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Molecular Cloning and Expression in *Escherichia coli* of *Pseudomonas aeruginosa lipase* gene

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Abstract: In this study genomic library was constructed from *P. aeruginosa* purified DNA and screened for these two lipases genes in an attempt to isolate both of these genes and subclone them on the same plasmid. Primary screening of a genomic library constructed on pTrcHis (A, B, C) plasmid showed a number of potential lipase positives clones when expressed in *E. coli* DH5α. Surprisingly, most of these potential positives showed a specific DNA insert with a molecular size of 1.2 kbp when cut with restriction enzyme. Pilot protein expression study showed a specific protein band of a molecular weigh of 15.0 kDa when expressed in *E. coli* DH5α that induced with IPTG this matched the corresponding molecular weight of the native enzyme that isolated from the parent strain. Moreover, a serine protease inhibitor, PMSF showed an inhibitory effect on the activity of the recombinant enzyme while EDTA showed slight inhibitory effect.

Key words: Lipases, *Pseudomonas aeruginosa* Ps-x, 16S rRNA

INTRODUCTION

Lipases (triacyl glycerol acylhydrolases, EC 3.1.1.3) constitute a diverse and ubiquitous family of enzymes that in biological system initiate the catabolism of fats and oils by hydrolyzing the fatty acylester bonds of acylglycerols (Sharma *et al*., 2001; Jose *et al*., 2004). Lipases are widely distributed in nature and have been found in many species of animals, plants, bacteria, yeast and fungi. Although their wide distribution, the enzymes from microorganisms are most interesting because of their potential application in various industries ranging from the use in laundry detergent to stereospecific biocatalysts (Maliszewska and Przemyslaw, 1992; Yadav and Devi, 2004). Since each industrial application requires specific properties of lipases, there is still an interest in additional lipases that could be used in new applications. One of the most recent applications of *Pseudomonas* lipases is the production of biodiesel fuel from soybean oil utilizing immobilized *Pseudomonas cepacia* lipase (Noureddini *et al*., 2005). Especially, lipases of bacterial origin received much attention for their ability to function in extreme environments, such as high temperatures and pH. Therefore, many bacterial lipases, such as those produced by *Pseudomonas* and *Burkholderia* species, have been characterized. The active site of these lipases is composed of the catalytic triad serine, histidine and an acidic residue, aspartic or glutamic, similarly to lipases from eukaryotic origin (El Khattabi *et al*., 2003). The cold activity (i.e., high catalytic activity at low temperatures) and thermostability of lipases and esterases can be a key to the success in some of their applications such as in laundry detergents for cold washing and catalysts for organic synthesis of unstable compounds at low temperatures (Suzuki *et al*., 2003). The bacterial genus *Pseudomonas* secretes a number of extracellular enzymes, which include lipases, in response to fluctuating external nutrients. Interest in *Pseudomonas* lipases stems either from their potential usefulness in a variety of biotechnological applications or from their detrimental effect on stored food products such as refrigerated milk (Zaliha *et al*., 2005). We previously isolated and purified two different extracellular lipases from *P. aeruginosa* Ps-x strain. In this study, we reported the isolation and cloning of *P. aeruginosa* lipase gene that encode a protein of a molecular weight of 15 kDa in *E. coli* strain. Moreover, partial characterization for the crude enzyme produced from *E. coli* was carried out.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial isolate used in this study was a gift from Dr. Yossry Gohar, Department of Microbiology, Faculty of Science, Alexandria University, Egypt. This isolate was tested for lipase production using nutrient agar medium, pH 7.5

