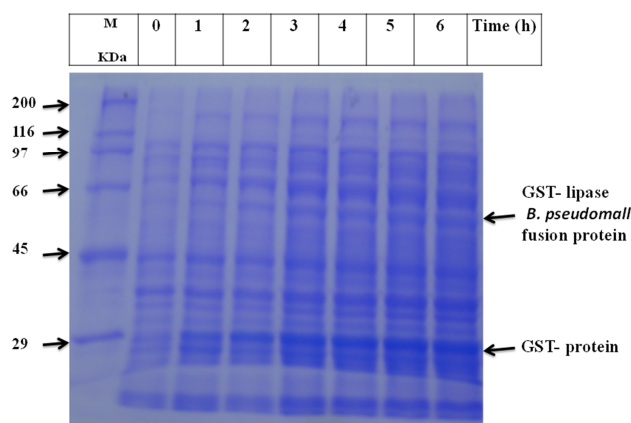


Figure 2. Induction time course for overexpression of the GST-lipase *B. pseudomallei* fusion protein. Early to mid-log culture of *E. coli lipBps* overexpressing GST-lipase *B. pseudomallei* fusion protein was induced at time 0 hours with IPTG at a final concentration of 1 mM and samples were taken and analysed by 10% SDS-PAGE gel at times indicated. (Lane 2-8) IPTG, protein marker (Lane 1) Sigma SDS6H2 (M.Wt 30,000-200,000 KDa).

3.4. Purification of GST-Lipase *B. pseudomallei* Fusion Protein

Bacterial cells from *E. coli lipBps* were lysed, and the GST- Lipase fusion protein was purified using glutathione sepharose. Before this nucleic acid was removed by applying the cell extract to a DEAE sepharose column. The lipase gene of bacterial strain *B. pseudomallei* was amplified by the PCR, and cloned into the pGEX-2T DNA vector under the strong *Taq* promoter. The *Taq* promoter allowed the lipase protein to be over expressed tagged with the GST protein to facilitate the purification process. It was noticeable from the protein profile in Figure 3 Lane 4 that the purification process led to the purification of 58 kDa protein corresponding to the GST-lipase fusion protein. The purification of GST-Lipase *B. pseudomallei* fusion protein was confirmed by Western blot using an anti-GST antibody (data not shown). As illustrated in Figure 3 the purified GST-lipase fusion protein of *B.*

pseudomallei appears to be homogeneous, yielding a strong homogenate protein band in polyacrylamide gel electrophoresis, which stained for protein. The apparent molecular weight of GST-lipase *B. pseudomallei* fusion protein is 58.0 kDa (Figure 3 lane 4). The molecular mass of GST is 26 KDa and the molecular mass of *B. pseudomallei* lipase is estimated 32 kDa. The molecular mass of lipase purified from *B. pseudomallei* is similar to that the molecular mass of lipases obtained from other bacterial strains as *Acinetobacter sp.* RAG.1 (33 kDa, [29]), *Pseudomonas sp.* KWI.56 (33 kDa, [30]) and *P. fluorescens* AK 102 (33 kDa, [31]). The molecular mass of lipases of *B. pseudomallei* is lower than that of the lipase protein purified from other microorganisms e.g. *Bacillus sp.* THLO27 (69 kDa, [11]). On the other hand, the molecular mass of lipase of *B. pseudomallei* (32 kDa) is higher than that of the lipase protein isolated from other sources e.g. *Acinetobacter calcoaceticus* (30.5 kDa, [30]) and *B. subtilis* 168 (19 kDa, [32]).

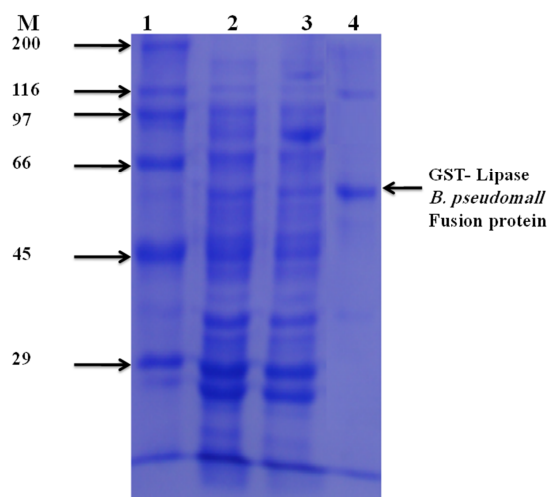


Figure 3. A figure showing the purification profile of GST- Lipase *B. pseudomallei* fusion protein.

