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THERMOSTABLE RECOMBINANT ESTERASE PRODUCTION IN 3-L STIRRED TANK BIOREACTOR, PURIFICATION AND CHARACTERIZATION

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ABSTRACT

Thermophilic lipases/esterases are currently attracting enormous attention because of their biotechnological potential. It was thus aimed in the present study to optimize enzyme on large scale production, to purify and characterize the produced enzyme. The *est* fragment of *Geobacillus* sp. AZ1 (ac: KM823656) was cloned directly by PCR into the pCYTEXP1 expression vector under the control of lambda promoter. Good intracellular expression of the studied gene was obtained in *Escherichia coli* DH5α. Optimization of the recombinant protein production was carried out using a 3L bench-top bioreactor with a stirred tank. The results showed that the fermenter condition and induction by shifting temperature from 37°C to 42°C at OD. 0.7 resulted in a 2-fold increase. The expressed protein was fused C-terminally with 6x-his tag to allow one step purification using IMAC (Immobilized Metal Affinity Chromatography). A 28kD protein was achieved. The kinetic characterization of the purified enzyme exhibited maximum activity at 50°C and pH 7.4. The enzyme had a considerable thermal stability. The percentage of remained activity after 1h exposure to 50, 55 and 60°C reached to 50, 42 and 27%, and retained more than 60% of its original activity at 65°C for 15min. However, exposure of crude enzyme to these conditions showed a complete stability and more than 90% of activity. The enzyme was also highly stable in a pH range of 3-10 for 24h. The enzyme activity was promoted in the presence of Co^{+2}, K^{+1}, Ca^{+2} and Fe^{+2} and was inhibited by Mn^{+2}, Mg^{+2}, Cu^{+2}, Hg^{+2}, Zn^{+2}. Diethyl-ether, and acetone but hexane enhanced the activity. On the contrary n-butanol, DMSO, ethanol, isopropanol, glycerol, methanol and chloroform, reduce the enzyme activity. EGTA, DTT, EDTA, PMSF and SDS decreased the enzyme activity, whereas the presence of urea, oxidizing and reducing agents, some non-ionic surfactants increased the enzyme activity. The values of K_m and V_{max} as calculated from the Lineweaver- Burk plot were 12.66 mM and 333.33 U/mg protein respectively.

KEYWORDS: Recombinant Esterase; Histidine Tag; Purification; Stirred Tank Bioreactor.

INTRODUCTION

Microbial sources are superior to plants and animals for enzyme production and this can be attributed to the ease with which they can be mass cultured and genetically manipulated (Hasan *et al*., 2006). Commercial microbial lipases are produced from bacteria, fungi and actinomycetes (Babu and Rao 2007). Lipases obtained from culture supernatants suffer various disadvantages like non-reproducibility of results, undesirable side-effects and demand tedious purification processes. Recombinant lipases however, overcome all these constraints and help in large scale production of pure lipases which may or may not be tailor-made (Schmidt-Dannert 1999). The popular expression host is *E.coli* (An *et al*., 2003; Long *et al*., 2007), while other efficient hosts include *Pichia pastoris* and *Saccharomyces cerevisiae* (Ramchuran *et al*., 2006; Mormeneo *et al*., 2008). The high-level expression of lipase in *E. coli* often results in formation of insoluble and inactive inclusion bodies (Akbari *et al*., 2010). The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier. Bacteria produce different classes of lipolytic enzymes, 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 phospholipids (Arpigny and Jagger, 1999). They catalyze the hydrolysis of ester bonds of triacylglycerols at the interface between an insoluble substrate and water. Lipases are thus of particular importance in biotechnology because of novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agrochemicals, and flavour compounds (Jaeger and Eggert 2002). Large amounts of such enzymes are required for fundamental studies as well as for biotechnological application. The results of production of fermentation products aerobically in shake flasks usually cannot be extrapolated to indicate possible performance in a fermenter. Both physical and biological factors are quite different in a fermentor and in shake flask. Moreover, controls on the reaction in a shake flask are extremely limited while in a fermentor such controls are almost limitless. Accordingly, results obtained in a shake flask should be taken only as preliminary indicators of the conditions necessary for successful industrial production and must be verified in studies carried out in a fermentor.

MATERIALS AND METHODS

Recombinant DNA techniques: DNA manipulations were performed as described by Sambrook *et al*., (1989). Restriction enzymes and other DNA-modifying enzymes were used according to the manufacturer' s (Fermentas International Inc, Canada) recommendations. *E. coli* cells were transformed by "heat shock" method. For cloning

the PCR reactions were repeated using *pfu*-polymerase for blunt end creation. The target product related to each gene was selected, cut and purified using a purification kit [EZ-10spin column, BioBasic INC. Canada]. The PCR products were cloned into p JET 1.2 cloning vector with blunt end, using CloneJET™ PCR Cloning Kit. After transformation some clones were selected and plasmid prepared for sequencing. The plasmid was isolated using Extreme Pure Plasmid Isolation Kit (GEBRI). The presence of insert in the plasmids was verified by applying the PCR reaction using both universal and specific primers. Accordingly, the right plasmid was selected for sequencing by the universal primer.

