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Characterization and solvent stable features of Strep-tagged purified recombinant lipase from thermostable and solvent tolerant *Bacillus* sp. strain 42

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Abstract - A 1.2 kb lipase gene (AY 78735) from solvent stable and thermostable *Bacillus* sp. strain 42 was overexpressed in a heterologous system that allowed for an extensive characterization of its solvent stability and thermostability. An overexpression was achieved using pET51b vector with *Escherichia coli* host strain BL21(DE3)pLysS, in which optimum expression was at 22-24 h incubation at 37 °C, with lipase activity reached at 80.0 U mL^{-1} (specific activity 160.0 U mg^{-1}), after induction by 0.5 mM IPTG. This expression was 11.5 fold higher and superseded the pQE-30UA/M15(pREP4) host-vector system, which only achieved at 17.0 U mL^{-1} (34.0 U mg^{-1}). The fusion lipase contains N-terminal Strep-tag II affinity tag that in one step of purification, the lipase was purified to homogeneity using Strep-tag II agarose column. The lipase was purified at 1.3 fold and 70% recovery with the elution fraction gave a band of 43 kDa in SDS-PAGE. The purified fusion lipase was most active at 70 °C and pH 8.0, and was stable in a broad pH range of 7-10. It showed hydrolysis preference towards olive, sunflower and corn oils. Based on solvent stability studies in 30 min pre-incubation in 25% v/v solvents with a shaking rate at 150 strokes per min, the purified Lip 42 showed a different residual activity profiles depending on solvents and temperatures. Lip 42 was found be stable in polar organic solvents such as DMSO, DMF, acetone, methanol, heptanol and octanol, which could make it as a potential biocatalyst for the use in industrial biodiesel production.

Key words: solvent stable lipase; overexpression; biodiesel; affinity chromatography; thermostable lipase.

INTRODUCTION

Lipases (E.C. 3.1.1.3) are triacylglycerols hydrolase which catalyze the hydrolysis of fat and oils at oil water interfaces. They have been commonly used in industries since they are thermostable, substrate specific, enantiomerically selective, regioselective and require no cofactor. Practical use of lipase in industries requires them to be slightly thermostable and solvent stable. Enzymatic reactions in non-aqueous solvent offer numerous possibilities that are desirable for production of useful chemicals, using reactions that are not feasible in aqueous media (Krishna *et al.,* 2002). In non-aqueous solvent, hydrolytic enzymes can carry out synthetic reactions and in this condition, some enzymes exhibit altered substrate selectivity, pH memory, improved activity and stability at elevated temperatures. Moreover, the regio-, enantio- and stereo-selectivity of enzymes could be affected by water activities.

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Lipases of microbial origin are generally more stable than lipases from animal or plant and for this reason; it has been a useful source for industrial enzymes. Therefore, they are the most versatile enzymes and known to bring about a range of bioconversion reactions (Vulfson, 1994; Haki and Rakshit, 2003). Amongst the microorganisms, *Pseudomonas* genera are of the major group (Reetz and Jaeger, 1998). Meanwhile, lipases from thermophilic *Bacillus* namely *B. stearothermophilus*, *B. thermocatenulatus*, and *B. thermoleovorans* usually exhibit maximal stabilities. In addition to stability at high temperature, thermophilic enzymes also possess great resistance against proteolysis, detergents and chaotropic agents (Sellek and Chaudhuri, 1999). In view of these reasons, great amount of attentions are given on their future uses in organic solvent.

A thermostable and solvent stable lipase (Lip 42) from *Bacillus* sp. strain 42 was previously isolated from palm oil mill effluent (Eltaweel *et al*., 2005) and shown to have high homology to a thermoalkalophilic lipase from *Geobacillus* sp. strain T1 (Leow *et al*., 2004, 2007). This lipase was then cloned and overexpressed in two types of heterologous expression systems. Firstly, it was cloned into the pQE-30 using *Escherichia*