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contained per liter: peptone, 10.0 g; NaCl, 5.0 g; $CaCl_2$, 0.1 g and agar, 20.0 g. The medium was sterile by autoclaving and let to cool to 45°C after that 1% of sterile Tween-20 or Tween-80 was added to the medium and mixed well then poured 20-25 mL into 100 mm petri dishes. The identification of the bacterial isolate was based on cell morphology, colony morphology, growth on nutrient broth and nutrient agar as well as several biochemical tests. The identification process was performed at the Fermentation and Biotechnology Center, El-Azhar University, Cairo. To confirm the biochemical tests results for isolate identification, 16S rRNA (rDNA) technique was carried out. DNA was isolated and purified according to Sambrook *et al.* (1989). Amplification of the 16S rDNA gene from the genome was carried out by polymerase chain reaction (PCR) using primers designed to amplify 16S rRNA gene. The forward primer was 5`-AGAGTTTGATCMTGGCTCAG-3` and the reverse primer was 5`-TACGGYTACCTTGTTACGACTT-3`. The polymerase chain reaction analysis was performed with 100 ng of genomic DNA in a final volume of 50 μL, including a reaction buffer 1×, 30 pmole of each primer and 2 units of *Taq* polymerase. Thermocycling consisted of an initial denaturation of 5 min at 94°C and of 30 cycles of 1 min at 94°C (denaturation), 1 min at 55°C (primers annealing) and 1.5 min at 72°C (extension). Polymerase chain reaction products were analyzed for purity check on 1% agarose gel by electrophoresis, stained with ethidium bromide (0.5 μg mL^{-1}) and visualized using ultraviolet transillumination. *Escherichia coli* DH5α host strain was obtained from Stratagene Co. and this strain used as a host strain for recombinant plasmids and in pilot protein expression study. The pTrcHis (A, B, C) plasmid cloning vector is a 4.4 kb in size and was used for construction of *P. aeruginosa* genomic library and for the expression of the recombinant protein. This plasmid was obtained from *Invitrogen* Corporation, USA.

Chemicals and enzymes: All chemicals used were of analytical reagent and molecular biology, chromatographic grade as appropriate. GeneClean II kit was obtained from Anachem. Co. Restriction endonucleases and DNA modifying enzymes were obtained from Promega (Southampton, UK), Boehringer Mannheim (Germany) and Stratagene, UK.

DNA sequencing: DNA was sequenced by the dideoxy chain termination method according to Sanger *et al.* (1977) using ABI Prism Ready Reaction Dye Terminator Sequencing Kit and analyzed on an ABI 377 automated sequencer. The nucleotide sequence obtained about 326 base pairs were then analyzed using nucleotide Blast search data base and have been deposited in the GenBank sequence data base and have the accession number AF419219.

Nucleic acid techniques: Genomic DNA and plasmid DNA purification, competent cells preparation, transformation and all other standard DNA methodologies were performed according to Sambrook *et al.* (1989).

Construction and screening of *P. aeruginosa* genomic DNA library: *P. aeruginosa* DNA was isolated and purified according to Sambrook *et al.* (1989) The prepared DNA was partially cut with *Eco*RI according to the following reaction conditions: purified DNA, 20 μg (25 μL); 10x *Eco*RI buffer (H), 5 μL; *Eco*RI enzyme 120 units (10 μL); d-H_2O up to 50 μL. The reaction was carried out at 37°C for 3 h after which the reaction was terminated at 70°C for 10 min and the product was analysed and resolved on agarose gel (1%) along with phage λDNA *Hind* III cut molecular weight. markers. DNA fragments of 1.5-3.0 kbp average size were separated from the agarose gel and was purified using GeneClean II kit and used for ligation reactions. The plasmid pTrcHis (A, B, C) vector used for ligation reaction and library construction was obtained from *in vitrogen* corporation, UK. This plasmid (4.4 kbp) contains a unique *Eco*RI site in the multiple cloning site that was used in ligation reaction. Ligation reactions utilizing all the three possible reading frames of the plasmid (A, B, C) and transformation was carried out according to Sambrook *et al.* (1989). Recombinant *E. coli* DH5α cells harbouring recombinant plasmids were screened separately utilizing LB-agar plates supplemented with ampicillin, IPTG and uric acid substrate at previously indicated concentration.