***Gene expression*:** In order to allow one-step purification by Immobilized Metal Affinity Chromatography (IMAC), the protein variants containing a c-terminal with His6-tag were expressed. *E.coli* DH5α carrying the plasmid (pCYTEX-*est*) containing the modified gene encoding the esterase, were grown in 50ml LB/ampicillin (100mg/l ampicillin) medium overnight at 37°C. Seed culture was prepared so as its final OD at 600nm reached 5.0-5.92 nm before inoculation into the cultivation system. Batch fermentation experiments were carried out in 3L bioreactor of 2L working volume, using the synthetic medium LB. After autoclaving, 2ml ampicillin (100mg/ml) was added, 1% of the working volume (20ml) was inoculated from the seed culture. The pH was uncontrolled. The electrode was calibrated before sterilization and checked afterwards during fermentation run. Foam was controlled manually by the addition of drop from antifoam reagent Galanopon 3002 (Bussetti & Co. Gesellschaft GmbH, Wien, Austria) to the bioreactor medium before autoclaving. The dO2 electrode was activated before autoclaving by electrolyte (Mettler-Toledo GmbH, process analytics Switzerland) and cultivated at 37°C and 170rpm until an OD_{600} of (0.4–1.0). Upon induction by a temperature shift to 42°C cells were cultivated additional 4h, samples (5ml) were taken each half an hour and finally cells collected by centrifugation (10min, 4000rpm, 4°C).

Enzyme assay: The esterase activity of the crude extracts was determined spectrophotometrically using p-Nitrophenyl laurate (Sigma-Aldrich Chemie GmbH, Germany) as substrate. All activity measurements were performed in triplicates and expressed as the arithmetic mean of estimations. A volume of 25µl appropriately diluted sample was dissolved in 725µl phosphate buffer (50mM, pH 7.2). The enzymatic reaction was carried out at 60◦C after addition of 100µl substrate solution (25mM p-Nitrophenyl laurate in absolute ethanol). After 10min of incubation, 250µl of 100mM Na_2CO_3 was added and the mixture was centrifuged at 13,000rpm for 10min. at 4◦C. The absorbance of librated p-Nitrophenol was measured at 420nm. One unit (U) of esterase activity is defined as the amount of enzyme that causes the release of

1µlmol of p-Nitrophenol/ min under test conditions (Becker *et al.*, 1997).

Purification of the recombinant protein: The pellet was dissolved in saline phosphate buffer pH 7.0, sonicated (3x1 min) and after centrifugation the supernatant containing the soluble protein fraction was separated and applied for purification. The purification procedure was performed at room temperature and the recombinant protein was purified under native conditions using TALON™ Metal Affinity Resin (BD Biosciences Clontech, Heidelberg, Germany) according to manufacturer's recommendations at pH 7.0 using imidazole for elution. An 8ml of the IMAC matrix was filled in a column and equilibrated with 3 volumes of saline phosphate buffer (50mM, pH 7.0). After applying the sample it was washed with saline phosphate buffer (50mM, pH 7.0). Fraction-wise elution of bound protein was obtained by applying 150mM imidazole in saline phosphate buffer (50mM, pH 7.0). Remaining imidazole was removed by gel filtration using a PD-10 column (Amersham Biosciences, Freiburg, Germany).

Determination of protein concentration: Protein concentration in crude and purified samples was determined according to Lowry *et al.*, (1951). To 1ml sample containing protein, 5ml of Lowry C were added and left to stand for 10minutes at room temperature. A volume of 0.125ml of 2N Folin Ciocalteau phenol reagent was added and mixed immediately. After 20min the developed blue color was measured spectrophotometrically at 750nm. A standard curve was prepared using crystalline bovine serum albumin.

***Protein electrophoresis*:** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the method of Laemmli *et al*, (1973) on 10% polyacrylamide gels with 0.1% sodium dodycyl sulfate (SDS) to establish the purity of the enzyme protein. For estimation of relative molecular weight 10% SDS-PAGE was run along with Spectra™ Multicolor Broad Range Protein Ladder, (Fermentas), with apparent molecular weights of 10-260kDa. Protein bands were visualized by staining with Coomassie brilliant blue R250.

Determination of optimal pH, temperature and stability: The optimal pH for the purified esterase was determined by incubating the enzyme–substrate at various pH from 3 to 10 in the following buffers: 50mM citrate buffer (pH 3.0-6.2), 50mM sodium phosphate buffer (pH 6.0-8.0), 50mM glycine-NaOH buffer (pH 7–9), and 50mM carbonate bicarbonate buffer (pH 9.2-10). The pH stability was determined by measuring the residual esterase activity with the purified enzyme previously incubating in each buffer at room temperature for 24h. Esterase activity of each sample in each buffer was measured using the standard assay as described previously. The optimal temperature for the purified lipase was determined by incubating the reaction mixture at various temperatures from 40 to 90ºC.

The thermal stability of the purified esterase was examined by measuring the residual activity after incubation the enzyme mixture at each desired temperature for 1h. Lipase activity of each sample at desired temperature was measured.

