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Full Length Research Paper

Production and optimization of alkalostable lipase by alkalophilic *Burkholderia cenocepacia* ST8

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A superior lipase-producing bacterium was isolated from forest soil samples in Setapak, Malaysia and it was identified as *Burkholderia cenocepacia* with 16S rRNA sequencing technique. Multifactor experimental design based on 'change-one-factor-at-a-time' approach was employed to optimize the production of *Bukholderia* lipase with submerged fermentation technique. Effects of carbon and nitrogen sources, metal ions as well as initial pH of medium on lipase production were extensively investigated. Optimal lipase activity was achieved in medium using combination of sunflower oil and Tween 80 (1% v/v each) as carbon sources. Simple sugars such as glucose and fructose, however, did not promote the production of lipase. Peptone (from meat) at 0.33% (w/v) was the most suitable nitrogen source for lipase production by this Gram-negative bacterium. The presence of Ca^{2+} in the cultivation medium possessed significant effect on lipase production while Mg^{2+}, Mn^{2+}, Na^{+}, Fe^{2+}, Cu^{2+} and Co^{2+} exhibited inhibitory effect towards the enzyme production. Initial culture pH in the range of 5 to 11 were found suitable for lipase production, with the maximum level of lipase activity recorded in the medium with initial culture pH of 9.0.

Key words: Alkalostable lipase, alkalophilic *Burkholderia cenocepacia,* optimization, multifactor experimental design.

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolase which catalyze both hydrolysis and synthesis reactions at lipid-water interface. Lipases also possess numerous unique characteristics (fatty acid specificity; thermostability; the reverse reaction in organic solvents; regio-, chemo-, stereo-selectivity and etc) which has made them become an important choice for biocatalyst in chemical, food, detergent, pharmaceutical, organic synthesis, bioremediation and cosmetics Industries (Jaeger and Reetz, 1998; Saxena et al., 1999; Jaeger and Eggert, 2002; Gupta et al., 2004). Lipases are widely distributed in plants, animals and microorganisms. Microbial lipases have obtained a special attention due to better stability compared to animals and plants; possibility of high enzyme yield; easy for genetic manipulation; various catalytic activity; inexpensive medium used; more convenient and safer for bulk production (Saxena et al., 1999; Gupta et al., 2004; Hasan et al., 2006).

Lipases from microorganisms are commonly found in bacteria, yeast and fungi (Treichel et al., 2010). Some commercially important lipase-producing genera include *Bacillus*, *Chromobacterium*, *Burkholderia*, *Pseudomonas*, *Rhizopus*, *Aspergillus*, *Candida* and *Geotrichum* (Jaeger et al., 1994; Gupta et al., 2004; Treichel et al., 2010). Lipases which originated from *Burkholderia* genus have been extensively studies for the past two decades, especially for industrial uses. *Burkholderia* lipases have been found in various industrial applications such as detergent, organic synthesis, cosmetics and flavors development (Jaeger et al., 1994; Jaeger and Reetz, 1998; Jaeger and Eggert, 2002; Gunasekaran and Das, 2004). This is due to the fact of their remarkable

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characterizations such as high alkaline and thermal stability, solvent tolerant ability, enantioselectivity and diverse substrate specificity (Rathi et al., 2001; Dandavate et al., 2009; Wang et al., 2009).

Most bacterial lipases are produced extracellularly with submerged fermentation (Abada, 2008; Treichel et al., 2010). Production of lipase was found greatly influenced by environmental factors such as medium composition, cultural conditions, pH and temperature, different types of carbon and nitrogen sources (Gupta et al., 2004; Hasan et al., 2009). Lipase productions were normally enhanced in the presence of lipid sources, nitrogen sources and other media components (Sharma et al., 2001; Gupta et al., 2004). From economic point of view, optimization of medium formulations and culture conditions for enzyme production is a crucial step for viable commercial application of microbial lipases.

This study was first initiated by isolation and screening of potential lipase-producing microorganism from various local samples. Identification of potential isolate was conducted through molecular technique. Optimization of nutrient requirements and culture conditions for enhancement of lipase production by the potential isolate were also conducted.

MATERIALS AND METHODS

Isolation and screening

Various sources of samples, including soil samples and water samples were collected from different locations in Setapak, Kuala Lumpur Malaysia. The samples (approximately 0.5 g) were suspended in 10 ml of sterile distilled water and heated at 80°C for 10 min to destroy non-spore-forming bacteria. The samples were then serially diluted before been streaked onto the tributyrin agar medium (TBA). The composition of TBA was as follows (g/L): peptone, 5; beef extract, 3; agar, 15 and tributyrin (1% v/v). The pH of TBA was adjusted to 9 with 3 N NaOH prior to sterilization at 121°C for 15 min.