Lipase assay: Lipase activity was determined colorimetrically according to Kordel *et al.* (1991), where two solutions were prepared for the assay. Solution 1 contained 90 mg of *p*NPP (*p*-nitrophenyl palmitate), dissolved in 30 mL propane-2-ol. Solution 2 contained 2 g Triton X-100 and 0.5 g gum Arabic dissolved in 450 mL (Tris-HCl 50 mM) buffer at pH 8.0. The assay solution was prepared by adding 1 mL of solution 1 to 9 mL of solution 2 drop wise to get an emulsion that remained stable for 2 h. The assay mixture contained 900 μL of the emulsion and 100 μL of the appropriately diluted enzyme solution. The liberated *p*-nitrophenol was measured at 410 nm using Novospek, Pharmacia spectrophotometer. One unit of enzyme was defined as the amount of enzyme that releases 1 μmol of p-nitrophenol from the substrate.

Protein analysis: Total protein concentrations were assayed by the method of Bradford (1976) using a calibration curve established with bovine serum albumin as a standard. Proteins in these preparations were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels according to the method of Laemmli (1970). Protein molecular weight markers for SDS-PAGE was SeeBlue® Plus2 prestained standard and obtained from Invitrogen life technologies.

Pilot expression study in *E. coli* cells: Recombinant protein was expressed in *E. coli* DH5α cells. Hundred militer of LB medium supplemented with ampicillin at a concentration 100 μg mL^{-1} were inoculated with 1 mL of an overnight culture of *E. coli* DH5α pTrcHis plasmids. The flasks were shaken at 200 rpm and at 37°C until the absorbance at 600 nm was 0.6 at which point IPTG (1 mM) was added to the induced the culture followed by continued incubation at 200 rpm and at 37°C. Samples were taken from each flasks at different time intervals along with uninduced *E. coli* DH5α cells pTrc His plasmids as a control. After that, the induced and uninduced cells were pelleted by centrifugation at 8,000 rpm and 4°C. The cells were then washed with 100 mM Tris-HCl, pH 7.4 and pelleted a second time. The cells were then resuspended in 4 mL of 100 mM Tris-HCl, pH 7.4 and sonicated using 4×15 sec pulses. Cells debris were removed by centrifugation at 10,000 rpm, 4°C for 10 min after which the supernatant was taken for protein analysis and for lipase assay.

Other Nucleic acid techniques: Genomic DNA and plasmid DNA purification, competent cells preparation, transformation and all other standard DNA methodologies were performed according to Sambrook *et al.* (1989).

Substrate gel using Tween-20: Zymogram using Tween-20 was carried out according to Talon *et al.* (1995). Unstained SDS-polyacrylamide gel was washed for 30 min in 2.5% Triton X-100 to remove SDS. The gel was laid on 1.3% agar plate in 20 mM Tris-HCl buffer pH 8.0 containing 0.4% Tween-20. The agar plate was then incubated at 37°C for 1-3 h. Lipase activity was detected by the formation of white precipitation zones.

Effect of PMSF and EDTA on the activity of recombinant lipase: To examine the effect of EDTA (ethylenediamine tetraacetic acid) and PMSF (phenylmethane sulfonyl fluoride) of recombinant lipase different concentrations of these compounds were prepared and the enzymes solution were pre-incubated with these compounds for 30 min on ice after which the residual activity was determined as described before using p-nitrophenyl palmitate substrate.

RESULTS

Identification of bacterial isolate: Based on cell morphology, colony morphology, growth on nutrient broth and nutrient agar as well as several biochemical tests, the bacterial isolate was identified as *P. aeuroginosa*. To confirm the biochemical tests results from bacterial isolate identification, 16SrRNA methodology was carried out. The sequencing data obtained using this strategy indicated that the isolate under study was 98% *P. aeruginosa* and the nucleotide sequence was deposited in the GenBank sequence database and given the accession number AF419219.