***Effect of some additives*:** The enzyme was pre-incubated with 1mM of metal ions ($CaCl_2$, $MgCl_2$, $HgCl_2$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, $FeSO_4$, $CoCl_2$, and KCl), alcohols (ethanol, methanol, butanol and isopropanol) and organic solvents (hexane, chloroform, diethyl-ether, acetone) in addition to DMSO at a concentration of (1%), EDTA, EGTA, phenylmethylsulphonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), urea and DTT at a concentration of (10%) and some surfactants (Triton X-100, Tween-20 and Tween-80) at a concentration 1mM for 15 min at room temperature. The activity was recorded at the optimal conditions of temperature and pH and then the residual activity was determined by comparing with untreated enzyme.

RESULTS

PCR screening for Lipase/Esterase gene: Three sets of degenerate primers: Fset1: 5`-ATGAAAKGCTGYCGGGT-3`, Rset1: 5`-TTAAGGCCGCAARCTCGCC A-3`; Fset2: 5'-ATGATGAAARTTGTT MCGCCGAAG-3', Rset2: 5'TTACCA ATCTAACKWTTCA AGC CGAAG-3'; Fset3: 5'TCCTATGGCT CCTTC CACTGTT-3', Rset3: 5'ATGAAAGC GGGAACACCGCA-3' were used for screening the presence of the Lipase/ Esterase gene in the genomic DNA of *G.thermodenitrificans* AZ1 (ac:KM 82 3655). The degenerate primers were designed based on the aligned sequences of closely related group of Geobacilli. The PCR products were separated and analyzed by gel electrophoresis, where there was no clear amplified band using the first set of tested primer. However, an amplified bands with approximate sizes 750 and 850bps using the second and third primer sets, respectively.

Cloning of PCR product related to the second primer set: The PCR-product (~750bp) with blunt ends was amplified using the second set of primer and *Pfu*-polymerase. The purified product was cloned into pJET-1.2 blunt vector. After ligation and transformation, the resulted plasmids carried the PCR fragment was isolated then subjected to sequencing using the universal primer of the cloning vector FpJE T328: 5'-CGACTCACTATAGGGAGA GCGGC-3' and RpJET422: 5'-AAGA ACATCGATTTTCCATGGCAG-3'. The obtained assembled sequence was submitted into Gen Bank under ac: KM823656.

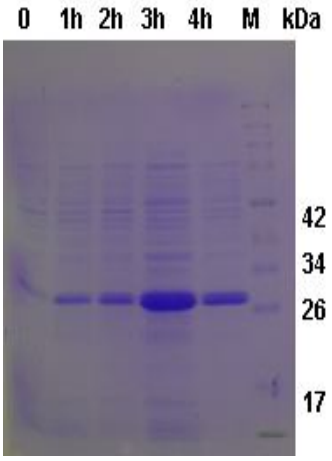
Sequence similarity and phylogeny: Sequence similarity of the coded amino acid for the cloned PCR product proved that it contains the Lipase/Esterase domain (*estAZ1*, ac: KM823656) and showed 99%, 89% similarity to *G.thermodenitrificans* NG 80-2 (ac:CP000557) and *G. thermoleovorans* YN (ac:DQ:288886), respectively.

Heterologus expression of thermostable EstAZ1: Primers for expression of the esterase gene (*estAZ1*) of *G. thermodenitrificans* AZ1 with *Nde*1/ *Eco*R1

sites were designed to express the gene into pCYTEX-P1under lambda promoter. The plasmid carrying the gene obtained from the previous step was used as a template in the PCR. PCR product purification, digestion, ligation and *E. coli* transformation were applied for cloning *estAZ1* into pCYTEX-P1 and creation of pCYTEX-*estAZ1*. The transformants were spread on LB-*amp* tributyrin plates to allow screening for esterase activity. The appearance of clear zone around active clones can be easily observed after induction by shifting the temperature to 42°C. The active clones picked and allowed to grow in LB-*amp* for extraction of their plasmids. The presence of target gene in these plasmids was verified by PCR using both of the universal primer of the expression vector [SDM1F: 5'CCAACA CTACTACGTTTTAACTGAAACAAAC TGG3' and SDM3R: 5'GCGA ACGCC AGCAAGACGTAGCCCAGC3'], as well the specific primer for the gene.

Fusion of C-terminal estAZ1 with 6x his tag: In order to purify the recombinant esterase, C-terminally 6x his-tag was fused to express protein of *estAZ1* gene in the tagged form. This was carried out again by PCR amplification using the former forward primer and a modified reverse primer containing the codon encoding the 6x his-tag. The PCR product was purified, subjected to restriction digestion with *Nde*I/*Eco*RI, and then ligated to respective sites of cut expression vector pCYTEX-P1 to create a new construct (pCYTEX-6x his *estAZ1*). After transformation the released clones were screened in LB/*amp* tributyrin agar plates, where the active clones showed a halo zone after induction by shifting the temperature from 37-42°C. The presence of 6x his in frame was verified by sequencing the plasmid (pCYTEX-6x his *estAZ1*) using SDM1 and 3 primers.

