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S5 Lipase : An Organic Solvent Tolerant Enzyme

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In this study, an organic solvent tolerant bacterial strain was isolated. This strain was identified as *Pseudomonas* sp. strain S5, and was shown to degrade BTEX (Benzene, Toluene, Ethyl-Benzene, and Xylene). Strain S5 generates an organic solvent-tolerant lipase in the late logarithmic phase of growth. Maximum lipase production was exhibited when peptone was utilized as the sole nitrogen source. Addition of any of the selected carbon sources to the medium resulted in a significant reduction of enzyme production. Lower lipase generation was noted when an inorganic nitrogen source was used as the sole nitrogen source. This bacterium hydrolyzed all tested triglycerides and the highest levels of production were observed when olive oil was used as a natural triglyceride. Basal medium containing Tween 60 enhanced lipase production to the most significant degree. The absence of magnesium ions (Mg^{2+}) in the basal medium was also shown to stimulate lipase production. Meanwhile, an alkaline earth metal ion, Na^{+}, was found to stimulate the production of S5 lipase.

Keywords: *Pseudomonas* sp. organic solvent lipase, nutritional factors

Many microorganisms, including bacteria, yeast, and fungi, have been determined to secrete lipases during their growth in hydrophobic substrates (Haba *et al.*, 2000). According to Gao *et al.* (2000), many researchers have found that among lipases of various origin (animal, plant, and microorganism), those from bacteria, particularly from *Pseudomonas* species, evidence the highest degrees of versatility, reactivity, and stability in the catalysis of reactions in organic phase. For example, Ogino *et al.* (1994) and Isken *et al.* (1999), reported the isolation of organic solvent-tolerant *Pseudomonas* strains which most effectively secreted lipolytic enzyme. These strains were designated as strains LST-03 and S12.

In addition, the ability of enzymes being active in the presence of organic solvents has received a great deal of attention over the past two decades (Sellek and Chaudhuri, 1999). In an organic solvent environment, some enzymes evidence enhanced thermostability, and it is possible to conduct reactions that are suppressed in water environments (Koops *et al.*, 1999). Substrates and products of lipase-catalyzed reactions are frequently insoluble in aqueous solution, and the enzyme tends to remain insoluble in organic solvents. Reactions catalyzed by lipases are normally conducted in organic-aqueous two-phase media, which are favorable because they render the separation of enzyme from substrates or products fairly easy. However, in general, enzymes are easily denatured, and their catalytic activities are abnegated in the presence of organic-solvents, even in cases in which they are almost water-insoluble (Ogino *et al.*, 1999).

Previously, we reported on the organic solvent-tolerant lipase secreted by the Benzene, Toluene, Ethyl-benzene, and *p*-Xylene (BTEX) degrading *Pseudomonas* sp. strain S5 (Baharum *et al.*, 2003; Rahman *et al.*, 2005a). Few studies regarding the effects of nutritional factors on lipase production have been published, and no reports have thus far become available regarding nutritional factors affecting the organic solvent-tolerant lipase. In this paper, we describe the nutritional factors affecting the generation of an organic solvent-tolerant lipase BTEX degrading bacterium, *Pseudomonas* sp. strain S5.

Materials and Methods

Bacterial strain

Pseudomonas sp. strain S5 was locally isolated from soil. This strain has been identified as a BTEX (Benzene,

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Toluene, Ethyl-Benzene, and Xylene) degrader (Baharum *et al.*, 2003). This strain generates a lipase which is stable in the presence of organic solvents including n-hexane, cyclohexane, toluene, and 1-octanol (Baharum *et al.*, 2003).

Assay of lipase activity

Determination of liberated free fatty acid (FFA) was conducted via the method of Kwon and Rhee (1986), with minor modifications. Olive oil was employed as a substrate. The reaction mixture was comprised of 1.0 ml of crude enzyme, 2.5 ml of olive oil emulsion, and 0.02 ml of 20 mmol $CaCl_2 \cdot 2H_2O$, and was incubated for 30 min at 37°C, with agitation at 200 rpm. The reaction was then halted via the addition of 1.0 ml of 6 N HCl and 5.0 ml of isooctane. The upper layer (4 ml) was pipetted into a test tube, and 1.0 ml of cupric acetate pyridine was added. The free fatty acids dissolved in isooctane were assessed via measurements of the absorbency of isooctane solution at 715 nm. Lipase activity was determined via measurements of the amount of FFA from standard curves of oleic acid. One unit of lipase activity (U) was defined as the rate of enzyme necessary to liberate 1 μmole of free fatty acid in 1 min at 37°C.

