Nutritional Requirements and Physical Factors Affecting the Production of Organic Solvent-Stable Lipase by *Acinetobacter baylyi*

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ABSTRACT

This study aims to find effective factors for the production of organic solvent-stable lipase from Acinetobacter baylyi that has been isolated from marine sludge in Angsila, Thailand. A study of physical parameters for lipase production by this strain revealed an optimum condition to be 25°C, pH 5.75 at 150 rpm. Maximum lipase activity and biomass contents were achieved after cultivation of the strain at the optimum condition for 15 h. Lipase production could be enhanced to nearly 2.5-fold by using glucose and ammonium sulfate at a concentration of 0.8% (w/v) and 0.4% (w/v) in the culture medium, respectively. Moreover, a 24- fold higher activity was observed with a combination of glucose and ammonium sulfate. When 0.8% (w/v) tryptone was included in the growth medium, lipolytic activity in the strain could be increased ~ 8.5-fold after 24 h of growth. The addition of either 5mM alanine or 0.1% (w/v) agar to minimal medium gave lipase production ~7-fold, approaching that obtained in the same medium. No significant lipase production was observed with the addition of hexadecane. Interestingly, the addition of 0.8% (v/v) of Tween 80 could enhance the enzyme production with 16,142- fold. A.baylyi lipase tolerated up to at least 75% (v/v) of short-chain alcohols, acetonitrile, heptane and decane and was also stable in the presence of 25% (v/v) DMSO, benzene, hexanes and hexadecane. This organic solvent-stable lipase could be used as a biocatalyst for enzymatic synthesis in the presence of organic solvent.

Keywords: Physical factors, Nutritional requirements, *Acinetobacter baylyi*, Lipase production

INTRODUCTION

A widespread use of lipases or triacylglycerol hydrolases (EC 3.1.1.3) as industrial biocatalyst has noticeably increased in the field of biotechnological applications (Arbige and Pitcher, 1989; Jaeger et al., 1994; Hasan et al., 2006). Most of lipases used in industry work are generally distributed in plants, animals and microorganisms (Arbige and Pitcher, 1989; Jaeger et al., 1994; Fang et al., 2006). Among them, lipases of microbial origin find immense applications, since they can catalyze a variety of hydrolytic or synthetic reactions (Jaeger and Reetz, 1998; Schmid et al., 2001). Each lipase has a number of unique characteristics such as substrate specificity, regio-specificity and chiral selectivity and some enzymes are industrially very important for the production of free fatty acids, synthesis of useful esters and peptides (Dordick, 1989; Jaeger et al., 1994). The demand for the production of highly-active preparations of lipolytic enzymes has led to research on lipase-producing microorganisms and on culture strategies.

Recently, the genus *Acinetobacter* is well represented among fermentable bacteria for the production of a number of extra-and intracellular economic products including lipases (Reisfeld et al., 1972; Kaplan and Rosenberg, 1982; Navon-Venezia et al., 1995; Haleem, 2003; Snellman and Colwell, 2004; Young et al., 2005). Contribution of this genus to biotechnology seems to be equally robust and versatile as *Pseudomonas* sp. However, it is safer because the genome of this organism contains very few traits that might be associated with pathogenesis (Young et al., 2005). Most of the lipases produced by Acinetobacter sp. have biochemical properties similar to those produced by *Pseudomonas* sp. and *Burkholderia* sp., and show stability and maximum activity under alkaline conditions at high temperatures (Bornscheuer et al., 2002; Snellman and Colwell, 2004). In addition, lipases produced by *Acinetobacter* sp. have been isolated from a variety of sources, including aquatic environments, soils, drugs and human skin; however, they were not closely studied until much later than those produced by *Pseudomonas* sp. and *Burkholderia* sp. (Haleem, 2003). Moreover, the information about nutritional requirements and physical parameters affecting the productivity has been documented a few (Chen et al., 1998; Liu and Tsai, 2003; Li et al., 2005). Here, we report the optimum condition suitable for high level of production of lipase from *A.baylyi* screened from marine sludge in Thailand.

MATERIALS AND METHODS

Bacterial strain

The bacterial strain used in this study was isolated from marine sludge in Angsila, Thailand and identified as *A.baylyi*. This strain was proven to be a benzene-tolerant bacterium which produces an organic solvent-stable lipase (Uttatree et al., 2010).