Construction and screening of *P. aeruginosa* genomic library: The expression efficiency of lipase in *P. aeuroginosa* wild type strain is low and reached to 55 units mL^{-1} after 24 h of inoculation (Saeed *et al.*, 2005). This low expression has limited its broad utilization and to increase the lipase production and yield and to characterize this enzyme, DNA recombinant technology has been used. Genomic library was constructed by ligating the *Eco*RI partially digested and purified DNA with average insert size of 1.5-3.0 kbp, onto *Eco*RI digested, Calf intestinal alkaline phosphatase (CIAP) treated pTrc His A, B, C plasmid expression vector. The pTrcHis A,B,C plasmid (Fig. 1) is a 4.4 kbp that carries a unique *Eco*RI site and a 6 histidine tag that facilitates the purification of the expressed protein using metal affinity chromatography. The ligation products were transformed into competent *E.coli* DH5α and the recombinant cells were plated onto LB agar plates supplemented with ampicillin as selectable marker and incubated overnight at 37°C. *E.coli* DH5α that harbouring pTrcHis A, B, C recombinant plasmids were subjected to more than one round of screening for the identification of potential positives lipase producing clones. The screening process was carried out on LB agar plates supplemented with ampicillin, IPTG and Tween-20. Results of the screening process showed that 3 potential positives clones were obtained and most of them were found to be on the pTrcHis C frame rather than the other two open reading frames A and B thus, they were given individual clones number C1-C3 (Fig. 2). Lipase producing recombinant

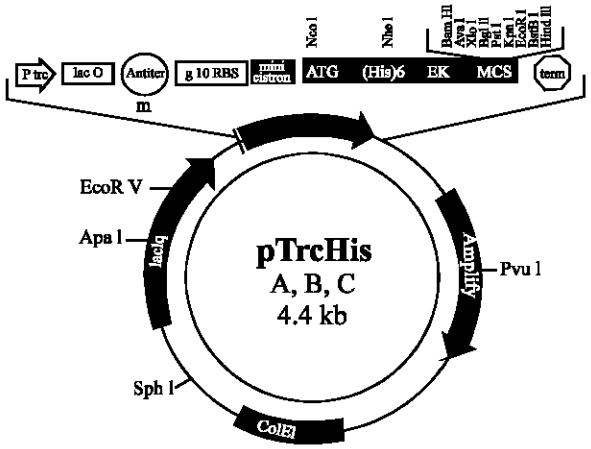

Fig. 1: Schematic representation of pTrc His A, B, C plasmid

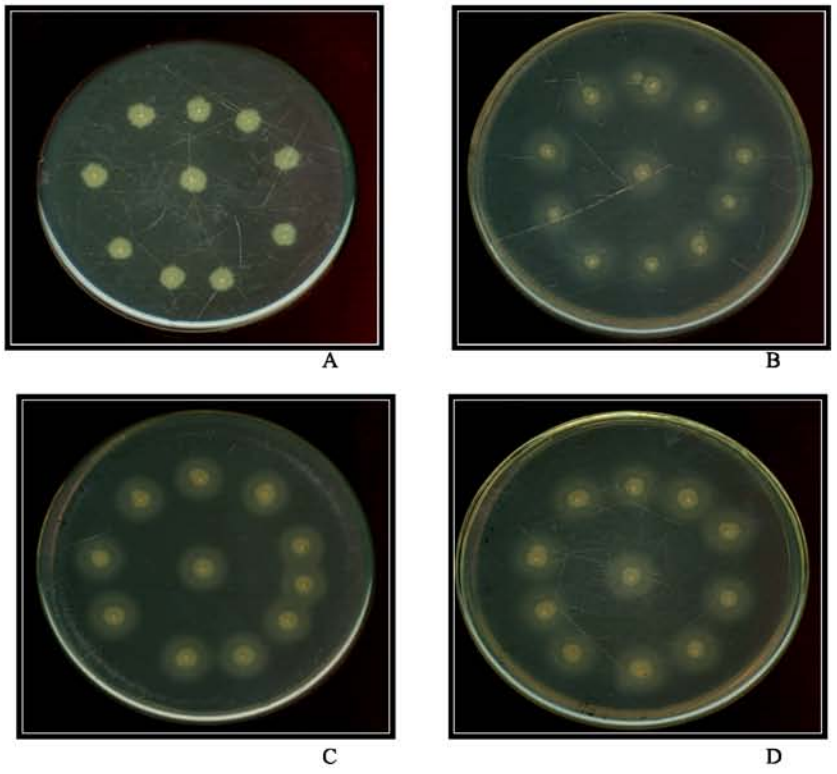

Fig. 2: Recombinant *E. coli* DH5α pTrc His C1-C3 (Panel B, C and D) on LB/agar/ampicillin/Tween-20 plates induced with IPTG. Panel A represents *E. coli* DH5α pTrc His C plasmid without DNA insert

clones characterized by the presence of clear zone around the bacterial growth colony which contain precipitation zones indicating the hydrolysis of Tween-20 and the formation of insoluble calcium salt (Fig. 2).