Statistical analysis

For statistical analysis, standard deviations for each of the experimental results were calculated using Excel Spreadsheets, with Microsoft Excel software.

16S rDNA sequence identification and phylogenetic tree analysis

Genomic DNA extraction was utilized as a template for the performance of PCR amplification for 16S rDNA identification with a set of universal primers that are highly conserved among prokaryotes and could amplify 1,500 bp. The universal primers used were as follows: Forward: 5'-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G-3'; and Reverse: 5'-CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACT T-3' for 3 min at 94°C, 30 PCR cycles (94°C 1 min, 58°C 2 min, and 72°C 2 min). This was followed by 1 cycle of 7 min at 72°C, and was maintained at 4°C. This reaction was then amplified in a thermocycler (GeneAmp PCR system 2400, Perkin Elmer, USA). The amplified products were evaluated via agarose gel electrophoresis. The PCR product, with a size of 1,500 bp, was purified and ligated into the TOPO TA cloning vector (Invitrogen, USA) in accordance with the manufacturer's instructions, and was then transformed into *E. coli*. The plasmid was then extracted and sequenced. A DNA homology search was conducted using the Genbank database (http://www.ncbi.nih.gov). A phylogenetic tree was constructed using TreeTop Phylogenetic Tree Prediction software (http://www.genebee.msu.su).

Growth curve and lipase production of Pseudomonas sp. strain S5

Bacterial growth and lipase production were determined in 250 mL of basal medium (M1) in 1 L flasks. The basal medium consisted of yeast extract (1.0 g/L), olive oil (5.0 ml/L), NaCl (2.0 g/L), $MgSO_4$ (0.4 g/L), $(NH_2SO_4)_2$ (0.5 g/L), K_2HPO_4 (0.3 g/L), and KH_2PO_4 (0.3 g/L), pH 7.0 (Lee *et al.*, 1999). Samples were harvested at 4 h time intervals for lipase production, bacterial count, biomass determination, and the pH of the culture medium. Biomass was spectrophotometically determined at an absorbance of 540 nm. The bacterial count was conducted via the plating of serial dilutions (10^{-7}, 10^{-8}, and 10^{-9}) of bacteria cultured on nutrient agar plates.

Effect of reaction time on enzyme activity in n-Hexane

The stability of this lipase in various organic solvents was determined in previous studies (Baharum *et al.*, 2003). Lipase activity was reported to be highest in n-hexane. In this study, n-hexane was added to the crude enzyme at a 1:3 ratio, then shaken at 150 rpm at 37°C. One ml of solution was extracted after 5, 10, 15, 20, 25, and 30 min for determinations of lipase activity.

Effect of nitrogen sources

The nitrogen sources used on this study were divided into two groups; namely, organic and inorganic nitrogen sources. In order to determine the effects of different organic nitrogen sources on lipase production, the yeast extract in the basal medium was replaced with peptone, corn steep liquor, soytone, tryptone, and casamino acid, each at a concentration of 5% (w/v). In this study, ammonium sulphate was eliminated from the basal medium.

The effects of inorganic nitrogen sources on lipase production were determined using basal medium absent of any organic nitrogen source. The inorganic nitrogen sources at 0.5% (w/v) used in this study were sodium nitrate, ammonium nitrate, ammonium sulphate, and ammonium chloride. The samples were incubated at 37°C, at a pH of 7.0, for 48 h under static conditions. Lipase production and bacterial growth determination were conducted as mentioned previously.

Effects of carbon sources

On the basis of the results regarding the effects of nitrogen sources, inorganic nitrogen sources were removed from the basal medium. The yeast extract was replaced with peptone, renamed as peptone medium, and used in further study.

In order to elucidate the effects of carbon sources on lipase production, olive oil in the peptone medium was replaced with 1% (w/v) of the following; glucose, sucrose, maltose, galactose, calcium carbonate, sorbitol, or starch, and was incubated at 37°C, at neutral pH for 48 h under static conditions. All carbon sources were separately filter-sterilized using a 0.22 μm membrane filter. Lipase production and bacterial growth determination were conducted as mentioned previously.