Lipase activity assay

Lipase activity was measured by a hydrolysis reaction using *p*-nitrophenyl

palmitate as substrate (Sigma, Germany) according to the method of Pencreac'h and Baratii (1996). One unit (U) of enzyme was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the assay conditions. The amount of *p*-nitrophenol was calculated from the *p*-nitrophenol (Sigma, Germany) standard curve. Protein concentration was determined spectrophotometrically according to the method of Bradford (1976), using the Bio-Rad assay reagent (Hercules, USA) and bovine serum albumin as the standard.

Growth curve and lipase production of *A.baylyi*

The growth and lipase production were investigated in nutrient medium (pH 7.0) in 1 L flask. Samples were withdrawn aliquots from the flask at 3 h intervals for lipase production and biomass determinations. Biomass was determined spectrophotometrically at optical density of 600 nm while the production of lipase was determined by hydrolytic activity as described above. All experiments were done in triplicate.

Physical parameters affecting the lipase production

Physical parameters used in this study were pH (2-12), temperature (20-45°C with 5°C intervals) and aeration (100-300 rpm with 50 rpm intervals). Throughout the study, the general procedures for cultivation were as follow: 5.0 ml of 24 h bacterial inoculums was inoculated into 100 ml of nutrient medium. Samples from the culture broths used in this study were taken from the late exponential phase of growth (OD_{600} ~ 0.8). Culture broth was centrifuged at 10,000 x g and 4°C for 20 min. The supernatant obtained was filtered through a 0.2 μm nylon membrane filter (Whatman, England) to collect the cell-free supernatant and used for enzymatic assay. Growth was monitored by an optical density of 600 nm. Each experiment was done in triplicate.

Effect of carbon source on lipase production

To test the effect of different carbon sources on the lipase production, a variety of concentration (0.2-1.0%) of carbon sources were added directly into the W-minimum medium (Kimbara et al., 1989). All carbon sources were filter-sterilized by 0.2 μm nylon membrane filter (Whatman, England). The following carbon sources were studied: glucose, sucrose, fructose and glycerol.

Effect of nitrogen source on lipase production

The effect of nitrogen source (0.2 -1.0% concentration) was investigated by adding directly into the W-minimum medium (Kimbara et al., 1989). Organic nitrogen sources used in this study were tryptone, peptone, yeast extract and urea while inorganic nitrogen sources were ammonium sulfate, potassium nitrate and ammonium nitrate.

Effect of amino acid on lipase production

Since some amino acids are typically involved in the catalytic site of lipase and enhance the synthesis (Arbige and Pitcher, 1989; Bornscheuer et al., 2002). To test the effect of additional amino acid in the W-minimum medium (Kimbara

et al., 1989), experiment was carried out by adding 5.0 mM of amino acids directly into the medium. The following amino acids were used: glycine, alanine, cysteine and histidine.

Effect of additives on lipase production

To determine the effect of polysaccharides, gum arabic, sodium alginate and agar were added directly into the W-minimum medium (Kimbara et al., 1989) ranging from 0.1 to 0.5% (w/v) concentration. Hydrocarbons as hexadecane and Tween 80 were also used for investigation at the concentration between 0.1 and 1.0% (v/v).

Effect of organic solvents on the stability of lipase

The tolerance of *A.baylyi* lipase against several organic solvents was tested in nutrient medium. The bacterium was cultured aerobically in the absence of organic solvent and removed from the medium by centrifugation at 10,000 × g and 4°C for 20 min. The cell-free supernatant was filtered with a 0.2 µm-pore size nylon membrane filter (Whatman, England). One milliliter of organic solvent was added to 3.0 ml of the cell-free supernatant and incubated at 37°C, 150 rpm for 6 h. The stability of lipase at different concentrations (0, 25, 50, 75%, v/v) of organic solvents was also examined. The remaining lipolytic activities were measured. Organic solvents chosen in this study were dimethyl sulfoxide, ethanol, acetonitrile, isopropanol, methanol, butanol, isoamyl alcohol, benzene, hexanes, heptane, decane and hexadecane. Each experiment was done in triplicate. Stability is expressed as the remaining lipolytic activity relative to the non-solvent-containing control (0%, v/v).