Heterologous over-expression of the tagged estAZ1: For expression of the tagged *estAZ1*, *E. coli* DH5α harboring the vector (pCYTEX-6x his *estAZ1*) was cultivated overnight in 5ml LB broth ampicillin. A portion of 50ml LB ampicillin medium in 250ml Erlenmeyer conical flask was inoculated with 500µl of overnight culture, incubated at 37°C with shaking (200 rpm) till OD_{600} 0.7-1.0. For induction, the temperature was shifted to 42°C, and then the samples were collected regularly for protein analysis and activity measurements. Expression analysis was performed for all samples taken before and after induction (temperature shift). SDS-PAGE revealed a band of the appropriate size (~30kD), only in samples taken after induction (Fig-1), whereas, un-induced culture (Lane 1) showed no band. Also, quantitative estimation of activity was recorded after disrupting the collected cells by sonication. A progressive increase in activity measurements is easily noticed by increasing the time and the maximum was attained after 3hrs induction (22.1U/ml/min). The amount of expressed protein also progressively increased by time (1-3h) after induction.



Fgure-1: SDS-PAGE analysis of total cellular proteins stained with Comassie brilliant blue. M: multicolor broad range protein ladder, Lane 1: cell extract before induction, Lane 2-5: cell extract after one, two, three and four hours induction.

Optimization of protein expression induction: For optimization of protein expression in an *E. coli* system; three factors were studied; the induction temperature, the OD at which the induction occurred and the induction medium. *E.coli* DH5α harboring the vector (pCYTEX-6x his *estAZ1*) was cultivated overnight in 50ml LB/ ampicillin broth. A portion of 50ml (different *E.coli expression* media)/ampicillin in 250ml Erlenmeyer conical flask was inocu-lated with 500µl of overnight culture, incubated at 37°C with shaking (200rpm) till OD_{600} 0.7. For induction, the temperature was shifted to 42°C and then the samples were collected regularly for activity measurements. Samples (5ml) were taken each half an hour centrifuged 10,000rpm for 10min, pellet was washed with phosphate buffer (pH 7.4) then dissolved in 1ml phosphate buffer (pH 7.4), sonicated and analyzed for enzyme activity and found that the media with LB composition is the best induction medium (Fig- 2) where after 3h from induction in LB the activity obtained was 22.19 U/min/ml whereas YT only 21U/min/ml. SOB the highest activity 21.4U/min/ml was obtained after 4h from induction. SOC the highest activity 11.11U/ min/ml was obtained after 2h from induction. Terrific broth the highest activity 13.77U/min/ml was obtained after 4h from induction. For scaling up the production; Seed culture was prepared so as its final O.D. at 600nm reached 5.0-5.92 nm before inoculation into the cultivation system. Batch fermentation experiments were carried out in 3L bioreactor of 2L working volume, containing synthetic medium LB. After autoclaving, 2ml ampicillin (100mg/ ml) was added, 1% of the working volume (20ml) was inoculated from the seed culture in parallel with (50ml) Erlenmeyer conical flask cultivation at 37°C with shaking.

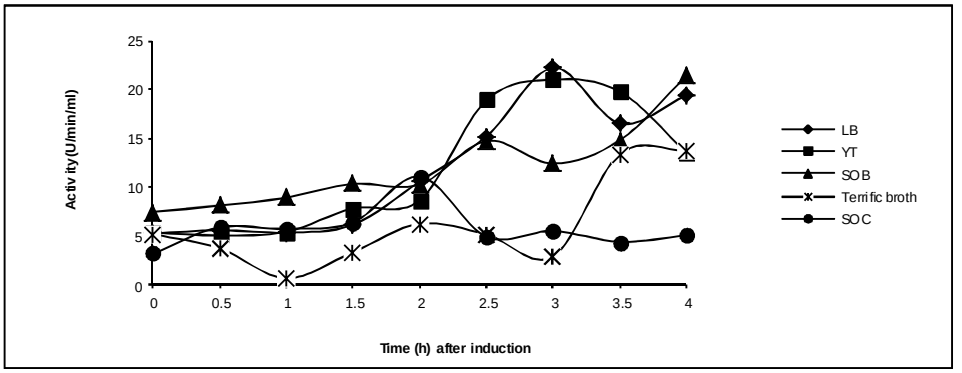


Figure- 2: Effect of medium variation on the expression of recombinant EstAZ1

After autoclaving, 2ml ampicillin (100mg/ml) was added, 1% of the working volume (20ml) was inoculated from the seed culture in parallel with (50ml) Erlenmeyer conical flask cultivation at 37°C with shaking (200 rpm) till OD_{600} 0.7, the temperature was shifted to 42°C, and then the samples were collected regularly for activity measurements from both the fermentor and the flask. Expression analysis was performed for all samples taken before and after induction (temperature shift). Figure-3 showed that the fermentor can be used to increase yields by 2-folds where after 3h from induction in fermentor the activity obtained was 51.70U/min/ml in contrast to flask only 22.19U/min/ml. For studying the induction temperature, 3L bioreactor of 2L working volume, containing the synthetic medium (LB/ampicillin) was inoculated with (20ml) from the seed culture, incubated at 37°C with shaking (200rpm) till OD_{600} 0.7, the temperature was shifted to 40, 42 and 44°C (In three separate runs), and then the samples were collected regularly for activity measurements.