Effect of lipids

Each of the lipid sources used was added to basal medium at a concentration of 0.5% (w/v), and was incubated at 37°C, at neutral pH under static conditions for 48 h. The lipid sources used were divided into three groups; natural triglycerides (olive oil, soybean oil, coconut oil, and palm oil), synthetic triglycerides (C2-C18) and fatty acids (oleic acid and palmitic acid). Lipase production and bacterial growth determinations were conducted as mentioned previously.

Effect of surfactants

The following detergents were added to the basal medium at 0.2% (w/v); sodium percholate, sodium deoxycholate, SDS, Triton X-100, Tween 60, Tween 80, and cetyltrimethylammonium bromide (CTAB). These mixtures were incubated at 37°C, at neutral pH for 48 h under static conditions. Lipase production and bacterial growth determination were conducted as mentioned previously.

Effect of metal ions

K^{+}, Zn^{2+}, Na^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ba^{2+}, and EDTA ions were added to the basal medium at a concentration of 1 mM, and were incubated at 37°C, at neutral pH for 48 h under static conditions. The effects of lipase production in the absence of K^{+}, Na^{2+}, and Mg^{2+} were also determined. Lipase production and bacterial growth determination were conducted as mentioned previously.

Results and Discussion

16S rDNA identification and phylogenetic tree analysis

Strain S5 was identified as *Pseudomonas* sp. on the basis of its biochemical properties (Baharum *et al.*, 2003). 16S ribosomal RNA was employed for further identification of the S5 strain. The 16S rDNA nucleotide sequence obtained for S5 was deposited into the Genbank database, and assigned the accession number AY738722. The phylogenetic tree analysis of *Pseudomonas* sp. strain S5 was constructed on the basis of comparison of the 16S rDNA sequence of this strain with other *Pseudomonas* sp. strains available in the NCBI Genbank database. The phylogenetic tree analysis of strain S5 was compared with 10 other *Pseudomonas* spp. sequences. It evidenced a high degree of homology with the majority of *Pseudomonas aeruginosa* strains, including ATCC 27853 (AY268175), SCD-1 (AF448038), BHP7-6 (AY162139), and PAO1 (AE004844). The results of our analyses indicated that *Pseudomonas* sp. strain S5 is phylogenetically distant from other *Pseudomonas* sp., such as strains H157 (AY074896) and X13 (AY631241). The phylogenetic relationship of closely-related *Pseudomonas* sp. is depicted in Fig. 1. On the basis of its morphological, cultural, biochemical characteristics, 16S rDNA strain S5 was identified as *Pseudomonas aeruginosa.* The bacterial pure culture was submitted to the German collection of microorganisms and cell culture (DSMZ), and was assigned the accession number DSM 17160.

Among the other *P. aeruginosa* strains listed in the phylogenetic tree, only *P. aeruginosa* PAO1 (AE004844) was reported to secrete lipase. Thus far, only one strain of *P. aeruginosa* has been reported to generate organic solvent-tolerant lipase. Ogino *et al.* (1994) reported the discovery, via screening, of an organic solvent-tolerant *P. aeruginosa* strain, which generated an organic solvent-tolerant lipase. This lipase proved to be naturally stable in organic solvents (Ogino and Ishikawa, 2001).