RESULTS AND DISCUSSION

Growth curve and lipase production of A.baylyi

A time course study was performed to determine the growth and lipase production of *A.baylyi* with respect to time. Figure 1 shows the different parameters determined at 3-h intervals. After inoculation, biomass of the strain was rapidly produced and reached stationary phase after 9 h incubation. The highest growth yield was obtained at 12 h incubation. Then, biomass production was slightly reduced and appeared to be constant after 21 h. On the other hand, lipase production was significantly detected after 3 h incubation and showed the maximum production at 15 h, and then the yield was gradually reduced and seemed to be constant after 18 h. These results could be noted that lipase production occurred in the late logarithmic phase of growth when the cell density was high. This is similar to the quorum sensing theory which describes that once the cell densities have reached certain threshold level, the expression of genes encoding extracellular enzymes and secretion systems is induced (Swift et al., 1996). A drop in lipase activity after 15 h incubation might be attributed to the reduction of biomass contents.

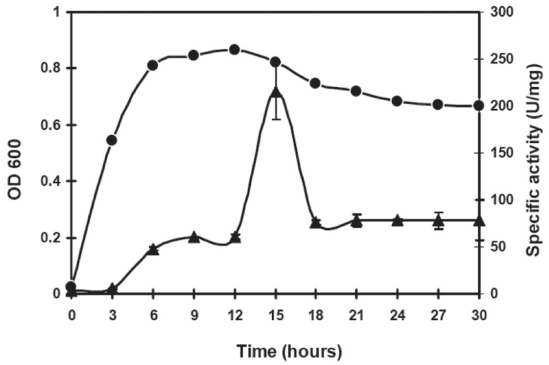


Figure 1. Growth curve and lipase production of *A.baylyi* on nutrient medium (pH 7.0) at 25°C, 250 rpm. Samples were taken at 3-hour intervals for lipase production (closed triangles) and biomass determination (closed circles). Specific activity was measured by hydrolytic activity towards *p*-nitrophenyl palmitate and expressed as the mean of three determinations with the standard derivations (mean±SD).

Effect of pH on the production of lipase

Effect of pH on the production of lipase was tested by assaying the lipolytic activity after growing the strain in nutrient medium at various pH values in the range of 2-12. As shown in Figure 2, the strain preferred to grow and produce lipase at weak acidic pH (5.5-6.0) and optimum pH for lipase production was 5.75. Below pH 5.5 and above pH 6.0, both growth and lipase production were suppressed. Most microorganisms could survive within the pH range 5 to 8.5 and exhibit maximum growth rates at close to neutrality (Stolp and Starr, 1981). In the case of *Acinetobacter*, they preferred to grow and secrete lipase at alkaline pH (Kok et al., 1995; Hong and Chang, 1998; Chen et al., 1998; Lin et al., 2001; Snellman et al., 2002; Liu and Tsai, 2003) and no document was found at acidic pH. Thus, it is of interest to note here that production of lipase at pH 5.75 seems to be the unique characteristics of *A.baylyi* and needs more investigation.

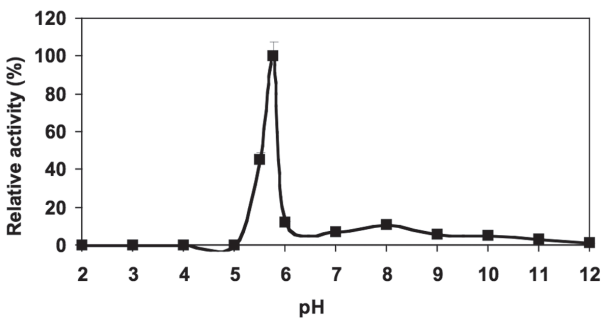


Figure 2. Effect of pH on lipase production by *A.baylyi.* The strain was cultivated in nutrient medium with each pH at 25°C for 24 h and 250 rpm. Percentages shown are relative to maximum activity and expressed as the mean of three determinations with the standard derivations (mean±SD).