The plates were incubated at 37°C for 48 h. Lipolytic isolates were selected based on the formation of zone of hydrolysis around the colonies. Following that, submerged cultivation of all positive isolates was conducted to determine the level of lipase production quantitatively. Liquid culture proposed by Chin et al. (2003) was used as basal medium for the cultivation of lipase-producer, with the compositions as follows: 1% (v/v) Tween 80; 1% (v/v) olive oil; 0.325% (w/v) nutrient broth; 0.05% $CaCl_2.H_2O$ (w/v) and gum Arabic (1% w/v) as emulsifier. The pH of the medium was adjusted to 9.0 prior to sterilization process. Following the inoculation with 10% (v/v) of inoculum, the flasks were incubated at 37°C and 200 rpm. Culture broth (3 ml) were withdrawn every 24 h for analysis. After centrifugation at 9000 rpm for 10 min, the supernatant was used for the determination of lipase activity while the pellet was used to investigate the growth of the strains.

Following that, identification of the most potential strain was conducted using molecular approach. In addition, the effects of several factors affecting lipase production by the potential strain were evaluated with submerged cultivation technique. The maximum lipase activity was determined as the highest level of enzyme activity achieved during the cultivation period and mean values of three independent experiments were calculated. Lipase productivity was determined by using Equation 1.

$$\text{Lipase productivty, P (U/ml/h)} = \frac{\text{Maximum lipase activity (U/ml)}}{\text{Time taken to obtain maximum activity (h)}}$$

(Equation 1)

Identification and characterization of potential isolate

Genomic DNA of the selected strain was extracted using a commercial kit (i-genomic CTB DNA extraction Mini kit, Intron Biotechnology, Inc, Korea). The extracted DNA sequences were submitted and sequenced by NHK Bioscience solutions Sdn Bhd, Malaysia. The procedures of DNA amplification and DNA sequencing were as followed. Template DNA (1 µl) was added to 20 µl of PCR reaction solution. The universal primer (27F/1492R) was used for this bacteria, and PCR was performed using 35 amplification cycles at 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s. DNA fragments were amplified about 1,400 bp in the case of bacteria. A positive control (*Escherichia coli* genomic DNA with 518F/800R as primers) was included in the PCR. Homology was analyzed through nucleotide BLAST (Basic Local Alignment Search Tool) from NCBI database (http://www.ncbi.nlm.nih.gov/). The sequences for *E.coli* primers were 518F-CCAGCAGCCGCG GTAATACG and 800R-TACCAGGGTATCTAATCC. The sequences for universal primers were 27F-AGAGTTTGATCMTGGCTCAG and 1492R-TACGGYTACCTTGTTACGACTT.

Effect of culture variables on lipase production

In order to investigate the effect of carbon sources on lipase production, olive oil and Tween 80 in basal medium were replaced by various carbon sources, such as, commercial oils (sunflower oil, rice bran oil, corn oil, soybean oil, used engine oil, palm oil), Tween 20, glucose and fructose. Subsequently, the effects of nitrogen source, either organic or inorganic, was also investigated. The replacement was done based on the level of nitrogen content in the basal medium. Various concentration of chosen nitrogen source was also examined. The effect of various metal ions (Mg^{2+}, Fe^{2+}, Cu^{2+}, Na^{+}, Co^{2+}, Mn^{2+}) with the same molarity of Ca^{2+} in the basal medium (pH 9) on growth and lipase production were also tested. The effect of initial culture pH on lipase production was studied in shake flasks at different initial pH values. The pH of the medium was adjusted to the desired value by using either 1 N NaOH or 1 N HCl prior to sterilization process. All the fermentation process was conducted in triplicate and the results presented are average values.

Analytical procedures

Lipase activity was conducted by colorimetric method using p-nitrophenyl laurate (p-NPL) as substrate (Castro-Ochoa et al., 2005). The assay mixture was carried out at 37°C for 30 min and p-nitrophenol released was determined at 410 nm. One unit (U) of lipase activity was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol from p-NPL per minute under the standard assay condition used. The cell pellet was used for determination of dry cell weight by using filtration and oven drying method.