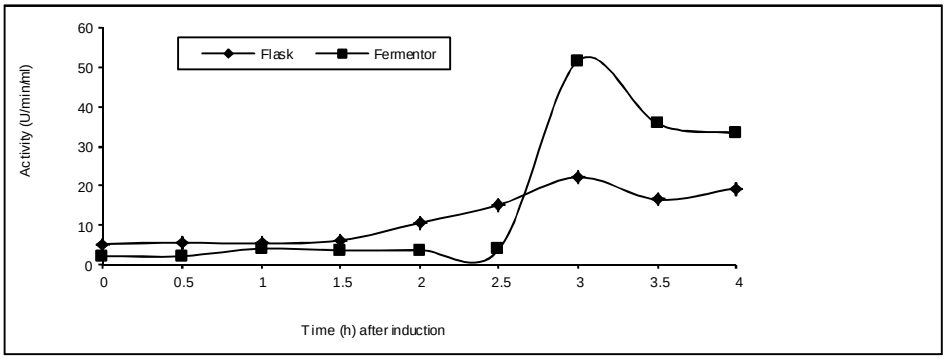


Figure- 3: Expression of recombinant EstAZ1 in flask and fermentor cultures.

Expression analysis was performed for all samples taken before and after induction (temperature shift) (Fig-4). The results suggested that the temperature shift to 42°C is the best where the enzyme activity obtained after 3h from induction was 51.7U/min/ml in contrast temperature shift to 44°C and 40°C resulted ted in 28.26U/min/ml after 2.5h from induction and 23.70U/min/ml after 3.5h respectively. For further optimization of the fermentor culture, growth was allo-wed up to OD_{600} of 0.4, 0.7 and 1 after which the temperature was shifted to 42°C cells were cultivated additional 4h, samples (5ml) were then collected regularly each half an hour and finally cells collected by centrifugation (10min, 4000rpm, 4°C).

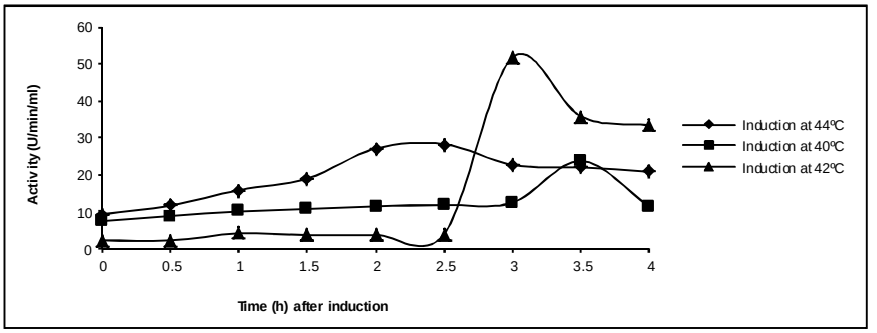


Figure- 4: Effect of induction temperature on EstAZ1 enzyme expression.

Expression analysis was carried out on all samples taken before and after induction (temperature shift) (Fig-5) and found that the induction at OD_{600} of 0.7 is the best where the enzyme activity obtained after 3h from induction was 51.7U/min/ml in contrast the induction at OD_{600} of 0.4 and 1 gave 15.73U/min/ml and 28.88 U/min/ml after 2h from induction.

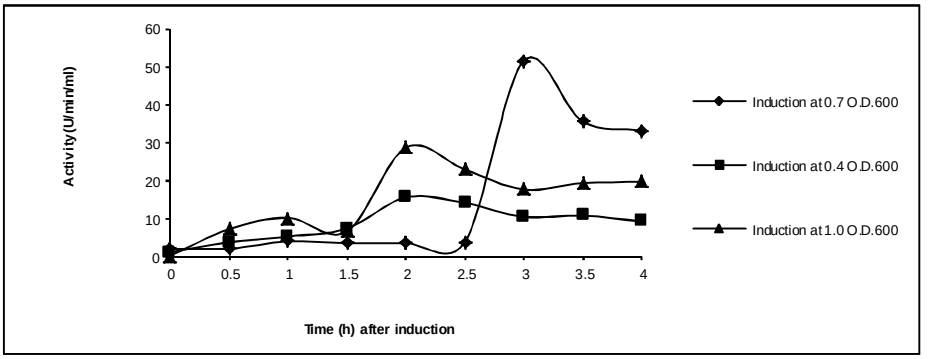


Figure-5: Effect of induction at different cell densities on EstAZ1 enzyme expression.

Purification of the tagged EstAZ1: The purification was performed using IMAC (Immobilized Metal Affinity Chromatography). The tight association between the tag and IMAC cobalt resin allowed contaminant to be washed away under stringent conditions (fraction numbers 1-21). The bound proteins were eluted gently by competition with imidazole (fraction numbers 22-42). Enzyme activity was determined in the collected IMAC washed eluted fractions (1-42) using pNP-laurate. It was noticed that there was no activity in the IMAC washed fractions. The activity started to appear in fractions 27-30 after elution using IMAC/ Elution buffer with gradual increasing from fraction 27 to 30. The purity was tested by SDS-PAGE and showed in Figure 6.