coli host strain M15(pREP4), and secondly, into pET51b vector using *E. coli* host strain BL21(DE3)pLysS, containing strong T7 promoter system. This article reports some characteristics identified for the purified fusion lipase, highly expressed from pET51b-BL21(DE3)pLysS host-vector system. Expression of lipases using pET expression systems have also been reported elsewhere, for instance, thermostable lipase from *Geobacillus thermoleovorans* Toshki (Abdel-Fattah, 2008), wild-type and its mutant from *Staphylococcus xylosus* (Mosbah, 2006), lipase from *Bacillus licheniformis* (Nthangeni *et al.,* 2001) and lipase from *Serratia marcescens* (Long *et al.,* 2007). In this work, however, this lipase was purified using strep-tag affinity system with more focus given to the characterization of its solvent stability. Many reports and studies on solvent stable lipases were based on crude extracts derived from wild type hosts. Solvent stability study on an overexpressed enzyme is not only resulted in a more reliable assay, but also the recombinant construct could be subjected to various gene manipulations for purification and future target of large scale production. Moreover, unlike Pseudomonas sp, which is commonly found in large-scale lipase productions, the use of Lip 42 in *E. coli* host would omit the requirement for helper protein necessary for optimizing the expression (Frenken *et al*., 1993; Gerritse *et al*., 1998). Additional stability in water miscible solvent such as methanol would propose this enzyme as a suitable source of industrial biocatalyst, especially in bio-diesel production and in polysaccharides methanolysis or acylation.

MATERIALS AND METHODS

Bacterial strain and manipulation. The microbial strain isolated from palm oil mill effluent was identified as *Bacillus* strain 42 based on 16S rDNA (Genbank accession nos. AY763118) (Eltaweel *et al*., 2005). The bacterium was grown on Tryptic Soya broth (TSB) medium and the genomic DNA was isolated and used as template for PCR amplification using forwards primer, 5′-GCATCCCTACGCGCCCATGAT-3′ and reverse primer 5′-TTAAGGCCGCAAGCTCGCC-3′, designed from consensus sequence generated from the alignment of several thermostable lipase genes. The PCR products was sequenced and predicted signal peptide was removed so that the mature sequence was then amplified using another set of primers (Lip42F: 5′-GCATCCCTACCGCGCCAATGAT-3′ and Lip42R: 5′-TTAAGGCCGCAAGCTCGCCAA-3′), spanning the mature lipase ORF.

Cloning and expression. The gene was amplified and cloned into the pQE-30 vector via QIAexpress UA Cloning Kit (Qiagen, Germany) and transformed into *E. coli* host strain M15(pREP4) (Qiagen). Colonies with lipolytic clearing zones were formed on kanamycin and ampicillin containing tributyrin plates. Extracted plasmid from the colonies was double-digested with restriction endonuclease *Eco*RI and *Bam*HI, producing two fragments of size 1251 and 3504 bp corresponding to insert and vector, respectively. The plasmid was then double-digested with *Bam*H1 and *Hind*III and ligated to pET51b vector (Novagen, USA) linearized with the same restriction enzymes. Following ligation, the transformed cell (*E. coli* strain Top 10, Invitrogen, USA) was plated onto Luria Bertani (LB) agar medium containing 50 µg/mL ampicillin, 1% tributyrin and 1% gum arabic. The plasmid from the transformant was extracted, sequenced and re-transformed into *E. coli* strain BL21(DE3)pLysS (Invitrogen). The transformant was inoculated onto LB agar plate containing 1% tributyrin, 1% gum arabic plus antibiotics, 50 µg/mL ampicillin and 35 µg/mL chloramphenicol. The presence of 1.2 kb insert in both *E. coli* strain Top 10 and BL21(DE3)pLysS transformants were then confirmed through PCR amplifications using T7 promoter and terminator primers (vector specific forward and reverse primers) and lipase specific primers (Lip42F and Lip42R).

Optimum isopropyl-β-D-1-thiogalactopyranoside (IPTG) concentration for expression. Inoculum from *E. coli* BL21(DE3) pLysS colonies harbouring the lipase gene was inoculated into 10 mL, and then to 200 mL LB broth containing 35 µg/mL chloramphenicol (Sigma) and 50 µg/mL ampicillin (Sigma), and incubated overnight at 37 °C. The liquid broths were incubated at 37 °C and 150 rpm shaking until the growth had reached OD_{600nm} of 0.5, at which IPTG at concentration of 0.5 mM was added. Following further incubation, 10 mL of aliquots were removed and pelleted at 4 h interval, for lipase assays.

Crude enzyme preparation. The cells pellets were washed and resuspended in 20 mM phosphate buffer, pH 7.0 then lysed by sonication for 2 min on ice. Cells debris was removed by centrifugation and the supernatant was filtered through a 0.45 µm membrane filter.