Effect of temperature on the production of lipase

A.baylyi could grow and produce lipase at a temperature between 25 and 37°C and exhibited the maximum activity at 25°C (Figure 3). At the temperature above 37°C, the growth and lipase production were reduced and the strain could not grow at 45°C. It has been reported that low temperature could decrease lipase export to the supernatant phase and high temperatures might possibly lead to a denaturation of the enzyme (Barbaro et al., 2001). The optimum growth temperature determined in this study is in agreement with the findings of others on the production of lipase by different *Acinetobacter* sp. (Chen et al., 1998; Wang and Chen, 1998).

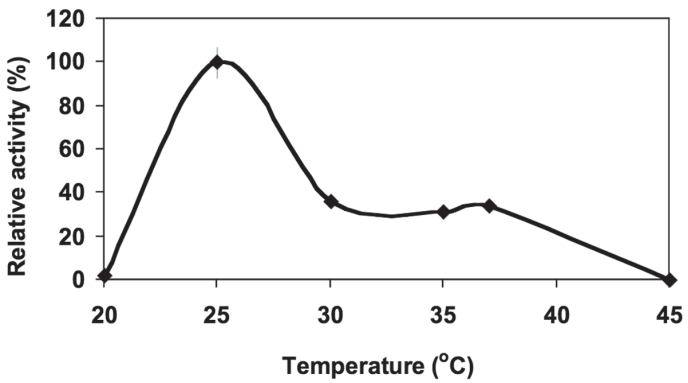


Figure 3. Effect of temperature on lipase production by *A.baylyi*. Cultivation was done in nutrient medium (pH 7.0) at different temperatures, ranging from 20-45°C with continuous shaking at 250 rpm for 24 h. Percentages shown are relative to maximum activity and expressed as the mean of three determinations with the standard derivations (mean±SD).

Effect of aeration on the production of lipase

Although in most cases, oxygen seems to favor lipase production, but low levels of aeration have also been reported to increase production of the enzyme (Freire et al., 1997; Chen et al., 1999; Corzo and Revah, 1999; Elibol and Ozer, 2000; Gulati et al., 2000). Optimum culture condition for lipase production by *A.baylyi* was continuous shaking at the speed of 150 rpm while lower and higher speed gave lower production of lipase (Figure 4). The lower cell growth and lipase production observed at 100 rpm suggested limitation of oxygen. In addition, the decrease in lipase production after 150 rpm was possibly due to cell removal from the medium caused by the formation of foam (Liu and Tsai, 2003). Increase in stirring speed to 300 rpm resulted in lowest production, probably caused by mechanical and/or oxidative stress.

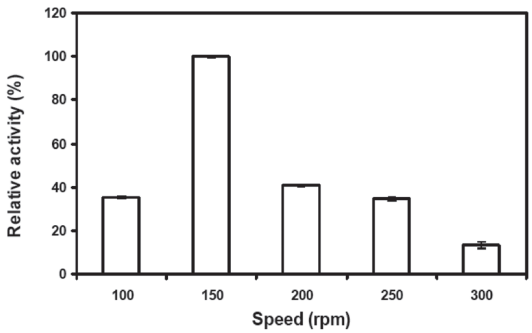
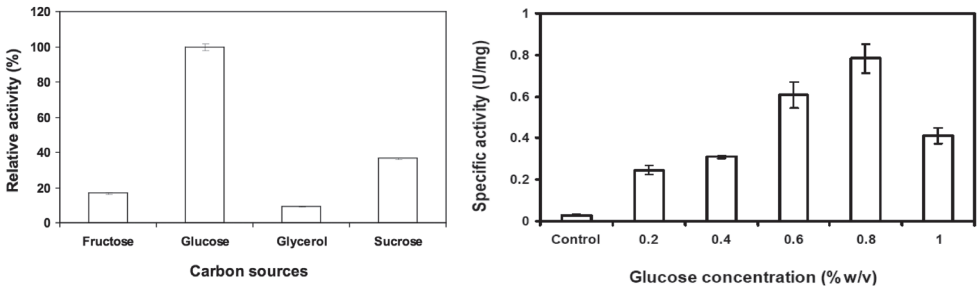


Figure 4. Effect of aeration on lipase production by *A.baylyi*. Cultivation condition was 25°C for 24 h in nutrient medium (pH 7.0) with continuous shaking at speed of 100-300 rpm. Percentages shown are relative to maximum activity and expressed as the mean of three determinations with the standard derivations (mean±SD).