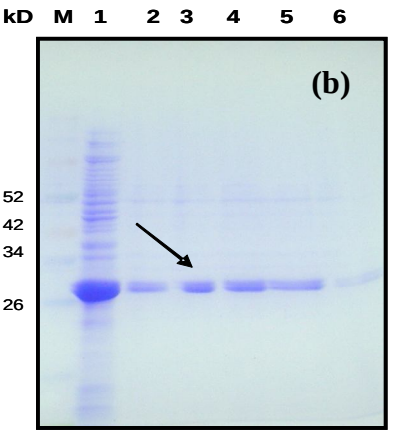


Figure- 6: SDS-PAGE analysis of cellular proteins stained with Comassie brilliant blue. M: multicolor broad range protein ladder, Lane 1: crude extract, Lane 2-6: eluted fraction numbers 27-31.

Characterization of the purified recombinant EstAZ1: The optimum temperature of the purified enzyme was determined at temperature range (40-90◦C), where the maximum activity (90.37U/min/ml) was obtained at 50°C and decreased by raising the temperature to 90°C. The thermal stability of the purified enzyme was determined and shown in Figure 7a. It was noticed that, the purified EstAZ1 was relatively thermostable at tested temperatures 50, 55 and 60°C. The percentages of remained activity after 1h exposure were 50, 42 and 27%, respectively. However, the exposure of crude enzyme to these conditions showed almost a complete stability and more than 90% of activity was kept. At temperature 65°C, the enzyme activity progressively decreased by time elongation but, more than 60% of activity remained within the first 15min exposure (Fig-7b). The optimum pH of the purified enzyme was determined at pH range (3-10), where the maximum activity (95.70U/min/ml) was reached at pH 7.4. The pH stability of the purified enzyme was tested at pHs 3,5,7,8,10. The purified EstAZ1 retained 122,121,96, 96 and 57% of its original activity at pH 7,8,5,10 and 3 respectively after 24h of incubation.

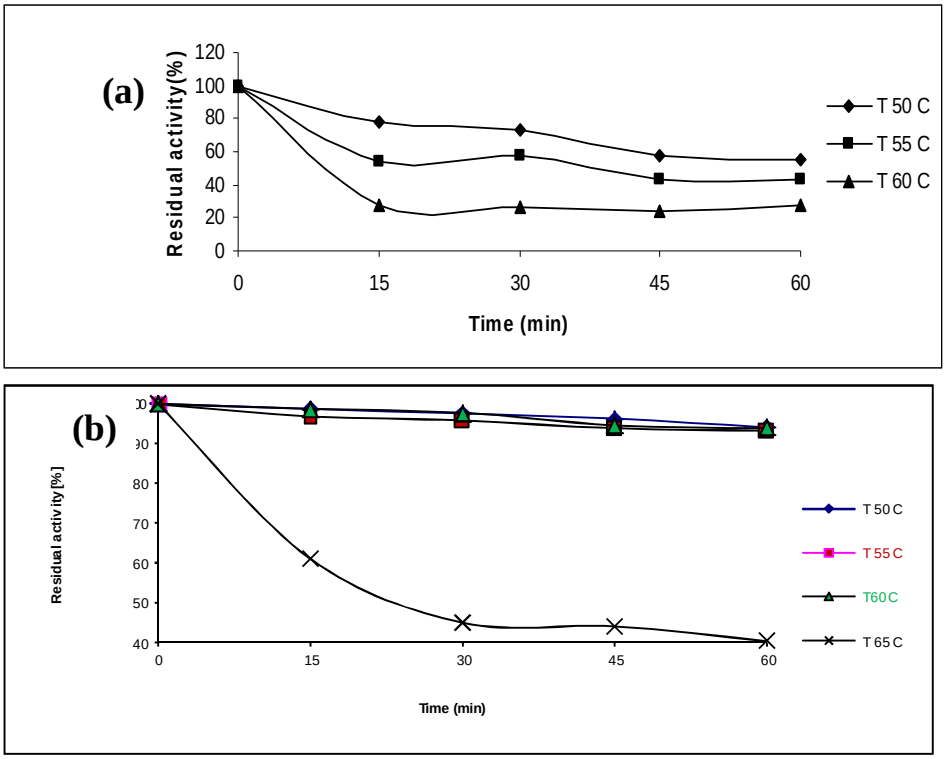


Figure- 7: Effect of temperature on the activity of the esterase:
(a) Thermalstability of purified enzyme at (50-60ºC) under the following reaction conditions: pNP-laurate as a substrate, pH 7.4 and temperature 50ºC, time interval for exposure 15-60 min.
(b) Thermalstability of crude enzyme at (50-65ºC) under the following reaction conditions: pNP-laurate as a substrate, pH 7.4 and temperature 50ºC, time interval for exposure 15-60 min.