Lipase assay. Lipase assays were conducted based on spectrophotometric detection of liberated fatty acid from hydrolysis of olive oil substrate, according to Kwon and Rhee (1986). Mixture was prepared with 2.5 mL of olive oil emulsion in 50 mM phosphate buffer pH 7.0 (1:1 v/v), and the addition of 20 µL of 20 mM $CaCl_2$, 0.05 mL enzyme dilution in 3.5 mL total volume of reaction. Reactions were agitated at 200 rpm at 60 °C for 30 min and terminated by addition of 1.0 mL of 6N HCl. Free fatty acid was extracted using 5.0 mL isooctane and this was vortexed. The upper layer (4 mL) was mixed with 1.0 mL cupric acetated pyridine reagent and vortexed. The 715 nm absorbance was measured and compared with oleic acid standard. One unit of lipase activity is defined as 1 µmole of fatty acid released per min at 60 °C assay condition.

Characterization of the purified lipase. Lipase assays were also carried out for stability and activities at different pH and temperatures. The effect of pH on lipase activity was investigated by emulsifying the substrate (olive oil) in buffers with different pH ranging from 4.0 to 12.0 and the effect on lipase stability was investigated by pre-incubating the purified enzyme in buffers of different pH range. The effect of temperature on the lipase activity was investigated by assaying the reaction mixtures at various temperatures ranging from 40 to 90 °C with intervals of 5 °C. The effect of temperature on the lipase stability was determined by pre-incubating the aliquots of the purified enzyme at various temperatures ranging from 60 to 75 °C (at intervals of 5 °C).

Lipase selectivity towards natural oils (sesame, coconut, palm, soybean, corn, rice-bran, sunflower and olive oils) which were emulsified in 50 mM Tris HCl pH 8.0 and used as substrates in the assay reaction as mentioned. The relative activities were expressed as a fraction of the highest activity obtained. Lipase activities in the presences of various metal ions (at 1 and 5 mM), and surfactant (at 0.1 and 1%) were similarly carried out (see results and discussions). The hydrolytic activities towards different triacylglycerols were determined by spectrophotometric assay based on the formation of copper soaps for the detection of free fatty acids according to Schmidt-Dannert *et al*. (1994).

The relative activity was expressed as a fraction of activity to the highest activity obtained.

Affinity chromatography. Purifications were carried out by selective binding of engineered streptavidin called Strep-Tactin, to Strep-tag II fusion protein (Skerra and Schmidt, 1999). The purifications were performed at 4 °C using XK 16/20 column (Amersham Pharmacia) packed with 5.0 mL column volume of Strep-Tag agarose resin (Novagen). The column was washed and equilibrated with the binding buffer (150 mM NaCl, 100 mM Tris-HCl and 1 mM EDTA, pH 8.0) and eluted with same buffer plus 2.5 mM *d*-desthiobiotin, at two column volumes and the fractions were collected at 2.0 mL per tube. The fractions with activities were pooled together and buffer-exchanged against 100 mM Tris-HCl buffer, pH 8.0, using 1.5 mL PD-10 Hitrap desalting column, G25 (Amersham Pharmacia). The concentration of protein was estimated by Bradford method (Bradford, 1976).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein samples from purified fractions were loaded and electrophoresed in 12% SDS-PAGE according to method by Laemmli (1970).

Effect of organic solvents. Purified enzyme aliquots were mixed with the solvents at 25% v/v and pre-incubated in vial tubes at 30, 40 and 60 °C for 30 min with shaking rate at 150 rpm. Following incubation, the mixtures were vortexed for 30 s and 100 µL aliquots were instantly taken for assay. Solvents (at 25% v/v) were selected based on their different in log *P* values: methanol (-0.76), ethanol (-0.24), acetone (-0.23), propanol (0.28), butanol (0.80), propylacetate (1.2), benzene (2.0), heptanol, (2.4), octanol (2.9), ethylbenzene (3.1), *n*-hexane (3.6), heptane (4.0), isooctane (4.5), dodecane (6.6) and *n*-hexadecane (8.8). The stability was expressed as the remaining lipolytic activity relative to that of the positive control containing buffer with no solvent. Blank control contained no enzyme, and all of the residual activities were measured in triplicate. Purified enzymes were concentrated using filter centrifuge (Amicon, Milipore, USA) up to 1 mg/mL. The buffer dilutions containing different compositions of organic solvents: dimethyl sufoxide (DMSO), acetone and 1-octanol, were prepared at 25, 50 and 75% v/v, with volumes adjusted at 1.9 mL using buffer. Before assay, 100 µL enzyme concentrate was added into each solvent dilution.