RESULTS AND DISCUSSION

Isolation and Identification of lipase-producer

A total of 20 bacterial strains were isolated from various

Table 1. Performance of 9 positive isolates during liquid fermentation.

Isolate code	Rate of Growth in medium	Maximum lipase activity (U/ml)	Lipase productivity (U/ml/h)	Maximum cell concentration (g/L)
ST1	**	8.47	0.53	0.73
ST2	*	0.08	0.01	0.09
ST3	*	0.17	0.01	0.11
ST4	*	0.17	0.01	0.55
ST5	*	0.15	0.02	0.17
ST6	**	4.25	0.43	0.89
ST7	*	0.24	0.01	0.27
ST8	***	120.18	0.77	4.40
ST9	*	0.28	0.02	0.12

* , Poor; ** , good; ***, very good.

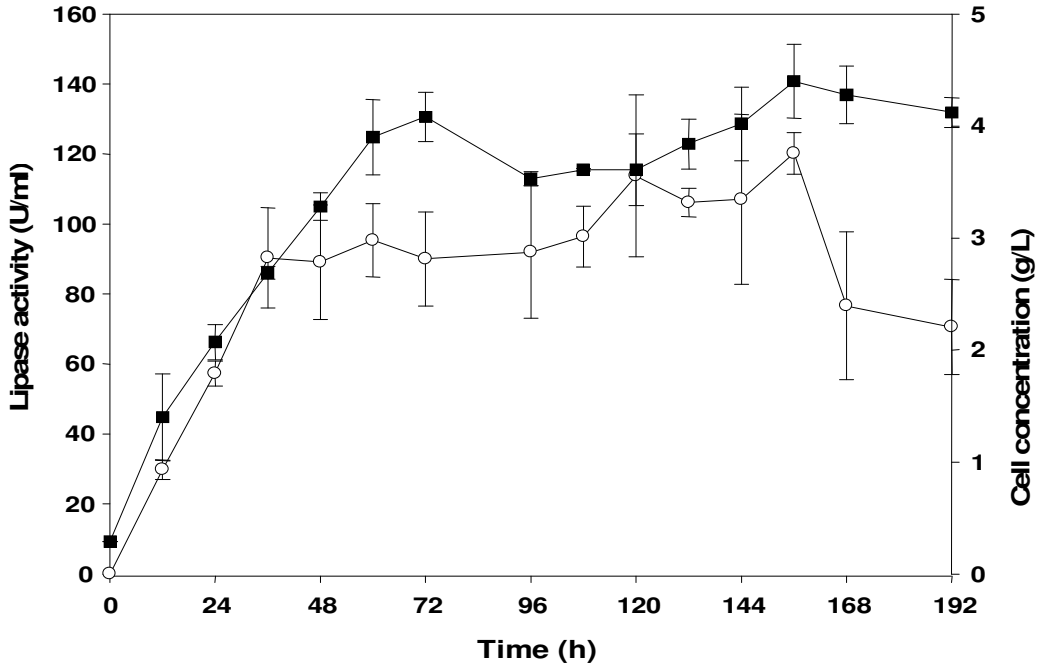

Figure 1. Time profile of lipase production of *B. cenocepacia* ST8 in basal medium containing Tween 80 and olive oil as carbon sources. (○), lipase activity; (■), cell growth.

forest soil samples and nine of them were found to be lipolytic. On the basis of lipase activity and productivity (Table 1), one strain designated as ST8 was selected for subsequent studies. Through biochemical tests, the isolate ST8 was a rod-shaped, motile and aerobic Gram-negative bacterium. Based on the 16S rRNA analysis, the gene sequences of strain ST8 matched well (100% identity) with *Burkholderia cenocepacia* [CCBAU 51299 (accession number GU433447); ESR100 (accession number EF602566); ESR99 (accession number EF602565); ESR 60 (accession number EF602557); HI2424 (accession number CP000458); AU1054 (accession number CP000378)] and *Burkholderia* sp. [ESR90 (accession number EF602562); ESR85 (accession number EF602560)]. With 100% similarity of gene sequence to those *B. cenocepacia* species available in GenBank, the strain ST8 was re-designated as *B. cenocepacia* ST8.

Time course of cell growth and lipase production by *B. cenocepacia* ST8 in basal medium are shown in Figure 1. Lipase production was noted from the early exponential growth phase and the lipase production attained maximum level during the late growth phase of the microorganism. Similar pattern of lipase production was also reported by using *Burkholderia cepacia* (Rathi et al., 2001) and *Pseudomonas* sp. MSI057 (Kiran et al., 2007)

Table 2. Fermentation performance of *B. cenocepacia* ST8 in shake flask using different type of carbon sources for lipase production.