Lanes 1: protein marker Sigma SDS6H2-1VL (M.Wt 30,000- 200,000 KDa).
Lane 2: Crude extract of *E. coli* strain overexpressing GST- Lipase *B. pseudomallei* protein.
Lane 3: GST- Lipase *B. pseudomallei* protein eluted from DEAE-sepharose column by using 100 ml gradient of 50-500 mM of NaCl in buffer A.

The results of various purification steps for the GST-lipase *B. pseudomallei* fusion protein homogenate are presented in Table 1. It is apparent that the protein content decreased progressively from 11.7 mg/ml in the crude extract to 0.81 mg/ml in the eluant of the glutathione S sepharose 4B step. This is accompanied by a gradual increase in the specific activity of the enzyme from 0.914 U/mg proteins in the crude extract to 117.2 U/mg proteins at the final purification step. As shown in Table 1 the final step of purification indicates that the enzyme activity is 128.23 fold enhanced compared to the specific activity of the enzyme in crude extract.

In view of lipase purification, it was purified from *B. stearrowthermophilus* MC7 to 19.25 fold with 10.2% yield and a specific activity of about 12 U/(mg protein) [12]. A

thermostable lipase formed by a thermophilic *Bacillus* sp. J33 was purified to 175 fold [33]. The 128.23 fold of purification for GST lipase *B. pseudomallei* (this work) is higher than that the fold of purification recorded for lipase from other bacterial species [34-36]. On the other hand, the

fold of purification of a GST lipase *B. pseudomallei* is lower than the lipase recorded from other species as *Pseudomonas mendocina* 240 fold [37]. The low fold of purification may be due to the premature translation signal beyond the *Taq* promoter in the pGEX-2T DNA vector.

Table 1. Purification of the lipase from *B. pseudomallei*. Results are the mean of triplicate assays.

Purification step	Volume (ml)	Protein (mg/ml)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	300	11.7	3208.14	0.914	100	1
DEAE Sepharose	70	9.4	2566.20	3.9	79.9	4.27
Glutathione S sepharose 4B	10	0.81	949.32	117.2	29.6	128.23

3.5. Immobilization of Lipase Protein

In the present work, we report that the lipase enzyme cloned from *B. pseudomallei* can be efficiently immobilized in calcium alginate gelatin composites in the presence of glutaraldehyde. The immobilized enzyme showed about 83 % of the activity of the native enzyme (data not shown), indicating that the method of immobilization is particularly suitable for lipase enzyme isolated from *B. pseudomallei*.

3.6. Biochemical Characterization of Free and Immobilized Lipase of *B. pseudomallei*

3.6.1. Effect of pH

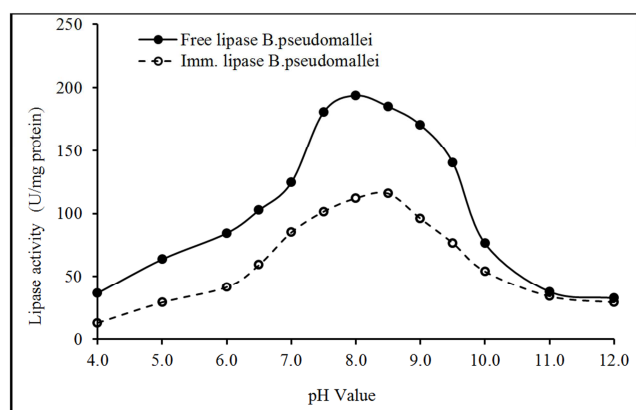


Figure 4. Effect of pH on the activity of the GST-lipase Bsp fusion protein free and immobilized lipase cloned from *B. pseudomallei*. Results are the mean \pm SD of triplicate assays.

The optimum pH of free and immobilized lipase is shown in Figure 4. The free and immobilized lipase activities were determined over a wide pH range from 4 to 12. It appears from Figure 4 that the free lipase activity is low at pH below 7 but it increases gradually from 7 to 7.5. The optimum pH value for the free enzyme is 7.5. After pH value of 7.5, the enzyme activity declines again. The optimum pH for the immobilized enzyme is recorded at the pH 8.5 (Figure 4). This means that the immobilization process shift the optimum pH from 7.5 to 8.5. Lipase purified from *Pseudomonas aeruginosa* had maximal activity at pH 8.5–9.0 [38]. Other *Pseudomonas* lipase, such as F.111 [39], has its maximal activity at pH values ranging from 6.0 to 10.0. On the other hand, lipases from *P. cepacia* and *Pseudomonas* sp.