RESULTS AND DISCUSSION

Amplification of the thermostable organic solvent-tolerant lipase gene

Sequence analysis of the lipase gene showed that it has an open reading frame (ORF) of 1251 bp in length which codes for 416 amino acids protein (Fig. 1). This included the Shine-Dalgarno sequence, the -35 and -10 promoter regions, upstream from the initiation codon ATG at position 1. The putative signal peptide cleavage site was predicted to be located between Ala-28 and -29 and as in many lipases there is a conserved pentapeptide, Ala-Xaa-Ser-Xaa-Gly. In non-Bacillus lipase, residue Gly usually replaces the first Ala in the pentapeptide. The ORF sequence was deposited to the GenBank library (Genbank accession nos. AY787835).

Cloning and expression pET51b/Lip42

Transformant of pQE-30UA-Lip42 plasmid produced clear zone lypolysis after 16 h incubation at 37 °C. For pET51b-Lip42, transformant (in *E. coli* Top 10), limited zone of lipolysis was observed on substrate plate due to poor expression, as in this host, the RNA polymerase do not recognize T7 promoter on pET vector. Moreover, in the absence of IPTG inducer, Lac repressor blocked the transcription of inserted protein (Muller-Hill *et al.,* 1968; Sørensen and Mortensen, 2005). Following the transformation of pET51b-Lip42 into *E. coli* strain BL21(DE3)pLysS, after 16 hours of incubation at 37 °C, active zones of lipolysis were produced which were further enhanced with the addition of IPTG. Therefore, increased in lipase expression in BL21(DE3) pLysS host was due to the presence of T7 RNA polymerase that interacts with the T7 promoter in pET51b vector (Studier and Mofatt, 1986; Studier *et al.,* 1990). Optimal expressions were found after about 22 h when the culture was induced with 0.5 mM IPTG, and the maximum lipase activity was recorded at 80.0 U mL^{-1} culture (approximately 160 U mg^{-1}). In contrast, the *Bacillus* sp. native host (in our previous work) which expressed at 0.6 U mL^{-1}, this level of expression had therefore increased

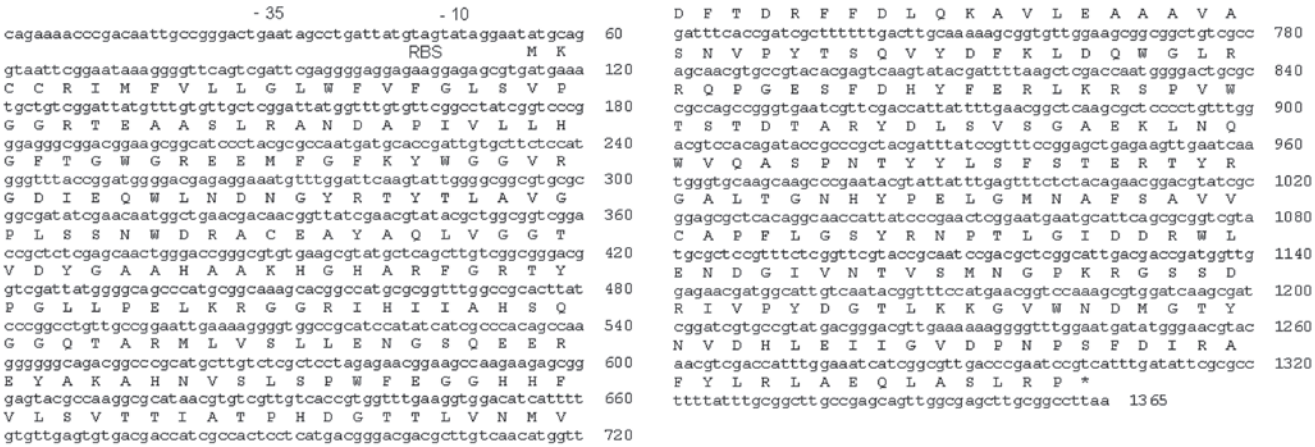

FIG. 1 - Nucleotide and deduced amino acid sequences of the thermostable organic solvent-tolerant lipase gene of *Bacillus* sp. strain 42. The predicted promoter region (-10 and -35 promoter) and ribosome binding site (RBS) are underlined. The arrow indicates the signal peptide cleavage site of the *Bacillus* sp. strain 42 lipase gene. A conserved pentapeptide is highlighted between brackets. Subsequent mature gene were amplified and cloned.