Carbon source	Maximum lipase activity(U/ml)	Lipase productivity (U/ml/h)	Maximum cell concentration (g/L)
Sunflower oil (1%, v/v) + Tween 80 (1%, v/v)	179.00^{a} ± 0.27	1.72^{a} ± 0.14	6.09^{c} ± 0.31
Sunflower oil (2%)	41.63^{g,h,i} ± 3.75	0.34^{e,f} ± 0.08	7.55^{a} ± 0.44
Rice bran oil (1%, v/v) + Tween 80 (1%, v/v)	159.47^{a,b} ± 16.19	1.15^{b,c} ± 0.20	5.09^{e} ± 0.36
Rice bran oil (2% v/v)	60.13^{e,f,g} ± 1.29	0.45^{e,f} ± 0.08	6.23^{c} ± 0.14
Corn oil (1%, v/v) + Tween 80 (1%, v/v)	143.64^{b,c} ± 17.54	1.41^{a,b} ± 0.20	5.92^{c} ± 0.07
Corn oil (2%, v/v)	108.20^{c,d} ± 18.47	0.90^{c,d} ± 0.15	7.67^{a} ± 0.07
Soybean oil (1%, v/v) + Tween 80 (1%, v/v)	142.41^{b,c} ± 0.22	1.40^{a,b} ± 0.22	5.84^{c,d} ± 0.21
Soybean oil (2%, v/v)	49.55^{g,h} ± 0.05	0.42^{e,f} ± 0.05	6.30^{c} ± 0.36
Olive oil (1%, v/v) + Tween 80 (1%) **	118.37^{c,d} ± 20.42	0.93^{c,d} ± 0.22	4.47^{f} ± 0.23
Olive oil (2%. v/v)	93.39^{d,e} ± 7.83	0.89^{c,d} ± 0.09	6.37^{c} ± 0.51
Used engine oil (1%, v/v) + Tween 80 (1%, v/v)	104.59^{d} ± 10.10	1.12^{b,c} ± 0.04	1.40^{j} ± 0.12
Used engine oil (2%, v/v)	10.29^{i} ± 2.57	0.22^{e,f} ± 0.05	0.87^{j} ± 0.06
Palm oil (1%, v/v) + Tween 80 (1%, v/v)	86.75^{d,e,f} ± 16.97	0.58^{d,e} ± 0.07	5.33^{d,e} ± 0.17
Palm oil (2%, v/v)	46.06^{g,h,i} ± 14.00	0.39^{e,f} ± 0.10	5.30^{d,e} ± 0.75
Tween 20(1%, v/v) + Tween 80 (1%, v/v)	53.65^{f,g,h} ± 7.06	0.42^{e,f} ± 0.06	2.36^{i} ± 0.09
Tween 20 (2%, v/v)	30.27^{g,h,i} ± 2.18	0.25^{e,f} ± 0.02	3.06^{h} ± 0.06
Coconut oil (1%, w/v) + Tween 80 (1 %, v/v)	44.67 ^{g,h,i} ± 5.39	0.37 ^{e,f} ± 0.04	6.84^{b} ± 0.20
Coconut oil (2%, v/v)	37.46 ^{g,h,i} ± 4.56	0.22 ^{e,f} ± 0.03	7.24^{a,b} ± 0.71
Tween 80 (2%, v/v)	142.08^{b,c} ± 20.01	1.28^{b,c} ± 0.15	3.85^{g} ± 0.01
Fructose (1%, w/v) + Tween 80 (1%, v/v)	48.97^{g,h} ± 8.14	0.37^{e,f} ± 0.08	3.06^{h} ± 0.03
Glucose (1%, w/v) + Tween 80 (1%, v/v)	20.84^{h,i} ± 5.10	0.15^{f} ± 0.04	2.44^{i} ± 0.10

$^{a-i}$, Means values in the same column not followed by the same letter are significantly different ($P < 0.05$) and values from three independent experiments were expressed as the mean ± SD.** Basal medium.

which suggested the growth-associated behavior of lipase production by these microorganisms. As observed from Figure 1, the production of lipase declined after 156 h of incubation which could be due to the nutritional limitation and the presence of inhibitor, such as protease (Rathi et al., 2002).