KWI.56 showed optimal pH values between 5.5–6.5 and 5.5–7.0, [40 – 41], respectively].

3.6.2. Effect of Temperature

The optimum temperature for the GST- lipase *B. pseudomallei* fusion protein for free and immobilized lipase activities were determined over temperature range of 20–80°C and the results are illustrated in Figure 5. It is apparent that the optimal temperature for the free enzyme activity is 65°C. Any further increase in the temperature leads to a significant decrease in the enzyme activity. The optimum temperature for the immobilized lipase is shifted to a higher temperature (70°C). The optimum temperature for the immobilized enzyme is higher than that for the free enzyme. The immobilization process increases the thermostability of the lipase protein of *B. pseudomallei*.

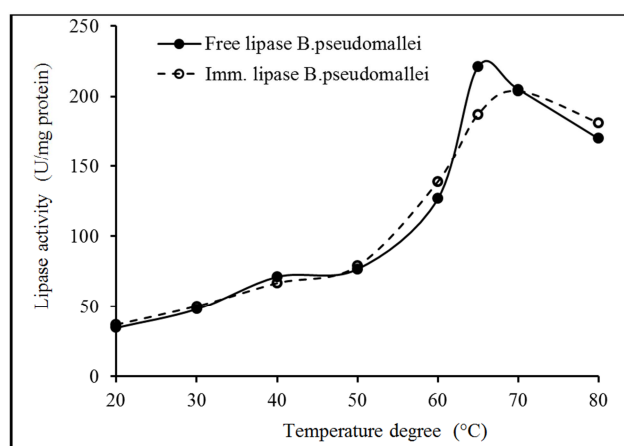


Figure 5. Effect of temperature on the activity of the GST- lipase *B. pseudomallei* fusion protein free and immobilized in 100mM Tris- HCl buffer (pH 7.5 for free lipase and pH 8 for immobilized lipase). Results are the mean \pm SD of triplicate assays.

It is well known that increasing the temperature causes an increase in the inherent energy of the system and more molecules can obtain the necessary activation energy required for reaction to take place. The optimum temperature of 65°C was recorded for free lipase of *B. pseudomallei* and that temperature is identical to that observed for the lipases isolated from *P. mendocina* 3121.1 [42] and *Bacillus* sp. strain 398 [43]. However the optimum temperature of the immobilized lipase of *B. pseudomallei* was 70°C which is similar to the free lipase isolated from *Bacillus* sp. THLO27 [11]. Many bacterial lipases show lower optimal temperature

than the ones for lipases of *Serratiamarcescens* (37°C) [44] and *S. haemolyticus* (28°C) [45]. On the other hand, higher optimum temperatures were reported for lipases from other sources e.g. *Burkholderia sp.* 90– 100°C [46-47] and *B. thermooleovorans* ID.1 75°C [48]. Raising the temperature above the optimum resulted in inhibition of the enzyme activity. As the temperature increases above the optimum, the energy of the system becomes sufficient to cause breakdown of hydrogen bonding and other non-bonding forces holding the tertiary structure of the protein.

3.6.3. Effect of Incubation Time

It is important to determine the period over which the enzymatic reaction varies linearly with time. Therefore, the free and immobilized GST-lipase *B. pseudomallei* fusion proteins activities were measured at different periods of incubation ranging from 15 to 120 minutes. The results of these measurements are shown in Figure 6. It is evident that the lipase activity is directly proportional to the time of incubation. The incubation period of 40 minutes was chosen for the free and immobilized GST-lipase *B. pseudomallei* fusion protein. The relationship between the lipase activity and the incubation period was linear up to 40 minutes. For other lipases, this incubation time can be as short as 10 minutes e.g. *Alcaligenes sp.* [30], 30 minutes *Pseudomonas sp.* [49], or as long as 1 hour *B. alcalophilus* [50].