TABLE 1 - Comparison of Lip 42 gene expressions in the two type host-vector expression systems

Vector	Heterologous *E. coli* host	Promoter type	IPTG inducer (mM)	Optimum expression time (h)	Affinity tag for fusion protein	Lipase expression (U mg^{-1})
pQE-30UA	M15(pREP4)	PT5	0.75	3	His-tag	34.0
pET51b	BL21(DE3)pLysS	T7	0.5	24	Strep-tag	160.0

TABLE 2 - Purification charts for Lip 42 usin Strep-tagII affinity chromatography

Sample	Volume (mL)	Protein content (mg/mL)	Enzyme content (U/mL)	Total protein (mg)	Total unit (U)	Specific activity (U/mg)	Purification factor	Recovery (%)
Crude	15.0	0.57	82.2	8.55	1233.6	144.2	1.00	100.00
Pooled purified fraction (26-34)	10.0	0.41	77.9	4.15	778.8	187.8	1.30	70.6

up to as much as 133 fold. This level of expression was still 11.5 fold higher compared to pQE-30UA/M15(pREP4) host-vector system in which expression was achieved only at 17 U mL^{-1} culture (approximately 34 U mg^{-1}). Table 1 summarizes the comparison of lipase expression between the two vectors.

Affinity purification of thermostable and solvent stable lipase 42

As shown on Table 2, pooled fraction recorded approximately 70% recovery and other losses were found to retain in low recovery fractions not included in the pool fraction. The affinity purification profile was shown on Fig. 2, and the pooled fraction has an overall purification factor of about 1.3 fold with protein content of about 0.41 mg mL^{-1}. Result from SDS-PAGE analysis for the purified fractions, as shown on Fig. 3, indicated the presence of band of size 43 kDa. Other organic solvent stable lipases were purified using multiple steps system, and these had contributed to a lower degree of recoveries (Karadzic *et al*., 2006; Yan *et al.,* 2007). By using Strep-tag purification system, single step purification could be achieved under mild condition (Skerra and Schmidt, 1999), effective, and producing protein almost as pure as epitope based systems tags (Lichty *et al.,* 2005).

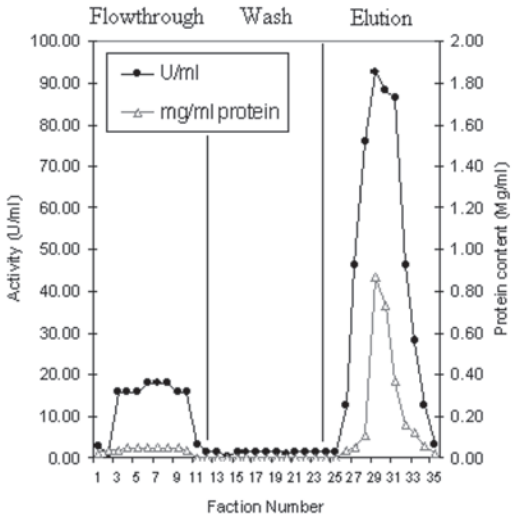

FIG. 2 - Purification profile for Lip 42 using Strep-tag II affinity chromatography.

Characterization of the purified fusion lipase

Various characterizations were carried out on purified Lip 42. This lipase showed an optimum activity at a moderate alkaline pH (7.0 to 9.5), a common feature shared by thermostable lipases from *B. strearothemosphilus*, *B. thermocatenulatus* and *B thermoleovorans* (Nthangeni *et al.,* 2001). Results shown on Table 3 completely summarize the pH activity and stability, the general affects of metal and surfactants, and its specificities for substrates and natural oils for the enzyme.

Solvent stability studies of purified Lip 42

Enzyme activity is usually inhibited in organic solvent concentration higher than 10 to 20% (Gupta *et al.,* 1997). As shown on Fig. 4, the residual activities after 30 min incubation at 25% v/v solvent composition, this fusion lipase showed varying degree of solvent stabilities, depending on temperature and the type solvents. The enzyme was more stable in water miscible solvents, whereby there were enhancements (5-40%) in water miscible solvents