Time course studies on bacterial growth and lipase production

A time-course study was conducted in order to determine

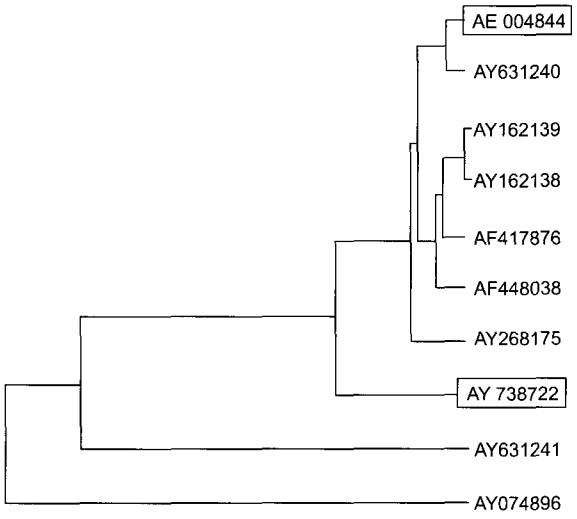

Fig. 1. Rooted phylogenetic tree showing the relationship of isolate S5 to other *Pseudomonas aeruginosa* strains.
Notes: AE004844 (*Pseudomonas aeruginosa* PAO1), AY631240 (*P. aeruginosa* X3), AY162139 (*P. aeruginosa* BHP7-6), AY162138 (*P. aeruginosa* MO2), AF417876 (*P. aeruginosa*), AF448038 (*P. aeruginosa* SCD-1), AY268175 (*P. aeruginosa* ATCC 27853), AY738722 (*P. aeruginosa* S5), AY631241 (*P. aeruginosa* X13), and AY074896 (*P. aeruginosa* H157). Accession numbers in boxes indicate extracellular lipase-producing bacteria.

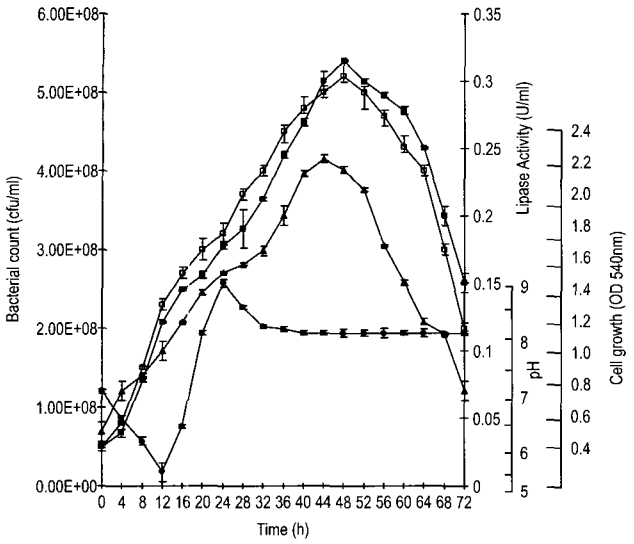

Fig. 2. Growth Curve and Lipase Production of *P. aeruginosa* strain S5.
Notes: Growth and lipase production were investigated in M1 at 37°C and pH 7.0. Samples were withdrawn at 4 h intervals for lipase production (■), bacterial count (□), pH of culture medium (●) and cell growth determinations (△). Results are expressed as the means of three independent determinations. The bar denotes standard deviation. When the error bar cannot be seen, the deviation is less than the size of the symbol.

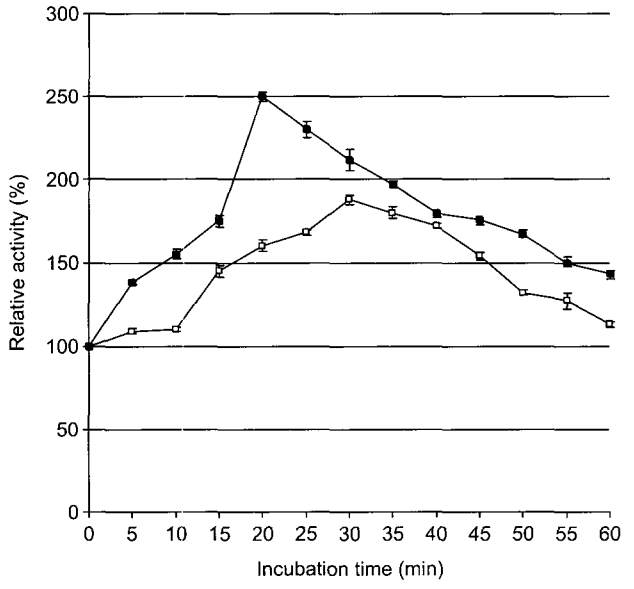

Fig. 3. Stability of S5 lipase in n-hexane.
Notes: The reactions were conducted at 150 rpm, 37°C for 1 h in the presence (■) and absence (□) of 50% n-hexane. Stability of S5 lipase in the presence and absence of n-hexane are shown as values relative to that at time 0. Data are expressed as the means ± standard deviation of three determinations, and indicated as error bars. When the error bar cannot be seen, the deviation is less than the size of the symbol.