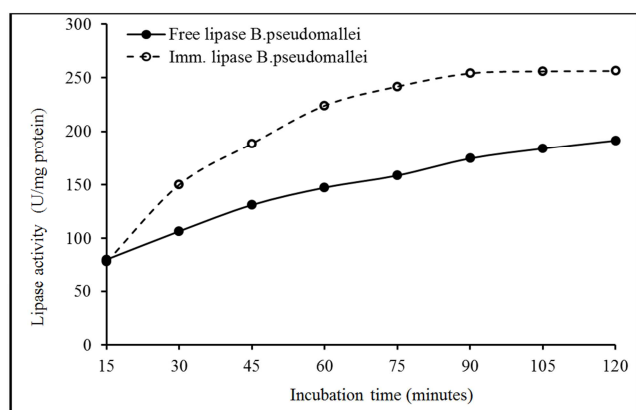


Figure 6. Effect of incubation time on the GST- lipase *B. pseudomallei* fusion protein activity of free and immobilized. Results are the mean \pm SD of triplicate assays.

3.6.4. Thermal Stability

Thermal stability of free and immobilized GST lipase *B. pseudomallei* activity were investigated at 70°C using *p*-NPP as a substrate (Figure 7). The free GST lipase *B. pseudomallei* protein is stable at 70°C for 45 minutes, and after that time the enzyme activity starts to decline, and reaches to 37% of its optimum activity after 180 minutes. While, the immobilized GST lipase *B. pseudomallei* activity at 70°C was stable and active for 120 minutes and beyond that time the enzyme activity begin to decline.

Thermal stability of both free and immobilized GST lipase *B. pseudomallei* proteins at 80°C were also studied (Figure 8). The free GST lipase *B. pseudomallei* was stable only for 30 minutes at 80°C, and after that time the enzyme activity start

to decline dramatically. The immobilized GST lipase *B. pseudomallei* protein when exposed to 80°C, the protein was stable for 60 minutes and after that time the protein activity start to decline.

On studying the heat of inactivation for the free and immobilized lipase enzymes from *B. pseudomallei* at 70 and 80°C it was found that the enzyme activity gradually decreased at least after half an hour of incubation. This demonstrates that lipase protein of *B. pseudomallei* is stable at high temperature beyond the optimal temperature for at least half an hour. Similar results were obtained for lipases from *B. licheniformis* strain H1 [51]. Thermal stability of lipase activity is obviously related to its configuration and subsequently, the melting point.

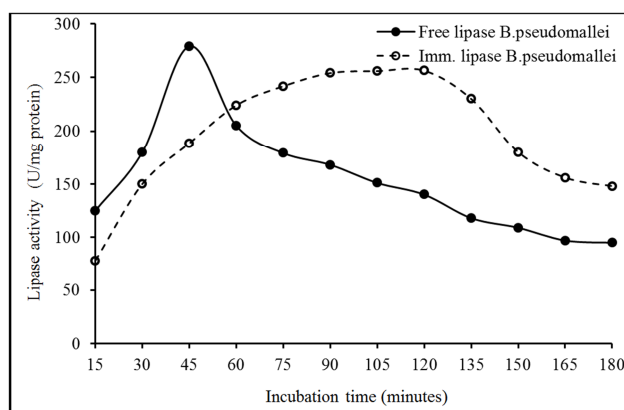


Figure 7. Thermal stability of free and immobilized GST lipase *B. pseudomallei* at 70°C. Results are the mean of triplicate assays.

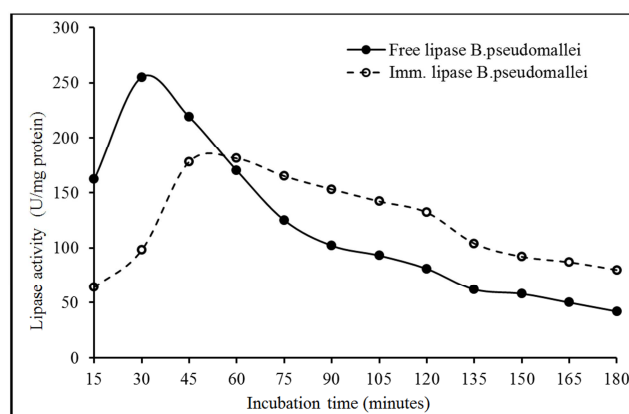


Figure 8. Thermal stability of free and immobilized GST lipase *B. pseudomallei* at 80°C. Results are the mean of triplicate assays.