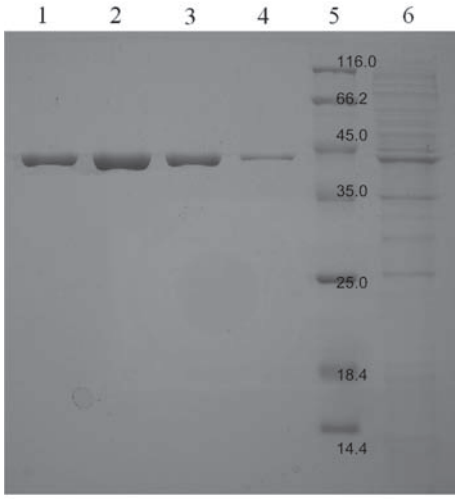

FIG. 3 - SDS-PAGE analysis of the crude and purified fractions from affinity chromatography. Lanes 1, 2, 3 and 4: purified fractions, showing an intense bands of size 43 kDa corresponding to the putative lipase; lane 5: standard molecular weight marker (Fermentas); β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase BSP981 (25.0 kDa), β-lactoglobulin (18.4 kDa) and Lysozyme (14.4 kDa). Lane 6: crude sample.

TABLE 3 - Characterization of the purified lipase Lip 42. Effects of pH, temperature for stabilities and activities, substrates, metals and surfactants

Characteristic	Purified lipase Lip 42	
pH optimum	8	
pH stability	7-9	
Temperature optimum	70 °C	
Temperature stability		
60 °C	315 min	
65 °C	120 min	
70 °C	45 min	
Substrate specificity		
Tricaprylin C_8	100%	
Tricaprin C_{10}	92%	
Olive oil	100%	
Corn oil	90%	
Sunflower oil	88%	
Metal	Concentration 1 mM	Concentration 5 mM
K	115%	128%
Zn	26%	0%
Mg	105%	113%
Ca	113%	118%
Fe	44%	36%
Surfactant	Concentration 0.1%	Concentration 1%
Tween 80	103%	15 to 25%
Tween 60	100%	

The '%' represents activities relative to maximal activity being measured. For effects of metal on enzyme, the '%' records the residual activity to that of control.

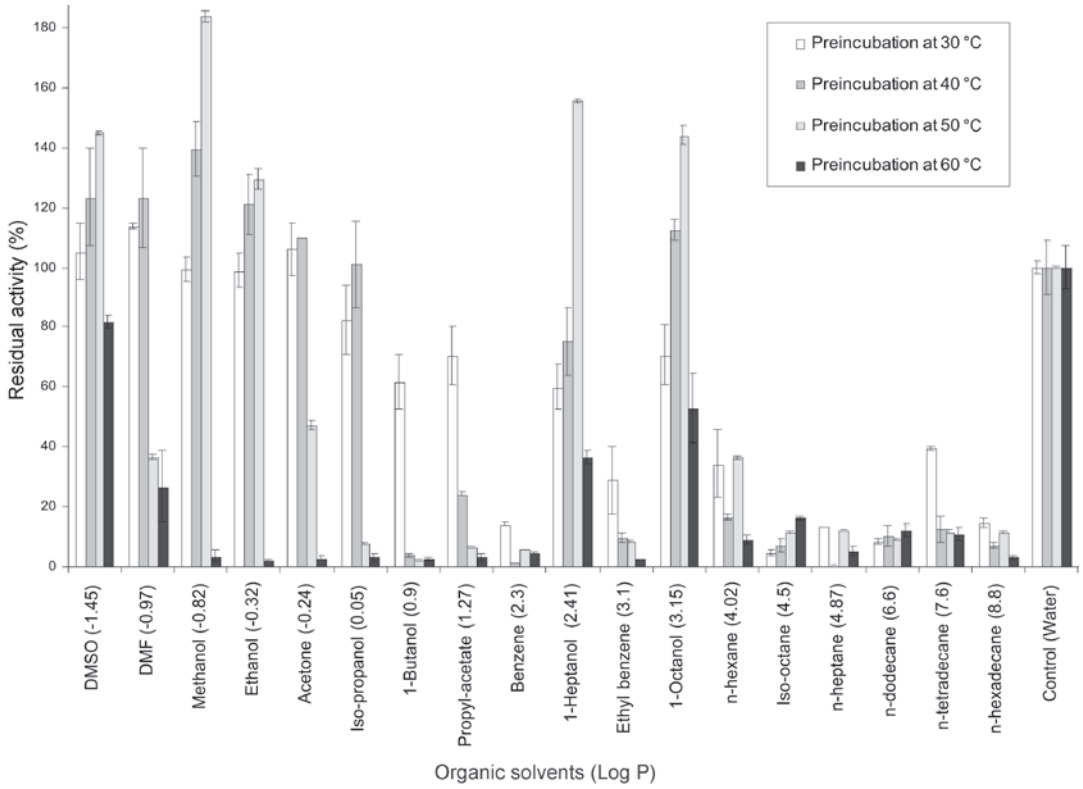

FIG. 4 - Solvent stabilities of lipase 42 in various organic solvents with range of log *P* values (in parenthesis). The purified enzymes were pre-incubated for 30 min at 25% v/v solvent with shaking rates at 150 strokes per min, and assayed for residual activity.