The effect of various cations (Ca^{+2}, Mg^{+2}, Hg^{+2}, Mn^{+2}, Zn^{+2}, Cu^{+2}, Fe^{+2}, Co^{+2} and K^{+1}) at a concentration of 1mM on the residual activity of the purified esterase were investigated. The results presented in Table 1a, shown that Co^{+2}, K^{+1}, Ca^{+2} and Fe^{+2} enhanced the activity with residual activities of 127, 122, 106 and 101% respectively; compared to the control. On the contrary Mn^{+2}, Mg^{+2}, Cu^{+2}, Hg^{+2} and Zn^{+2} showed inhibitory effects with 82, 74, 65, 45 and 48% residual activities, respectively. The effect of some organic solvents: ethanol, methanol, butanol, isopropanol, hexane, chloroform, diethyl-ether, acetone and DMSO at a concentration of (1%) on the residual activity of the purified esterase was investigated. The results presented in Table 1a, showed that diethyl-ether, acetone and hexane enhanced the activity with percentages of 121, 120 and 108% respectively; compared to

the control. On the contrary n-butanol, DMSO, ethanol, isopropanol, methanol and chloroform showed inhibitory effects with 93, 88, 88, 55, 47 and 43% residual activities, respectively. The enzyme was quite stable in some non-ionic surfactants and retained 568, 284 and 29% of its maximum activity in the presence of Triton X-100, Tween-20 and Tween-80, respectively (Table-1a). The effect of EDTA, EGTA, PMSF, urea, DTT and SDS at a concentration of 1mM on the residual activity of the purified esterase were investigated. The results presented in Table 1b, which showed that urea enhanced the activity and the percentage reached to 132. On the contrary EGTA, DTT, EDTA, PMSF and SDS showed inhibitory effect and the residual activity reached to 91, 87, 58, 50 and 47%, respectively. The enzyme was stable with tested oxidizing and reducing agents as shown in Table 1b. The enzyme retained 125 and 106%, of its maximum activity in presence of oxidizing agents ammonium persulfate (1mM) and potassium iodide (1mM). However, in presence of reducing agent ascorbic acid (1mM) and 2-mercaptoethanol (2%), the enzyme retained 88 and 171% of its activity, respectively.

Table-1a: Effect of different divalent metal ions and solvents on the activity of the purified EstAZ1 recombinant protein.

Treatment	Concentration used	Residual activity [%]
Ca^{+2}	[1mM]	106
Mg^{+2}	[1mM]	74
Hg^{+2}	[1mM]	48
Mn^{+}	[1mM]	82
Zn^{+2}	[1mM]	45
Cu^{+2}	[1mM]	65
Fe^{+2}	[1mM]	101
Co^{+}	[1mM]	127
K^{+1}	[1mM]	122
Ethanol	[1%]	88
Methanol	[1%]	47
Isopropanol	[1%]	55
n-Hexane	[1%]	108
Chloroform	[1%]	43
Diethylether	[1%]	121
Butanol	[1%]	93
Acetone	[1%]	120
DMSO	[1%]	88

Table 1b: Effect of detergents, anionic surfactant, chelating agent, some reducing and oxidizing agents on the activity of the purified EstAZ1 recombinant protein.

Treatment	Concentration used	Residual activity [%]
Triton X-100	[10%]	568
Tween 20	[10%]	284
Tween 80	[10%]	29
SDS	[1mM]	47
EDTA	[1mM]	58
EGTA	[1mM]	91
PMSF	[1mM]	50
Urea	[1mM]	132
DTT	[1mM]	87
β-mecaptoethanol	[2%]	111
Ascrobic acid	[1mM]	98
Ammonium per-sulphate	[1mM]	100
KI	[1mM]	99

In order to establish the optimum substrate concentration of pNP-laurate (C12) substrate, the relationship between the substrate concentration and reaction velocity (U/mg protein) was determined, where the specific activity of the enzyme increased progressively in a concentration dependent manner up to rather high substrate concentration (25mM), after that saturation was reached. When the initial velocity of hydrolysis of pNP-laurate (C12) was measured as a function of substrate concentration and plotted as double reciprocals in accordance with the Lineweaver-Burk analysis (Fig- 8), the plot gave a K_m value of 12.66 mM and *Vmax* 333.33U/mg protein.

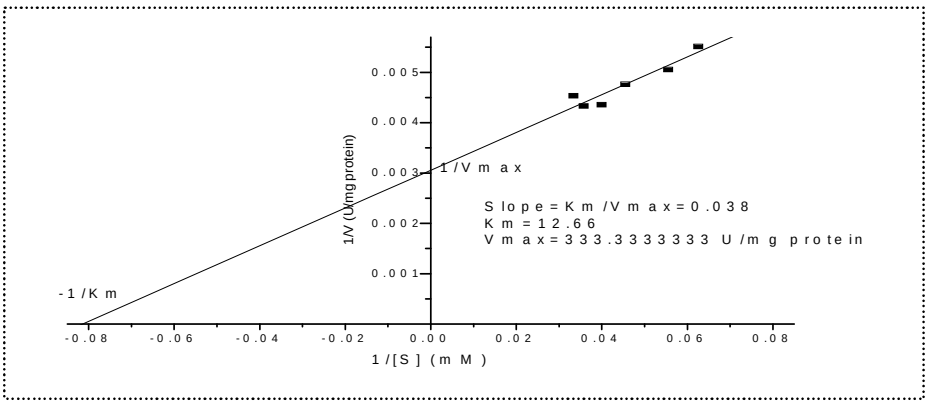


Figure- 8: Lineweaver-Burk plot for the purified EstAZ1 recombinant enzyme.