Plasmid DNA preparation and insert size determination: Plasmid DNA was prepared from recombinant *E. coli* DH5α pTrcHis-C3 and analyzed for DNA insert size by digestion of the prepared plasmid using *Eco*RI enzyme. It was found that the cloned DNA fragment has a size of 1.2 kbp (Fig. 3).

Monitoring the expression of the cloned lipase gene: A preliminary pilot expression study using *E. coli* DH5α pTrcHis-C3, the clone that harboring the 1.2 kbp DNA insert showed that, clear and discrete and protein bands of molecular weight 15 kDa was obtained in IPTG induced cells compared to the uninduced *E. coli* DH5α strain that harboring the same recombinant plasmid under the same and identical experimental conditions as shown in Fig. 4. To complement what has been investigated before, lipolytic activity of the recombinant *E.coli* DH5α pTrcHis-C3 induced and uninduced cultures were tested on substrate gel. Figure 5 shows that the result obtained

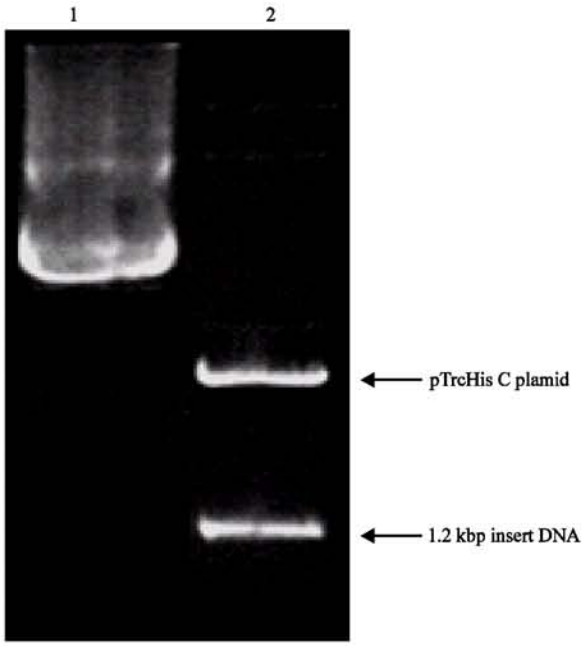

Fig. 3: Agarose gel (1%) electrophoresis for pTrcHis C3 plasmid; uncut (Lane 1) and *Eco*RI cut (Lane 2)

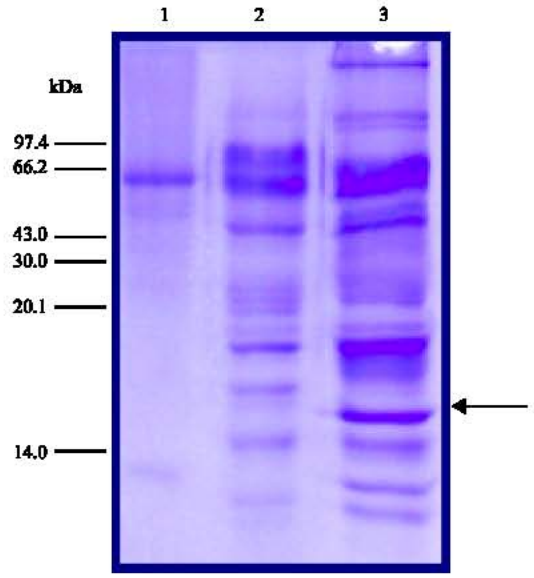

Fig. 4: Sodium dodecyl sulphate polyacrylamide gel (10%) (SDS-PAGE) electrophoresis of uninduced (Lane 2) and IPTG induced (Lane 3) *E. coli* DH5α pTrc His C3 cells. Lanes 1 represents molecular weight markers proteins. Arrows indicate the location of the expressed protein