such as DMSO, dimethylformamide (DMF), methanol and ethanol. However, residual activities were rather drop in solvent with middle range log P, but recorded higher again with heptanol and octanol, especially at temperature of 50 °C. Solvents with log *P* values of 4 or higher showed lower residual activities. In contrast, previous work on crude form of this lipase, however, showed high stability in benzene and *n*-hexane (Eltaweel *et al.,* 2005). This indicated that cellular milieu of crude mixture could affect enzyme stability in organic solvent and give rise to a different stability profile.

By using a different assay system, other reports on thermostable lipases had, however, shown a rather similar trend. Sugihara reported that the activity of purified thermostable lipase from *Pseudomonas cepacia* was not impaired during 21 h storage at pH 6.5 in 40% water miscible solvent and the addition of DMSO or acetone to the assay mixtures in the range of 0-35% had stimulated the enzyme, whereas benzene or *n*-hexane gave inhibitory effects (Sugihara *et al*., 1992). Activities for thermo and solvent stable extracellular lipase from a *Pseudomonas* sp. AG-8 was enhanced in 20% v/v ethanol, methanol and acetone by 2.9, 3.6 and 4.5 fold, respectively and at 90% v/v DMSO it was enhanced by 5.7 fold (Sharma *et al*., 2001). Generally, the lower the log *P* value the greater the polarity of solvent, the more toxic they are to the enzyme (Kamini and Iefuji, 2001). However, the ability of this lipase to retain significant residual activities in water miscible solvents such as methanol or ethanol would enhance its industrial use. For instance, in biodiesel production, methanol and ethanol have been used to reduce the viscosity of tryglycerides. Excessive presence of solvent such methanol or ethanol significantly reduced lipase activities, and the use of solvent tolerant lipase could reduce the production cost (Fukuda *et al.,* 2001). Moreover, the presence of hydrophilic solvent together with other non-aqueous solvents was thought to improve the activity of enzyme (Tsuzuki and Kitamura, 2001). The presence of denaturant such as DMSO could induce conformational flexibility on enzyme in non-aqueous media.

It is very difficult to conclude the general trend of lipase stabilities in different type of organic solvents. Meanwhile, purified solvent stable lipase from *Pseudomonas aeruginosa* LST-03 exhibited high levels of activities in the presence of *n*-decane, *n*-octane, DMSO, DMF and stable in the presence of *n*-decane, ethylene glycol, DMSO, *n*-octane, *n*-heptane, isooctane, and cyclohexane than in the absence of an organic solvent (Ogino *et al.,* 2000). Crude organic solvent tolerant lipase from *Staphylococcus saprophyticus* M36 showed decrease in residual activity with the decrease in log *P* value (Fang *et al.,* 2006). Purified organic solvent tolerant and thermostable lipase from fungus *Galactomyces geothrichum* YO5 showed stabilities in both water miscible and water immiscible solvents (Yan *et al.,* 2007).

Solvent stabilities at different pre-incubation temperatures showed increases in residual activities from 30 to 50 °C. For instance, for DMSO, methanol and ethanol, the residual activities increased from 30 to 50 °C, however dropped at 60 °C. This trend can be related to the results obtained by Karadzic *et al.* (2006) for alkaline lipase in which it was generally stable in organic solvents at 50 °C but dropped at 60 °C. Enzyme generally showed to improve in thermal stability in organic solvent. However, enhance stability may usually be accompanied with a lower activity (Klibanov, 2001).

Stabilities of Lip 42 at different solvent compositions

Results for solvent stability studies at different solvent compositions carried out in the presence of DMSO, acetone, and 1-octanol were shown in Fig. 5. In general, stabilities were retained within the region of 25% v/v in the most of the solvents shown. At 50% v/v solvent composition, there was a drop in residual activity in 1-octanol. Residual activities in DMSO and acetone were rather enhanced (~60%) at 25% v/v composition compared to other solvents, but then these were also drop at 75% v/v. Similar trends of bell shape curves displaying peaks of activity at 15 to 45% v/v, followed by activity drops at 50 to 60% v/v were observed in hydrolysis of some other lipases in DMSO (Tsuzuki *et al.,* 2001). Activity enhancements for lipases were commonly observed in low solvent compositions. For a thermostable extracellular lipase from *Bacillus megaterium* AKG-1, 20-70% increases in lipase activities were observed in the presence of 20% v/v acetone, 20% v/v DMSO and10% v/v isopropanol (Sekhon *et al.,* 2005).