the growth and lipase production characteristics of *P. aeruginosa* strain S5 with regard to time. Fig. 2 shows the different parameters determined, including bacterial growth, lipase production and pH. Maximum growth was achieved after 44 h of incubation at 37°C, similar to optical density observations at 540 nm evidencing higher OD readings at 44 h. In basal medium, the logarithmic phase commenced at 8 h and continued for another 12 h. Lipase production was detected in the late logarithmic phase (after 20 h), and increased until optimum production was achieved, after 48 h of incubation. The production of degradative enzymes tends to occur during the late logarithmic phase of growth, when cell density is high. This production occurs via a phenomenon referred to as quorum sensing. Quorum sensing normally involves the activation of specific genes at high cell densities in response to certain chemical signals released by *P. aeruginosa* (Rahman *et al.*, 2005a). This suggests that the lipase production effected by S5 occurred in a growth-dependent fashion.

The pH of the culture dropped from the initial neutral pH to a pH of 5.3 during the first 12 h of incubation. This might be attributed to the production of acids during bacterial growth. However, the pH of the culture medium increased gradually to a pH of 8.5 after 24 h. The pH then gradually dropped, and remained unchanged at pH 7.8 after 48 h. The rise in pH after 12 h of incubation may be due to the utilization of organic acids or the production of alkaline compounds during this period.

Stability of S5 lipase in n-hexane

On the basis of the results of previous studies, S5 lipase exhibited the highest levels of activity in n-hexane, as compared to the other tested organic solvents (Baharum *et al.*, 2003). The stability of crude lipase in n-hexane was also determined. Lipase activity in the cell-free supernatant was measured. The stability of lipase in the presence and absence of n-hexane is shown in Fig. 3. Lipase activity in the presence of n-hexane increased rapidly after 20 min of incubation. However, activity began to gradually decrease after 50 min of incubation. S5 lipase was not only stable, but was also activated in the presence of n-hexane. This may be attributable to the ability of these organic solvents to increase the solubility of substrate (olive oil), thus facilitating the reaction. The observed stability of S5 lipase is suggestive of the notion that the solvent maintains the enzyme in open conformation; the lid of the enzyme does not cover the active site crevice, and thus keeping maintains the flexible conformation (Klibanov *et al.*, 2001).

Enzymes are known to maintain their structural conformations via intra-molecular interactions, including

hydrophobic interactions among component amino acid residues. Meanwhile, non-solvent tolerant enzyme molecules will unfold in the presence of an organic solvent, as the organic solvent will effect a reduction in the polarity of medium surrounding the enzyme molecules (Ogino and Ishikawa, 2001).

Effects of nitrogen sources on lipase production

Table 1 shows the effect of organic nitrogen sources on lipase production. S5 evidenced higher degrees of lipase production (0.335 U/ml) in cases in which peptone was added to the basal medium, followed by cases in which tryptone (0.314 U/ml) and yeast extract (0.258 U/ml) were added. Peptone was determined to be the best organic nitrogen source for S5 lipase, showing 94% and 130% increases in relative activity as compared to what was observed with tryptone and yeast extract, respectively. Peptone was reported to increase lipase production for some microorganisms. For instance, Chander *et al.* (1980) reported that the optimal nitrogen source for *Aspergillus wentii* extracellular lipase production was 5% peptone, followed by tryptone. Peptone as the complex media with a high percentage of nitrogen (14%) supplies the bio-elements required by the microorganism for optimal lipase production. It was not possible to compare these results with the production levels of other organic solvent-tolerant lipases, as no similar studies have yet been undertaken.

Table 1. Effect of nitrogen sources on lipase production

Nitrogen sources	Lipase production (U/ml)
Soytone	0.112 ± 0.002
Peptone	0.327 ± 0.001
Corn steep liquor	0.089 ± 0.003
Tryptone	0.314 ± 0.002
Casamino acid	0.145 ± 0.002
Yeast extract	0.258 ± 0.003
Ammonium sulphate	0.123 ± 0.001
Sodium nitrate	0.106 ± 0.004
Ammonium nitrate	0.071 ± 0.002
Ammonium chloride	0.122 ± 0.003

Note: ± indicates the standard deviation (SD)