Effect of carbon source on lipase production

Various carbon sources at 1% (w/v) were added to the medium to determine their ability to influence lipase production. The best carbon source for lipase production by *A.baylyi* was 0.8% (w/v) of glucose while sucrose and fructose gave minimum lipase yield. No significant of the activity was found in the presence of glycerol (Figure 5). It has been reported that glucose stimulated both the enzyme production in different microorganisms and the secretion of lipase accumulated inside the cells (Macfarlane and Macfarlane, 1992; Mehrotra et al., 1999; Dalmau et al., 2000; Boekema et al., 2007). On the contrary, the repression of enzyme synthesis in the liquid medium by sucrose and other readily- metabolized carbon source was referred to as catabolite repression, the paradigm of cellular regulation for the low preferential carbon source (Stülke and Hillen, 1999; Brückner and Titgemeyer, 2002; Deutscher, 2008). In the presence of sucrose, for example, catabolite repression in *A.baylyi* might serve as an autoregulatory device to keep sucrose utilization at a certain level that led to the lower production of lipase rather than to establish preferential utilization of glucose.

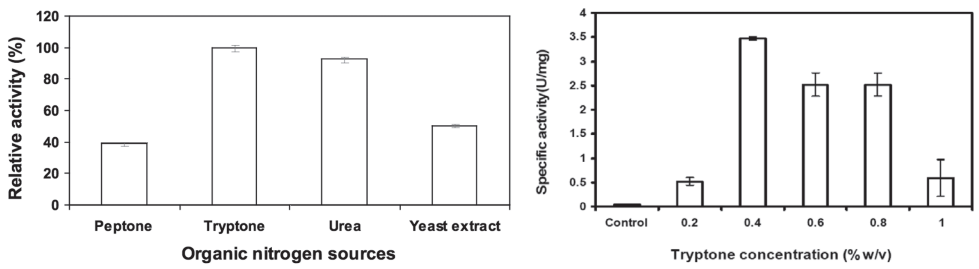


(a) (b)

Figure 5. Effect of carbon sources (a) and concentration of glucose (b) on lipase production. Different carbon sources at 1% (w/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the glucose supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).

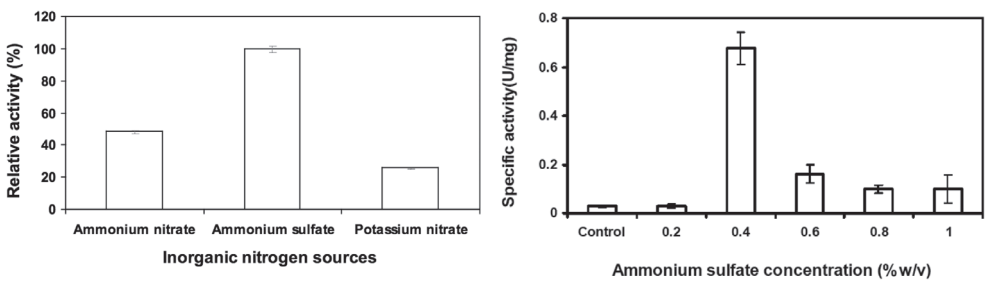
Effect of nitrogen source on lipase production

In most microorganisms, either inorganic or organic nitrogen sources are metabolized to produce amino acids, nucleic acids, proteins and cell wall components. The ability of *A.baylyi* to produce lipase in medium was examined in different nitrogen sources. Maximum lipase production was found with 0.4% (w/v) of tryptone (Figure 6). Others, in decreasing order of activity were yeast extract and peptone. For inorganic nitrogen sources, 0.4% (w/v) of ammonium sulfate seemed to be the best, however gave low yield (Figure 7). This observation suggested that complex nitrogen sources were not essential for growth and lipase production by this strain although the growth and enzyme productivity was low. The lipase yields obtained with higher concentration of ammonium sulfate were considerably lower than those observed at 0.4% (w/v). This phenomenon might be due to the repression of enzyme synthesis by rapidly-metabolizable ammonium ion concentration in the medium which interfered with the utilization and metabolism of peptides through catabolite repression (Giesecke et al., 1991; Snowden et al., 1992).