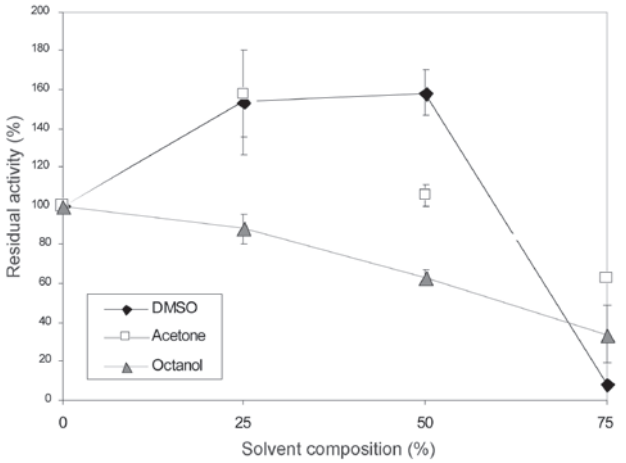

FIG. 5 - Solvent stability studies in different solvent compositions. The measurements are based on pre-incubation at 40 °C, with 150 strokes per min.

Solvent can affect enzyme by denaturation, stabilization or inhibition (Sellek and Chaudhuri, 1999). It is the nature of protein-solvent interactions that determines whether denaturation or stabilization could take place. The stabilization is resulted from compaction of the enzyme as water is excluded from the enzyme hydration layer. Meanwhile, denaturation is due to preferential binding via interaction of the solvent with non-polar residues and the concomitant disruption of the electrostatic interactions or H-bonds (Timasheff, 1993). This phenomenon has been used to explain the effects of solvent such as glycerol, acetonitrile and DMSO on proteins (Gekko and Timasheff, 1981; Kita, *et al.,* 1994; Mozhaev *et al.,* 1996; Gekko, *et al.,* 1998).

In case of DMSO and methanol, it was observed that residual activities depleted dramatically especially at 75% v/v solvent composition. It was shown that DMSO exhibited a transition from preferential exclusion to preferential binding as concentration increased (Sellek and Chaudhuri, 1999). It has been commonly found that some hydrophilic solvents could induce secondary structure rearrangement that resulted in the stabilization of secondary structure elements such as α-helix in protein. For instance, alcohol based co-solvent such as trifloroethanol (TFE) was reported to form such structural elements by destabilizing hydrophobic interaction whilst stabilizing hydrogen bonds (Buck, 1998; Hamada and Goto, 2005). Some proteins exhibit the formation of molten globule at high solvent compositions instead of undergoing denaturation.

CONCLUSIONS

In this work, thermostable and organic solvent stable lipase gene from *Bacillus* sp. strain 42 was successfully cloned and overexpressed into expression vector pET51b, using heterologous *E. coli* host strain BL21(DE3)pLysS that employs T7 expression mechanism. This level of expression superseded the pQE30-Top 10 host-vector system. The fusion enzyme was successfully purified to homogeneity using Strep-tag affinity tag, and shared many common features found in thermostable alkaline lipases. It has a molecular weight of about 43 kDa, and is most active at 70 °C and pH 8.0, and stable in a broad pH range 7-10. This lipase was shown to be highly thermostable with a half life of 315 min at 60 °C, 125 min at 65 °C and 45 min at 70 °C. It has hydrolysis preference towards some of the natural oils such as olive, corn and sunflower oils. Lip 42 showed enhancement in 25% v/v DMSO and acetone, and was able to retain at least 100% residual activity in 50% v/v DMSO. Since it was stable in organic solvent with low partition coefficient, this lipase has great potential for the use, for instance, in biodiesel production. Compared to pQE30-M15(pREP4) system, this fusion construct could give a higher expression level and require a lower level of IPTG inducer. With an improved recovery of purification, Strep-tag fusion system gives better specificity than His-tag system. Moreover, compared to other host systems, the use of *E. coli* would also require a simple large scale design. With this features, this lipase construct could represent an example of recombinant enzyme that is amenable to future rational design and scale up strategy, for the benefit in non-aqueous catalysis.

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