Effect of carbon sources

As shown in Table 2, by replacing olive oil with sunflower oil, lipase production was greatly enhanced (179 U/ml) in the medium supplemented with Tween 80. Moreover, medium containing various types of oils along with addition of Tween 80 generally promoted higher enzyme production as compared with system using individual oil without Tween 80 (Table 2). These phenomena might be due to the fact that addition of Tween 80 induces lipase synthesis by increasing cell permeability and also facilitate the export of certain compounds across the cell through its membrane in order to improve the availability of substrate for the microorganism (Dalmau et al., 2000). Similar results were reported for *Burkholderia glumae* (Boekema et al., 2007) and other moderate thermophilic bacterial strains (Fakhreddine et al., 1998).

In this study, addition of Tween 80 (1% v/v) to the medium with sunflower oil (1% v/v) resulted in the highest lipase productivity and it was 1.5-fold of the increment as compared to basal medium using the basal medium, that is, olive oil and Tween 80 as carbon sources (Table 2). The compositions of fatty acids in sunflower oil were: linoleic acid (~70%); oleic acid (~10%); palmitic acid (~10%) and stearic acid (~10%). Babu and Rao (2007) explained that polyunsaturated fatty acids component in sunflower oil might support high level of lipase synthesis

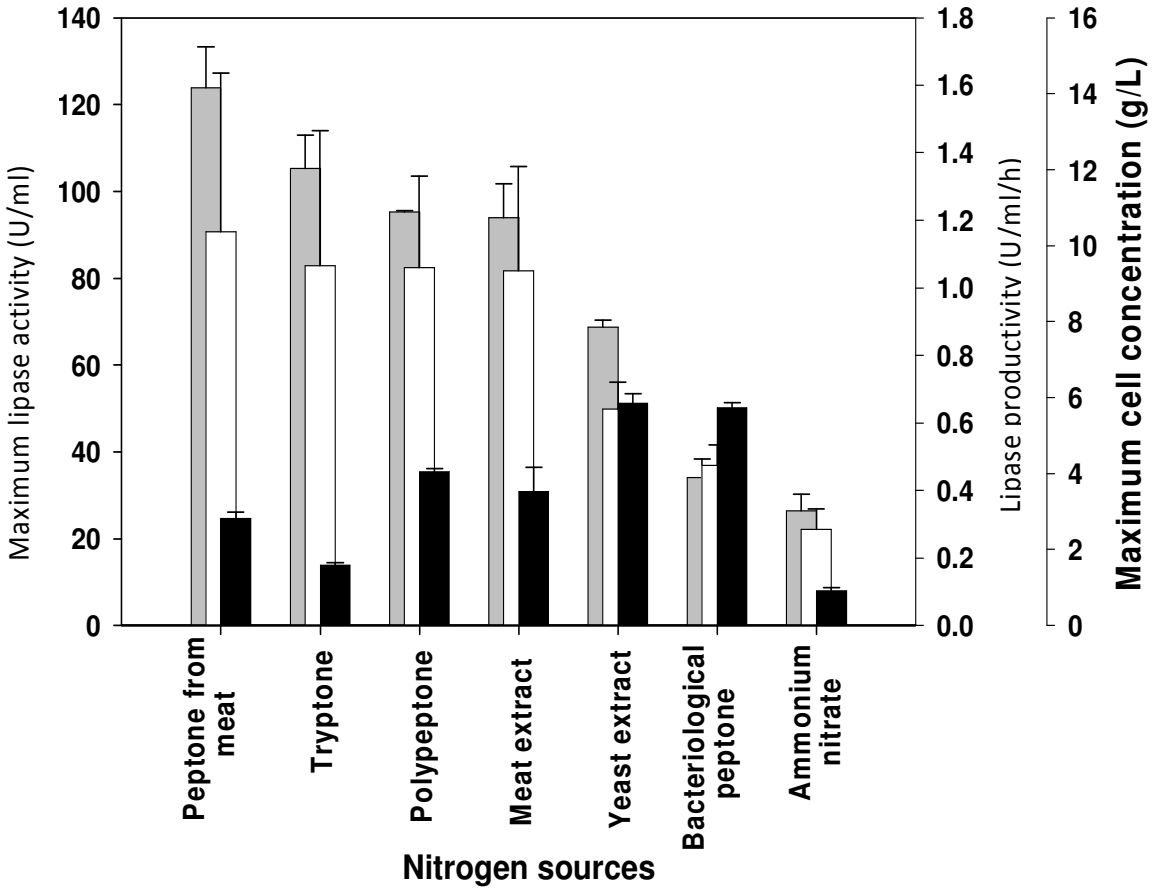

Figure 2. Lipase production by *B.cenocepacia* ST8 in shake flask using different type of nitrogen sources. Bars: (▒), maximum lipase activity; (□), lipase productivity; (■), maximum cell growth. Data shown are the means of three independent experiments with error bars.