(a) (b)

Figure 6. Effect of organic nitrogen sources (a) and concentration of tryptone (b) on lipase production. Different organic nitrogen sources at 1% (w/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the tryptone supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).



(a) (b)

Figure 7. Effect of inorganic nitrogen sources (a) and concentration of ammonium sulfate (b) on lipase production. Different inorganic nitrogen sources at 1% (w/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the ammonium sulfate supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).

Effect of amino acid on lipase production

Experiments done by supplementing the medium with 5mM amino acid indicated that the presence of alanine stimulated the highest lipase production while orders of low productivity were found with cystein, histidine and glycine (Figure 8). The inhibitory effect of glycine has been found in both amylase and protease production (Ikura and Horikoshi, 1987).

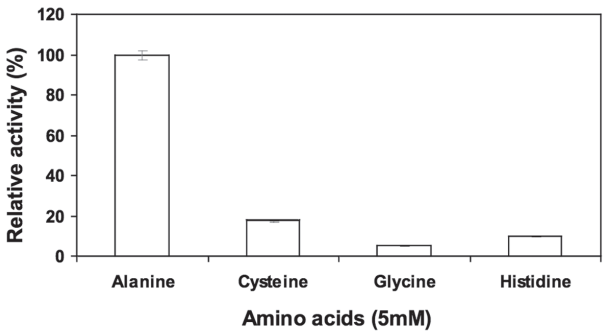
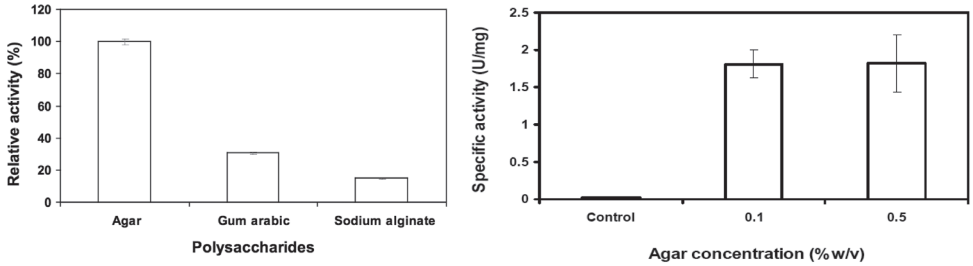


Figure 8. Effect of 5mM amino acid on lipase production. Different amino acids were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the alanine supplement and expressed as the mean of three determinations with the standard derivations (mean±SD).

Effect of additives on lipase production

Three polysaccharides, gum arabic, agar and sodium alginate were added separately to the medium at different concentrations. Among them, agar gave the highest lipase activity (Figure 9a) and 0.1% (w/v) concentration promoted maximum lipase production per unit of the growth of *A.baylyi*, similar with 0.5% (w/v) (Figure 9b). From economic point of view, the concentration at 0.1% (w/v) agar was selected to be the suitable concentration. The 7-fold increase in lipase production by the addition of agar might due to this compound enhance mechanically liberation of the enzyme at the cell surface (Winkler and Stuckmann, 1979; Mahler et al., 2000; Martinez and Nudel, 2002). On the other hand, Tween 80 was the best hydrocarbon source for the lipase production in W-medium compared with hexadecane (Figure 10a). The production of lipase in W-medium supplemented with 0.8% (v/v) of Tween 80 showed the highest lipase activity at 4,842.53±7.01 U mg^{-1} (Figure 10b). There was no significant production of lipase when the strain was grown on the medium containing hexadecane. This is in agreement with the conclusion of Kok et al., (1996) that some alkanes, such as hexadecane, have been shown to repress lipase expression. Enhancement of lipase productivity by Tween 80 might be due to the involvement of a fatty acyl ester bond that functions as an inducer of the lipase operon (Boekema et al., 2007). However, together with carbon or nitrogen source in the medium, Tween 80 much less increased the lipase activity of *A.baylyi* (data not shown). This might be because the expression

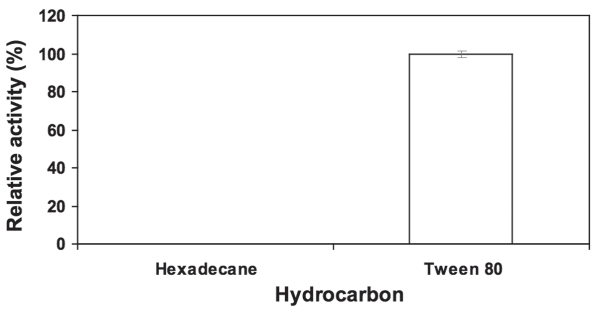
on the operon in the presence of other sources is prone to catabolite repression (Stülke and Hillen, 1999; Brückner and Titgemeyer, 2002; Deutscher, 2008).