3.6.5. Effect of Metal Ion on Lipase Activity

The chloride salts of the following cations were tested: Hg^{+2} , Cu^{+2} , Fe^{+2} , Ni^{+2} , Mn^{+2} , Mg^{+2} , Ca^{+2} , Co^{+2} , Li^{+} , Cs^{+} , K^{+} and Na^{+} . These cations were used in concentration of 1 and 5mM. The effects of metal ions on free and immobilized lipase activity are summarized in Table 2. Free and immobilized GST- lipase *B. pseudomallei* fusion protein were activated by 1mM of Ba^{+2} , Ca^{+2} and Na^{+} whereas the Free GST- lipase *B. pseudomallei* fusion protein was activated by 1 mM of Cr^{+2} , Li^{+} , Cs^{+} and Mg^{+2} . The immobilized GST lipase *B. pseudomallei* fusion protein was stimulated by 5 mM Fe^{+2}

and Co^{+2} but inhibited by 1mM of the same cations as represented in Table 2. The inhibitory effect on the enzyme was observed for Ni^{2+} , Mn^{2+} , and Cu^{2+} , and its with the maximum for Hg^{2+} .

Table 2. Effect of various cations on the activity of both free and immobilized GST-lipase *B. pseudomallei* fusion protein. Results are the mean of triplicate assays.

Lipase Activity	Free Cell		Immobilize Cell	
	1 mM	5 mM	1 mM	5 mM
Metals				
Control	100	100	100	100
Ba^{++}	137	118	108	46
Ca^{++}	141	74	134	48
Co^{++}	62	119	30	128
Cr^{++}	119	100	39	59
Mg^{++}	134	82	43	47
Cu^{++}	34	57	65	44
Fe^{++}	51	314	46	128
Hg^{++}	53	25	86	37
Mn^{++}	51	24	34	4
Ni^{++}	14	48	75	56
Li^{+}	111	192	43	137
Na^{+}	124	91	117	35
K^{+}	95	168	16	84
Cs^{+}	133	100	66	47

According to Voet *et al.*, [52], nearly one third of all known enzymes require the presence of metal ions for their catalytic activity. This type of enzymes includes the metal enzymes, which hold tightly bound metal ion cofactors. The most commonly transition metal ions are Fe^{2+} , Ni^{2+} , Cu^{2+} , Mn^{2+} , and Zn^{2+} . The metal activated enzymes, in contrast, loosely bind metal ion from solution, usually the alkali and alkaline earth metal ions Na^{+} , K^{+} , Mg^{2+} , and Ca^{2+} . It is important to mention that many enzymes need the presence of a mono or divalent cation (or more) to increase the enzyme activity. Cations generally form complexes with ionized fatty acids, changing their solubility and behavior at interfaces [53].

Among the various tested cations for their effects on free and immobilized lipase enzyme of *B. pseudomallei*, Ca^{+2} is the best activator (Tables 2) and this is in agreement with those for lipase protein from *B. alcalophilus* [50]. A probable elucidation of this observable fact is that Ca^{2+} has a particular enzyme activating effect that it exerts by concentrating at the fat water interface. Therefore, calcium ions may achieve three distinct roles in lipase action: removal of fatty acids as insoluble Ca^{2+} salts in certain cases, direct enzyme activation due to resulting from concentration at the fat water interface, and stabilizing effect of on the enzyme. The free lipase protein of *B. pseudomallei* was stimulated with Ba^{+2} . Indeed, [54] similar effects of Ba^{+2} on lipase protein from *B. sp.* was also observed. The other cations namely Mn^{+2} and Cu^{+2} expressed considerable inhibitions effect on lipase protein of *B. pseudomallei*. The behavior of lipase from *B. pseudomallei* in the presence of different concentration of Hg^{+2} indicates that any increase in Hg^{+2} concentrations is association with corresponded by a remarkable inhibition in the enzyme

activity. These results indicate the presence of thiol and histidine as active site residue of the enzyme and these results are in harmony with that of Sugihara *et al.* [41].

In conclusion, we have described the cloning and characterization of a thermophilic lipase from bacterial strain *B. pseudomallei*. The expression level of the recombinant enzyme in *E. coli* was fairly high and allowed the production of sufficient material for successful purification in only a two purification steps (DEAE Sepharose Glutathione S sepharose 4B) with subsequent characterization. Therefore, results of the present study suggest the possibility of production of thermostable lipase protein of *B. pseudomallei* using molecular biology techniques. This lipase protein was purified and effectively immobilized on the Ca alginate composite. The free and immobilized protein was stable at moderately alkaline pH and temperature around 60°C. The supplementations of the enzyme preparations with Ca^{+2} increase the enzyme activity. The addition of Ca^{+2} could significantly improve the cleaning performance towards different stains.

Acknowledgements

The author appreciates King Abdulaziz City for Science and Technology General Directorate of Research Grants Programs, Kingdom of Saudi Arabia for the financial support Project No. 08- Bio13-6.

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