when they revealed that maximum lipase production by *Yarrowia lipolytica* NCIM 3589 was achieved in the presence of sunflower oil. Nevertheless, high level of lipase synthesis was also achieved by using other types of oils by different bacterial species; for example, *Burkholderia multivorans* cultivated on olive oil (Gupta et al., 2007), *B. cepacia* cultivated on palm oil (Rathi et al., 2001), *Bacillus coagulans* BTS-3 cultivated on refined mustard oil (Kumar et al., 2005).

In this study, repression of lipases synthesis (21 U/ml) occurred in the liquid medium supplemented with glucose. The phenomenon might be due to catabolite repression effect which caused the inhibition of lipase production. Glucose repression in lipase production was reported by using *Pseudomonas fluorescens 2D*, *Bacillus alcalophilus* and *B. multivorans* as lipase-producer (Makhzoum et al., 1995; Ghanem et al., 2000; Gupta et al., 2007). There are, however, some reports on stimulation of lipase production by using glucose as carbon source, such as by using *Pseudomonas fragii* (Alford and Pierce, 1963) and *B. cepacia* (Rathi et al., 2001).

The results of this study clearly illustrated that the lipase production by *B. cenocepacia* ST8 was dependent on lipid sources such as Tween 80 and oils which could function as carbon source and also as inducer for lipase production. In contrast, non-lipid substrates (fructose and glucose) did not possess significant effect on enhancement of lipase production.

Effect of nitrogen sources

In this study, majority of the organic nitrogen sources tested were found able to support high level of lipase production with enzyme activity higher than 50 U/ml (Figure 2). However, lipase activity was extremely low with ammonia nitrate as sole nitrogen source (26.34 U/ml). As observed from Figure 2, the medium containing peptone (from meat) yielded the highest lipase activity (123.91 U/ml) and productivity (1.17 U/ml/h). Similar nitrogen source was used for lipase production by using *Geotrichum* sp. (Stransky et al., 2006). Through literature survey, we noticed that requirements on the types of

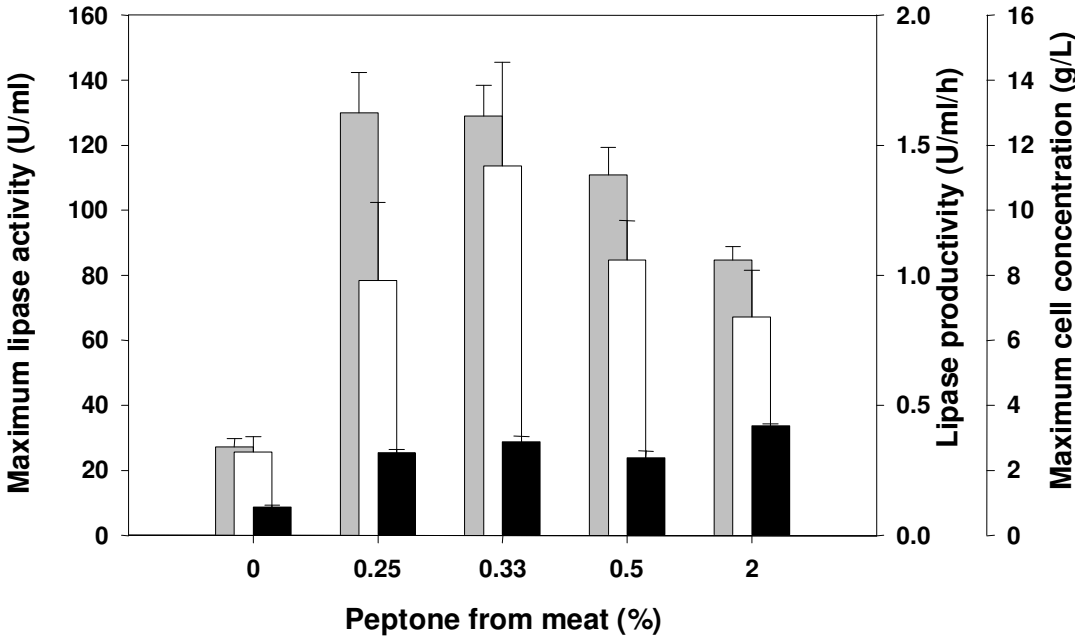

Figure 3. Lipase production by *B. cenocepacia* ST8 in shake flask using different concentration of peptone from meat. Bars: ( ), maximum lipase activity; ( ), lipase productivity; ( ), maximum cell growth. Data shown are the means of three independent experiments with error bars.