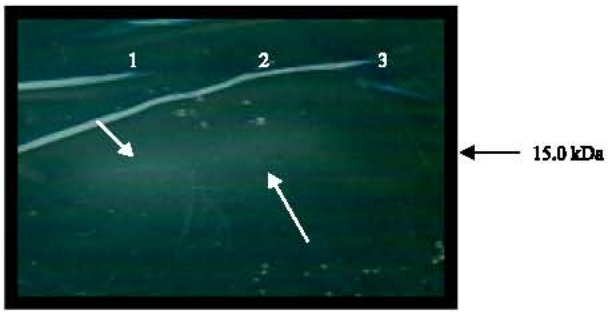

Fig. 5: Substrate gel (zymogram) showing white precipitation zones (arrows) on agar/Tween-20 plate. 100 µg of total *E. coli* DH5α pTrc His C3 proteins were loaded onto 10% SDS-PAGE and after the completion of the electrophoresis process, the proteins on the gel were renatured and overlayed ibto agar/Tween-20 plate at 37°C till the precipitation zones appeared.

utilizing the zymogram technique. It was found that, a white precipitation zones were formed at the sites where lipase protein specific bands were found (Fig. 5 Lanes, 1 and 2) which completely missed in uninduced bacterial culture (Fig. 5, Lane 3). Protein expression studies on the crude recombinant enzyme was conducted to monitor the

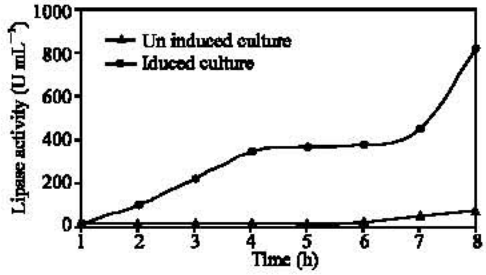

Fig. 6: Monitoring the expression of lipase gene of *E. coli* DH5α pTrc His C3 IPTG induced and uniduced cultures

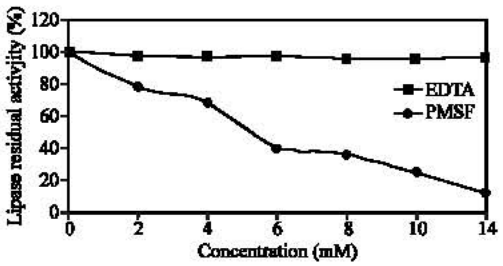

Fig. 7: Effect of EDTA and PMSF on the activity recombinant lipase produced from *E. coli* DH5α pTrc His C3

expression of lipase gene over 8 h after induction with 1 mM IPTG (Fig. 6). It was noticed that the lipolytic activity started to appear after 2 h of induction and reached its maximum level after 8 h compared to the uninduced bacterial culture using the same amount of total proteins and under identical assay conditions (820 units mL^{-1} versus 150 units mL^{-1}) (Fig. 6).

Effect of EDTA and PMSF on lipase activity: Figure 7 showed that the divalent metal-chelating agent, EDTA at a concentration of 10 mM showed slight inhibitory effect on the activity of the recombinant lipase, on the other hand, PMSF at a concentration of 10 mM showed a drastic inhibitory effect on lipase activity as shown in Fig. 7. The residual activity was found to be 25% at 10 mM PMSF and as the inhibitor concentration increased the residual activity decreased.