The effects of inorganic nitrogen sources were determined by removing the organic nitrogen source (yeast extract) from the basal medium. The inorganic nitrogen source (ammonium sulphate) in the basal medium was then replaced with sodium nitrate, ammonium chloride, and ammonium nitrate. All tested inorganic nitrogen source were determined to reduce bacterial growth, as well as lipase production. Similar lipase production was observed in media containing ammonium sulphate and ammonium chloride. However, lower levels of lipase production were detected in media containing ammonium nitrate as an inorganic nitrogen source, and this was also true of media containing sodium nitrate. Our results showed that peptone was the best organic nitrogen source for S5 lipase production. As the presence of inorganic nitrogen source reduces lipase production, it was removed from the basal medium. Yeast extract in the original medium was replaced with peptone. The new production medium, referred to as peptone medium, was then used for further study.

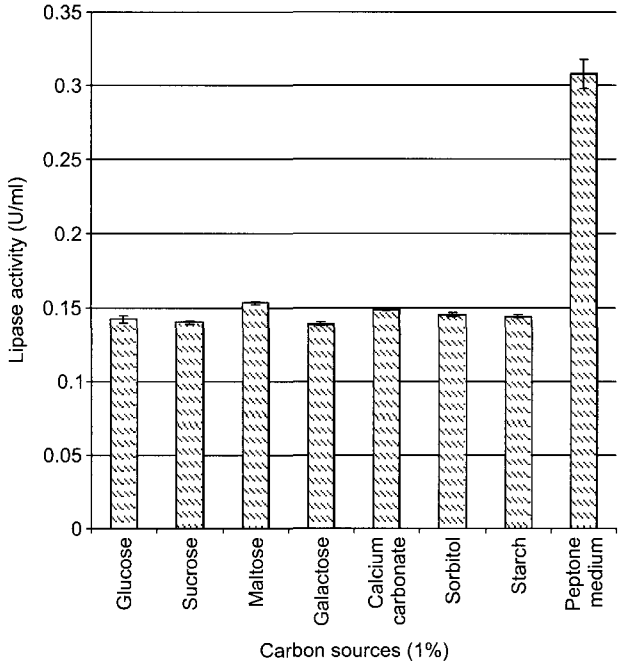

Fig. 4. Effect of carbon sources on lipase production.
Note: Results are expressed as the means of three independent determinations. The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is less.

Effect of carbon sources on lipase production

Carbon sources are important substrates for energy production in microorganisms. In order to investigate the effects of carbon sources on lipase production by strain S5, glucose, sucrose, maltose, galactose, sorbitol, calcium carbonate, and starch were replaced with olive oil in the basal medium. Fig. 4 shows that the addition of carbon sources at 1% to peptone medium resulted in a reduction of lipase production. Glucose effected an approximately 46% reduction of initial lipase production as compared to that of peptone medium. Lipase production in peptone medium was approximately 50% higher than when maltose, galactose, and starch were each added as carbon sources.

A similar result was previously reported by Tsuzuki

et al. (1999) who investigated the inhibitory effects of glucose on the production of lipase. Dalmau *et al.* (2000) reported that all tested carbon sources inhibited lipase production by *Candida rugosa*. In addition, Shabtai and Mishne (1992) demonstrated that lipase production in *P. aeruginosa* YS-7 was quite low when glucose and glycerol were employed as the sole carbon sources.

Utilizing olive oil as a carbon source in peptone medium resulted in superior lipase production, due to the fact that lipase is better hydrolyzed with long chain carbon-length substrates, as opposed to short chain carbon-length substrates. Similar results were reported by Fadiloglu and Erkran (2002), with higher lipase activity occurring in the media supplemented with olive oil than in media without olive oil. Gilbert *et al.* (1991) reported that the lipase produced by *Pseudomonas* sp. was induced strongly by triglycerides and detergents. None of the carbon sources tested in this study enhanced lipase production by S5. Peptone, when utilized as a sole nitrogen and carbon source, proved sufficient to support lipase production by S5 to maximum levels, and may thus prove an advantage, from an economic perspective.