DISCUSSION

Use of recombinant DNA technology facilitates the economic production of large quantities of almost pure lipases and the engineering of tailored-made

enzymes for specific applications (Schmidt-Dannert, 1999). Because of the great stability of hydrolases from thermophiles (Herbert, 1992 and Jaeger *et al*, 1994), pCYTEXP1 was chosen as a suitable vector for expression of *estAZ1* of the experimental bacterium. The esterase gene was cloned directly by PCR then expressed into pCYTEXP1. The same vector was successfully used by Mc Carthy, 1991; Rua *et al.*, 1997 and Soliman *et al.*, 2007 for expression and producing large amounts of an active enzyme. However, PLE was unsuccessfully expressed using pCYTEXP1vector (Lange *et al*, 2001), this may be because of the eukaryotic origin of PLE; otherwise, it was successfully expressed using yeast expression vector. In this study, expression of *estAZ1* of the experimental bacterium was performed under the regulation of a strong λ promoter in *E. coli* DH5α. A good intracellular expression was obtained after induction. Similarly, both of *B. subtilis* and *B. stearothermophilus* esterases were cloned and efficiently expressed in *E. coli* using an L-rhamnose-expression system (Henke & Bornscheuer, 2002) with higher yield compared to expression in *B. brevis* (Amaki *et al*, 1992 and Tulin *et al*, 1993). Sequencing and comparative similarity search of putative *estAZ1* of the experimental bacterium suggest that it encodes a polypeptide of 248 amino acid residues including the conserved pentapeptide motif GXSXG. BLAST search revealed 99%, 89% similarity to *G. themodentrificans* NG80-2 (ac: CP000557) and *G.thermoleovorans* YN (ac:DQ:288886) esterases, respectively. Production of fermentation products aerobically in shake flasks usually cannot be extrapolated to indicate possible performance in a fermentor (Dewitt *et al.*, 1989). Controls on the reaction in a shake flask are extremely limited while in a fermentor such controls are almost limitless. Accordingly, results obtained in a shake flask should be taken only as preliminary indicators of the conditions necessary for successful industrial production and must be verified in studies carried out in a fermentor . The dissolved oxygen (DO) rapidly decreased during exponential growth phase because of respiration of the cells. During the stationary phase (DO) levels started increasing probably because of a decrease in the respiration rate of cells. Meesters *et al*, (1996) attributed this increase of dissolved oxygen to the lack of substrate and used the increase of (DO) as signal to feed the fermentor. Enzyme purification is usually carried out by classical methods; these methods were applied for purification of lipolytic enzymes from bacilli (Kugimiya *et al*, 1992; Lesuisse *et al*, 1993; Dannert *et al,* 1994; Rua *et al*, 1997 and Lee *et al.*, 2001). The main disadvantage of those methods are that, they require long time, great effort and is usually associated with a low yield of purified protein. Thus for purification of the expressed protein the strategy of fusing the protein with 6x his tag can be applied to avoid these

problems and purify the recombinant protein under well-defined, generic conditions (Nthangeni *et al*, 2001). The preparation of purified and concentrated enzyme solution permitted a study of some factors affecting enzyme activity. It is known that metal ions have an important role to maintain the enzyme in active and stable structure by binding to amino acid residues with negative charge in specific sites (Çolak *et al.*, 2005). The effect of various cations (1mM) on the activities of the purified esterase was tested. Co^{+2}, K^{+1}, Ca^{+2} and Fe^{+2} enhanced the activity. On the contrary Mn^{+2}, Mg^{+2}, Cu^{+2}, Hg^{+2}, Zn^{+2} showed inhibitory effect. Zn^{+2} showed strong inhibitory effect on the activity of the purified recombinant esterase of the experimental bacterium agreed with the findings of (Lee *et al.*, 2001 and Nthangeni *et al.*, 2001). Stability and activity in the presence of organic solvents and detergents are important properties of an enzyme if it is used as a biocatalyst in the industry. When the effect of some organic solvents and inhibitors (1%) was tested, the results showed that diethyl-ether, acetone, hexane and urea enhanced the activity. On the contrary n-butanol, DMSO, ethanol, isopropanol, methanol, chloroform, EGTA, DTT, EDTA, PMSF and SDS showed inhibitory effects. The inhibiton of esterase activity in the presence of EDTA can be attributed to its metal chelating effect. The inhibition of esterase activity by PMSF may be a result of the presence of serine residues responsible for the hydrolytic reaction in the active site since this inhibitor is known to interact selectively and irreversibly with the serine hydroxyl groups. Several hyperther-mophilic carboxylic ester hydrolases have been tested. The esterase from *Pyrobaculum calidifontis* (Hotta *et al*, 2002) displays high stability in water-miscible organic solvents, and exhibited activity in 50% solutions of DMSO, methanol, acetonitrile, ethanol, and 2-propanol. In addition, the enzyme retained almost full activity after 1h incubation in the presence of the above mentioned organic solvents at 80% concentration.

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