nitrogen sources for lipase production varied among types of microorganisms. For instance, *Burkholderia* sp. GXU56 favored the combination of yeast extract and peptone for maximum lipase production compared to inorganic nitrogen source (Wei et al., 2008) while *P. fluorescens* NS2W preferred ammonium dihydrogen phosphate as nitrogen source (Kulkarni and Gadre, 2002).

In the study, concentrations of chosen nitrogen source, that is, peptone (from meat) had pronounced effect on lipase production (Figure 3). The production of lipase was enhanced with the increase of peptone concentration from 0 to 0.33% w/v. Peptone concentration at 0.33% (w/v) recorded the highest lipase productivity (1.42 U/ml/h). In contrast, significantly low lipase activity was observed when the peptone concentration was further increased to 2% (w/v). These peptone-dependence results were similar to that reported in the study using *Fusarium solani* FS1 when 0.50% of peptone almost abolished lipase production (Maia et al., 1999).

Addition of various metal ions

Metal ions can either stimulate or inhibit microbial enzyme production (Gupta et al., 2004). As illustrated in Figure 4, Ca^{2+} ion was the most suitable metal ion for lipase production (129 U/ml). The metal ions might be involved in the formation of complexes with ionized fatty acids, resulting in activation of enzymes (Gulomova et al., 1993). In this study, most of the metal ions under investigation, such as Mg^{2+}, Mn^{2+}, Na^{+} and Fe^{2+} had adverse effect on lipase production and Cu^{2+} and Co^{2+} exhibited strong inhibition on lipase production (less than 5 U/ml).

Ions of calcium, magnesium and sodium were reported to greatly enhance the lipase production from different type of microorganisms. For instance, lipase production by *B. multivorans* was enhanced in the presence of Ca^{2+} (Gupta et al., 2007); improvement of lipase production was done by *P. fluorescens* NS2W in the medium containing Mg^{2+} and Ca^{2+} (Kulkarni and Gadre, 2002); stimulation of lipase production using *Burkholderia* sp. GXU56 by addition of Mg^{2+} was observed (Wei et al., 2008); and, addition of 1.5% NaCl to the medium induced high level of lipase production by *P. fluorescens* NS2W (Kiran et al., 2007).

Effect of initial culture pH

In the study, initial culture pH had prominent effects on the cell growth and lipase production by *B. cenocepacia* ST8 (Figure 5). Initial pH of culture medium ranging from 5 to 11 promoted good growth and lipase production with the initial pH of 9 as the optimal pH for lipase production. In contrast, extreme low or high initial culture pH such as 3 and 13 repressed the growth and lipase production by