Effect of lipids on lipase production

The effects on lipase production of three different groups of triglycerides consisting of natural triglycerides and synthetic triglyceride were tested in peptone medium. Olive oil in the peptone medium was replaced with the tested triglycerides. In general, S5 lipase preferred natural triglycerides, compared to synthetic triglycerides. S5 lipase hydrolyzed all tested triglycerides, with the highest degree of affinity to olive oil as a natural triglyceride (Fig. 5). With regard to the group of natural triglycerides, lower lipase production was observed when coconut oil (0.05 U/ml) was used. Soybean oil evidenced 33% relative activity as compared to olive oil, followed by palm oil (30%). These results showed that S5 lipase was more selective towards long-carbon chain natural oils (olive oil, groundnut oil, sesame oil, soybean oil, and palm oil). By way of contrast, coconut oil (C12:0) was less preferred, possibly as the result of shorter fatty acid carbon chain-lengths. One characteristic typical of lipases is interfacial catalysis, or a sharp increase in lipase activity that is observed when the substrate begins to form an emulsion.

The lipid preference of the crude enzyme differed slightly from that of the purified lipase (Rahman *et al.*, 2005a). Purified S5 lipase was determined to exhibit the highest degree of activity against palm oil and coconut oil. This may be attributable to impurities in the crude enzyme which affected the positive results. However, these enzymes evidenced the same trend, and clearly preferred long-carbon chain natural oils. Similar results have been reported by Haba *et al.* (2000), with *Pseudomonas* sp. evidencing higher levels of lipase production with olive oil after 72 h of incubation.

Effect of surfactant on lipase production

In order to determine the effects of surfactants on lipase production, three different groups of surfactants were tested, consisting of ionic (sodium percholate, provinyl alcohol and SDS); non-ionic (Tween 60, Tween 80, and Triton X-100) and cationic (CTAB) surfactants. Peptone medium containing Tween 60 and Tween 80 was shown to enhance lipase production after 48 h of incubation (Fig. 6). S5 lipase production rapidly decreased when CTAB was used as a cationic detergent. We detected no lipase production when SDS was used. Peptone medium to which sodium percholate, sodium deoxycholate, and Triton X-100 were added reduced lipase production to approximately 75 to 85% of previous values. According to Wu and Tsai (2004), higher levels of lipase production were observed when the substrate formed an emulsion, thereby presenting an interfacial area to the enzyme.

Effects of metal ion on lipase production

The effects of a variety of inorganic salts on lipase production were evaluated. The production of extracellular lipase by *P. aeruginosa* S5 was enhanced

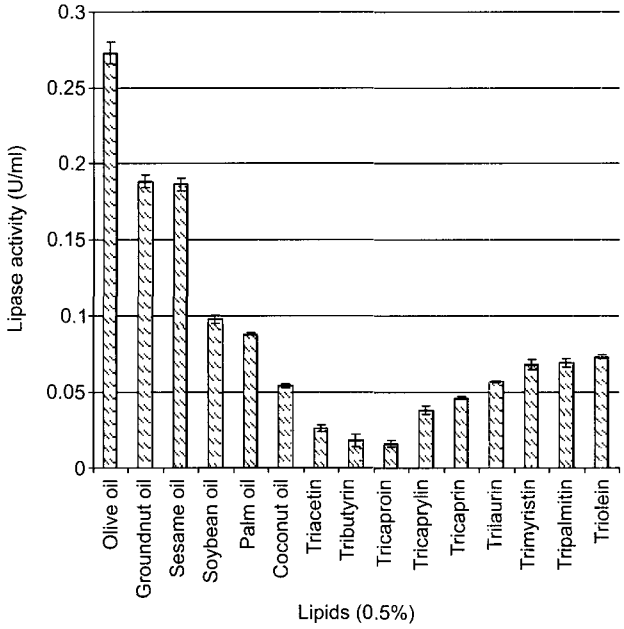

Fig. 5. Effect of lipids on lipase production
Note: Results are expressed as the means of three independent determinations. The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is less.

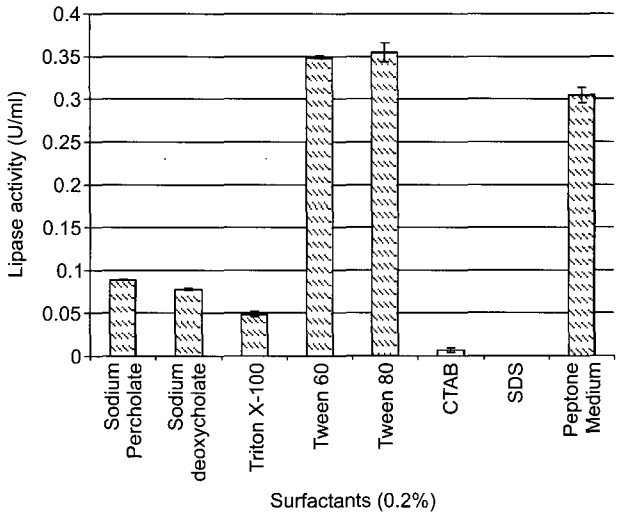

Fig. 6. Effect of surfactants on lipase production.
Note: Results are expressed as the means of three independent determinations. The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is less.