(a) (b)

Figure 9. Effect of polysaccharides (a) and concentration of agar (b) on lipase production. Different polysaccharides at 1% (w/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the agar supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).

(a)



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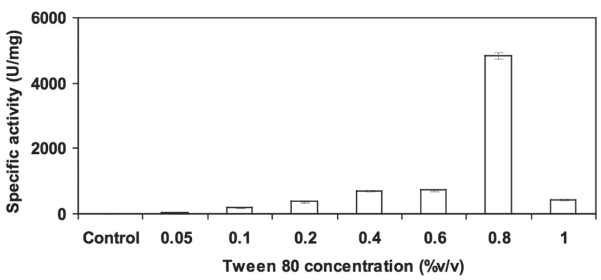
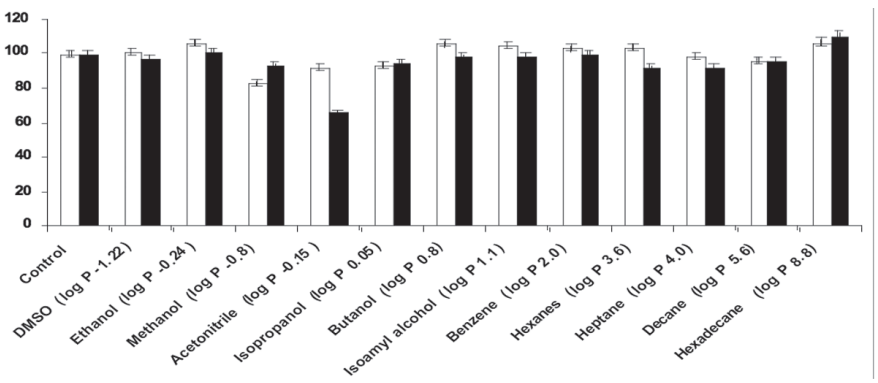


Figure 10. Effect of hydrocarbons (a) and concentration of Tween 80 (b) on lipase production. Different hydrocarbons at 1% (v/v) were added into the W-medium and cultivated the strain at 25°C with continuous shaking at 250 rpm for 24 hours. The relative activity was based on lipase activity in the culture media relative to the Tween 80 supplement. Specific activity was expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to W-medium (control).

Effect of organic solvents on the stability of lipase

Stability in the presence of organic solvents is a requisite property of enzymes used in organic synthesis in non-aqueous systems. *A.baylyi* lipase appears to be ideally suited for such syntheses since its activity was stable in the presence of many organic solvents as follows. As described in Figure 11a, after 6 h incubation in 25% (v/v) of solvents, the lipase from A.baylyi seemed to highly resist to all solvents except acetonitrile (log $P_{o/w}$ -0.15) that gave 66% remaining activity. When the concentration reached 50% (Figure 11b), slight reduction was found in the presence of acetonitrile, hexanes (log Po/w 3.6), heptanes (log $P_{o/w}$ 4.0), hexadecane (log Po/w 8.8) and short-chain alcohols. In addition, at 75% concentration (Figure 11c), *A.baylyi* lipase appeared to be stable in the presence of methanol, butanol and isoamyl alcohol for 1 h. However, the activity was suddenly dropped in the presence of all solvents for 6 h except benzene and heptane (58% residual activity). These results are similar to the facts that hydrophobic solvents hinder efficient interaction between enzymes and substrates (Laane et al., 1987) while hydrophilic solvents are capable of dissolving enzyme, resulting in invariable inactivation (Sugihara et al., 1991). Also, low solubility in oils of short-chain alcohols might lead to an inactivation of the enzyme (Shimada et al., 1999). However, tolerance to benzene of *A.baylyi* lipase at all concentrations seems to be the unique interesting property of this enzyme and needs more investigation.