DISCUSSION

Several intra- and extracellular lipases have been fully or partially characterized from various strains of *Pseudomonas*. Commercial lipases are usually manufactured from culture supernatants of lipase secreting strains such as yeasts, fungi or bacteria thus,

allowing for the inexpensive production but in limited quantities. Use of recombinant DNA technology, however, facilitates the economic production of large quantities of almost pure lipases and the engineering of tailored-made enzymes for specific applications (Schmidt-Dannert, 1999). This work described the construction and screening of *P. aeruginosa* genomic library for two different lipases previously isolated and purified by Saeed *et al.* (2005). Primary screening resulted in three positive clones (Fig. 2) which subjected to several round of screening to be sure that all of them are produced recombinant lipase upon induction with IPTG. It is worthwhile noting that most of *E. coli* DH5α pTrc C positive clones were capable of producing a recombinant lipase at quite the same level of expression since most of the recombinant clones produce the same size hydrolytic zones as shown in Fig. 2. Moreover, since *E. coli* cells normally does not produce extracellular recombinant enzyme in the surrounding medium, it was suggested that the produced recombinant enzyme found its way through the *E. coli* periplasmic space and membrane to the outer medium and this explain the clear zones and precipitation zones around the recombinant colonies that resulted from lipase action on its Tween-20 substrate. These results indicated that background lipase activities from *E. coli* host strains was extremely unlikely to account for the results produced upon using recombinant clones. All of these positives clones were found to encode a DNA insert of 1.2 kbp which larger enough to encode a protein of molecular weight of 15.0 kDa that previously was isolated and purified from *P. aeruginosa* parent strain. Pilot protein expression study on SDS-PAGE indicated that *E. coli* DHα5 pTrcHis C3 clones produced a recombinant protein of a molecular weight 15.0 kDa (Fig. 4) after induction with IPTG compared to the uninduced culture. The level of recombinant protein expressed in *E. coli* was much higher than that of the native *P. aeruginosa* strain (14.5 times). To confirm that the protein specific band represent recombinant lipase, a substrate gel was carried out (Fig. 5). It was found that, the renatured recombinant lipase was able to catalyze the enzymatic degradation of Tween-20 substrate that was incorporated into the SDS-polyacrylamide gel given a white precipitation zones around the protein of interest. Again these precipitation zones were of a molecular weight 15.0 kDa (Fig. 5). Time course upon induction with IPTG was carried out to measure the maximum level of expression of the recombinant lipase gene (Fig. 6). It was found that the recombinant protein was started to induced after 3 h of IPTG addition and reached its maximum level after 8 h (800 units mL^{-1}) compared to the uninduced culture filtrate which remain constant through out the time course. The effect of EDTA and PMSF was also studied to partially characterize the crude recombinant enzyme. Fig. 7 showed that the divalent metal-chelating agent, EDTA at various concentrations showed slight inhibitory effect on the activity of recombinant lipase. It was reported that lipase enzyme produced by *P. aeruginosa* EF2 not affected by metal chelating agent, EDTA which indicate that this kind of lipolytic activity independent of metal ions in contrast to some other metal ions dependent *Pseudomonas* lipases (Gibert *et al*., 1991; Fox and Stepaniak, 1983; Yamamoto and Fujiwara, 1988). Since most of the microbial true lipases contain a catalytic triad Ser-Asp-His (Jaeger *et al*., 2003; Kojima *et al*., 2003; Ewis *et al*., 2004), inhibition study using PMSF was carried out. PMSF at a concentration of 14 mM showed a drastic inhibitory effect on lipase activity (residual activity was 12%) as shown in Fig. 7. This inhibitory effect of PMSF also exhibited for some *Pseudomonas* sp. (Van Oort *et al*., 1989; Schrag *et al*., 1991; Svendsen *et al*., 1995).

In conclusion a fragment of 1.2 kbp was isolated from *P. aeruginosa* genomic library that encode a protein of a molecular weight of 15 kDa. Based on partial characterization of the crude enzyme it was found that this protein equivalent in characters to the native protein previously isolated and characterized from the native parent strain (Saeed *et al*., 2005). Partial sequencing of the 1.2 kbp pair fragment (data not shown) indicated that the lipase gene has some sequence similarity with other lipases produced from *Pseudomonas* sp. However, much effort will be needed to complete this sequence and to isolate the other lipase gene from the same library in an attempt to express both of them from the same plasmid as a fusion protein and characterize this protein in term of kinetic studies towards different lipase specific substrates and to classify both of these enzyme among *Pseudomonas lipases*.

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