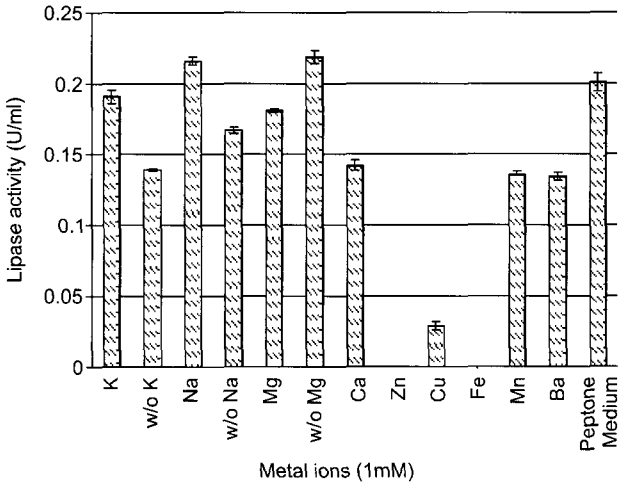

Fig. 7. Effect of metal ions on lipase production.
Note: K (K^+), w/o K (without K^+), Na (Na^{2+}), w/o Na (without Na^{2+}), Mg (Mg^{2+}), w/o Mg (without Mg^{2+}), Ca (Ca^{2+}), Zn (Zn^{2+}), Cu (Cu^{2+}), Fe (Fe^{2+}), Mn (Mn^{2+}), and Ba (Ba^{2+}). Results are expressed as the means of three independent determinations. The bar denotes standard deviation (SD). When the error bar cannot be seen, the deviation is less.

in cases in which the peptone medium was supplemented with Na^{2+} (Fig. 7). The exclusion of the sodium ions from the medium induced an approximately 25% reduction in lipase production. By way of contrast, the exclusion of the magnesium ions from the medium resulted in an enhancement of S5 lipase production. This result showed that the addition of Mg^{2+} did not significantly stimulate lipase production. Meanwhile, other metal ions, including Ca^{2+}, Mn^{2+}, Ba^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} exerted inhibitory effects. However, lipase production was decreased slightly, to approximately 5%, with the addition of K^+. However, a 30% decrease was observed in lipase production by S5 in an absence of potassium ions. The results of the study showed that *P. aeruginosa* S5 lipase required only a simple production medium, to which sodium ions have been added, to enhance lipase production.

Na^+ was determined to stimulate S5 lipase, thereby suggesting that S5 lipase was a metal-activated enzyme, in which the ions often function in a structural, rather than a catalytic, role. The ions bind to the enzyme and alter the conformation of the protein, to counter greater enzyme stability. Glusker *et al.* (1999) previously suggested that metal ions function as electrophiles, which seek the opportunity to share electron pairs with other atoms, such that a bond or charge-charge interaction might be formed. Meanwhile, transition metal ions, such as Fe^{3+}, Cu^{2+}, Mn^{2+}, and Zn^{2+}, change the conformation of the protein, rendering it less stable as the result of ion toxicity. According to Dong *et al.* (1999), this effect was attributable to the interaction between salt ions and the enzyme surface charge, which might markedly affect the ionization of some amino acid residues, thus changing the enzyme conformation and altering enzyme activity.

The preference for metal ions of the crude enzyme differed from that of the purified lipase (Rahman *et al.*, 2005a). Purified lipase S5 was determined to evidence the highest level of activity against Ca^{2+} and Mg^{2+}. However, this result was not suitable for comparison, due to different exposure times. In the case of the purified lipase, this enzyme was exposed to the metal ion only for 30 min. Meanwhile, in the case of crude enzyme, metal ions were added to the production medium, and incubated for 48 h prior to the measurement of lipase production.

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