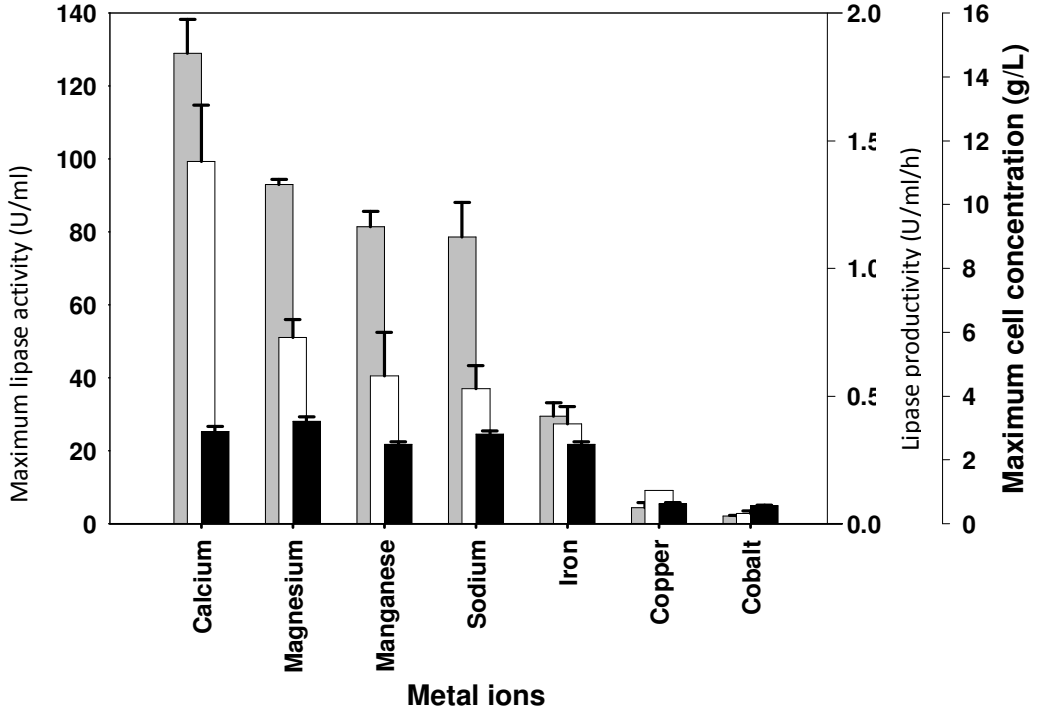

Figure 4. Lipase production by *B. cenocepacia* ST8 in medium containing different type of metal ions. Bars: (▒), maximum lipase activity; (□), lipase productivity; (■), maximum cell growth. Data shown are the means of three independent experiments with error bars.

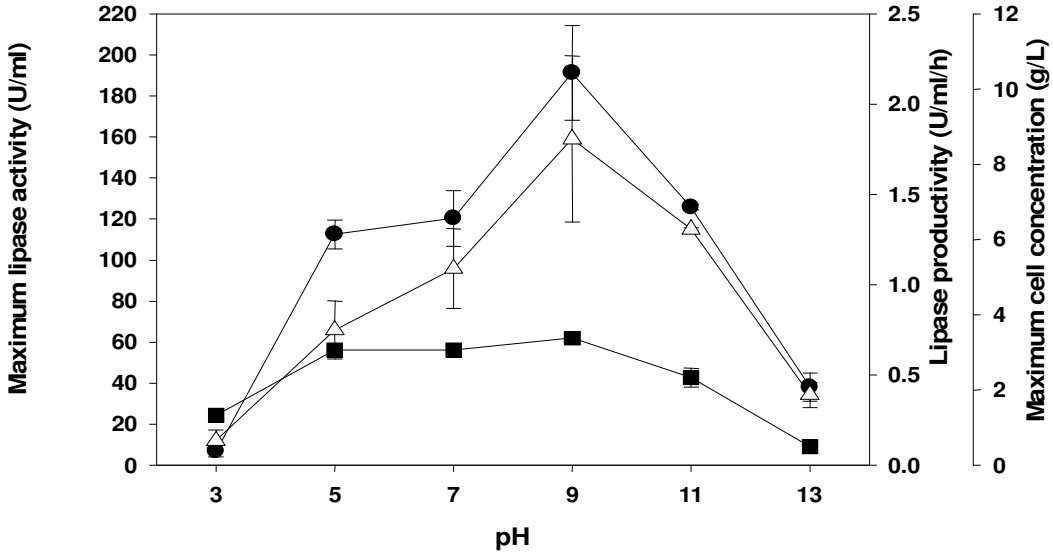

Figure 5. Lipase production by *B. cenocepacia* ST8 in medium with different initial culture pH. Symbols: (●), maximum lipase activity; (□), lipase productivity; (■), maximum cell growth. Data shown are the means of three independent experiments with error bars.

this local isolate.

As reported, maximum lipase production by *B. coagulans* BTS-3 (Kumar et al., 2005) and *Pseudomonas* sp. MSI057 (Kiran et al., 2007) occurred at pH 8.5 and 9.0, respectively. Lipase-producers such as *B. cepacia* and *Bacillus stearothermophilus* AB-1 preferred pH around 7.0 for optimal growth and lipase production (Rathi et al., 2001; Abada, 2008). Hence, initial culture pH

is an important factor which affects the lipase production and it varies with the types of microorganisms.

Conclusions

An indigenous *B.cenocepacia* was proven to be a potential lipase-producing bacterial strain. Lipase production by this strain is influenced by various physical and nutritional factors. From the study, maximum lipase production of 191 U/ml was achieved in the cultivation medium containing 1% (v/v) of sunflower oil supplemented with 1% (v/v) of Tween 80, 0.33 (w/v) of peptone (from meat) and initial culture pH of 9.

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