(a)



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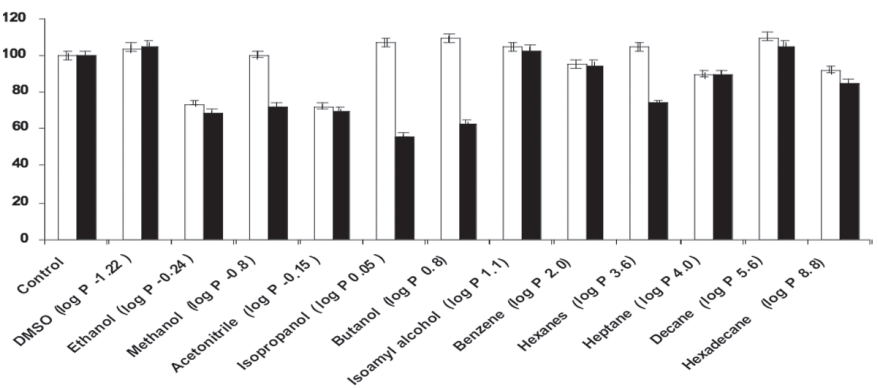

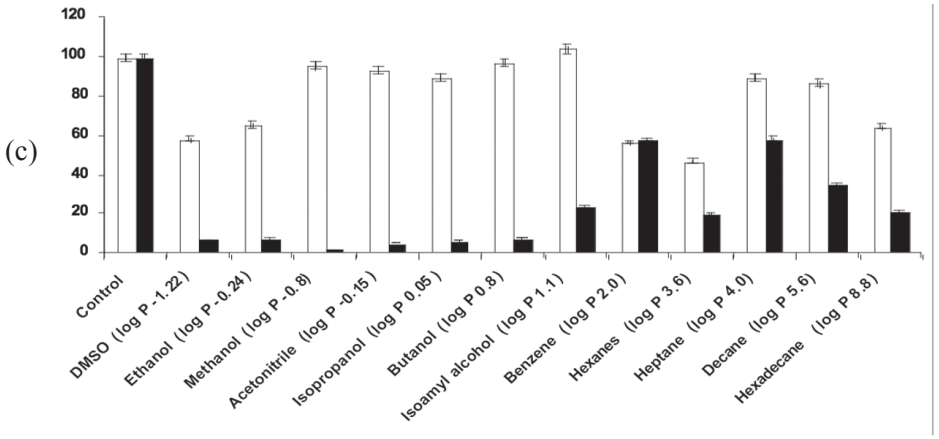

Figure 11. Effect of organic solvents on lipase activity (a) 25% (b) 50% (c) 75% concentration. The crude lipase was incubated at 37°C in the presence of organic solvents for 1 (white bar) and 6 h (dark bar). The remaining lipase activity was measured and expressed as the mean of three determinations with the standard derivations (mean±SD) comparing to control (without organic solvent).

CONCLUSION

The growth and lipase production in *A.baylyi* were found to be influenced by various nutritional and environmental factors such as culture media, pH, temperature, aeration and growth periods. Optimum condition for lipase production was found to be 25°C, pH 5.75 at 150 rpm for 15 h and the addition of 0.8% (v/v) of Tween 80 could enhance the enzyme production by 16,142-fold compared with those in minimum salt medium. The 2.5-fold higher production of lipase was found in the medium containing either 0.8% (w/v) of glucose or 0.4% (w/v) of ammonium sulfate while a combination of these compounds gave 24- fold higher activity. When 0.8% (w/v) tryptone was included in the growth medium, lipolytic activity in the strain could be increased ~ 8.5-fold after 24 h of growth. The addition of either 5mM alanine or 0.1% (w/v) agar to minimal medium gave lipase production ~7-fold, approaching that obtained in the same medium. No significant lipase production was observed with the addition of hexadecane. A.baylyi lipase tolerated up to 75% (v/v) of short-chain alcohols, acetonitrile, heptane and decane and was also stable in the presence of 25% (v/v) DMSO, benzene, hexanes and hexadecane. This organic solvent-stable lipase could be used as a biocatalyst for enzymatic synthesis in the presence of organic solvent.

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