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Production of Lipase by *P. fluorescens* (NRLL B-2641) under Submerged Fermentation Conditions

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Lipases are industrially important enzymes produced by a great variety of microorganisms. In this study, the production of extracellular lipase by *Pseudomonas fluorescens* (NRLL B-2641) using submerged fermentation was studied. The effects of initial pH, carbon source, nitrogen source and metal ion addition on lipase activity were investigated using batch experiments. The highest enzyme activity and biomass production obtained were 2.50 U mL^{-1} and 2.95 g cell dry wt. L^{-1}, respectively, using a fermentation medium with 1% corn oil as the carbon source, 0.4% peptone as the nitrogen source and an initial pH of 6.0. The addition of calcium increased the enzyme activity to 3.30 U mL^{-1}. Thus, initial lipase activity, which was 0.92 U mL^{-1}, increased 356%. Partial characterization of crude lipase was performed to determine the optimum pH and temperature. The crude lipase displayed high activities at pH 6.0-8.5 and 35-65°C, with maximum activity at pH 8.0 and 50°C.

Key words: Lipase, *P. fluorescens*, submerged fermentation, thermophilic and alkaline lipase.

Lipases, which are activated only when adsorbed to an oil-water interface, represent a group of enzymes with the ability to hydrolyze triacylglycerols. Lipases act as an interface and catalyzes the hydrolysis of fats and mono- and di-glycerides to free fatty acids and glycerol (Silva *et al*. 2005; Olusesan *et al*. 2011). These enzymes have the ability to hydrolyze ester bonds, transesterify triglycerides and allow the resolution of racemic mixtures. They also synthesize ester bonds in non-aqueous media (Acikel, 2010).

Due to the increasing demand for these biocatalysts, the number of available industrial lipases has increased considerably since the 1980s (Silva *et al*. 2005). Lipases, especially of microbial origin, are used in commercial applications such as additives in food (flavor modification), fine chemicals (synthesis of esters), detergents (hydrolysis of fats), wastewater treatment (decomposition and removal of oil substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather working (removal of lipids from animal skins) and analytical tools (blood triglyceride assay) (Eltaweel, 2005).

Due to the extensive usage of lipases for different industrial application, enhancing lipase production during the cultivation process is essential. Different environmental factors have been extensively studied and are known to increase lipase productivity: these factors include, carbon source, nitrogen source, pH and temperature (Maia, *et al*. 2001; Azeredo, *et al*. 2007; Dalmau *et al*. 2000). Lipases can be produced by animals, plants and microorganisms. Because microbial lipases are more stable compared with plant and animal lipases and they can be obtained cheaply, microbial lipases currently have a broad spectrum of industrial applications (Ellaiah *et al*. 2004). The *Pseudomonas* genus produces a variety of lipases and is a well-known psychrophilic lipase-producing bacteria (Kiran *et al*. 2005).

The aim of the present work is was to determine culture conditions for maximum lipase

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production by *P.fluorescens* (NRLL B-2641) a gram-negative bacterium. The crude enzyme was characterized with respect to optimal pH and temperature.

MATERIALS AND METHODS

Microorganism and growth conditions

P.fluorescens (NRLL B-2641) obtained from American Type Culture Collection was used in the study. The nutrient growth medium contained the following ingredients (%w/v): glucose, 0.5; yeast extract, 0.1; peptone, 0.1; K_2HPO_4, 0.05; KH_2PO_4, 0.05; $(NH_4)_2SO_4$, 0.05 and $MgSO_4 \cdot 7H_2O$, 0.0005. The pH of the medium was adjusted to 7, and the medium was sterilized by autoclaving at 121°C for 45 min. The microorganism was incubated at 30°C in an orbital shaker (150 rpm) for 24 h. This culture was used as inoculum (in 1:10 ratio) in further studies by re-preparing before each fermentation.

Lipase production studies

The fermentation basal medium composition (%w/v) was as follows: olive oil, 0.1 (v/v); yeast extract, 0.1; K_2HPO_4, 0.05; KH_2PO_4, 0.05 and $MgSO_4 \cdot 7H_2O$, 0.0005. However, the medium composition was altered to determine the effect of different carbon sources (olive oil, corn oil, soybean oil, sunflower oil, tributyrin and glucose), nitrogen sources (yeast extract, peptone, $(NH_4)_2SO_4$, $(NH_4)H_2PO_4$) and metal ion additions (NaCl and $CaCl_2.2H_2O$) on lipase production. After the sterilization (at 121°C for 20 min), the pH of the medium was adjusted to the desired value. Then, 15 mL of growth medium was added aseptically as inoculum to 500 mL Erlenmeyer flasks containing 150 mL of fermentation medium. The fermentation was conducted at 30°C with orbital shaking at 150 rpm for 72 h. The cell-free supernatant obtained by centrifugation at 5,000 rpm for 10 min was measured for lipase activity.

Lipase activity determination

Lipase activity was determined titrimetrically using an olive oil emulsion method without the addition of surfactants according to the method of Rosu *et al*. (1997) with some modifications. Briefly, for free enzyme, 1 mL of olive oil was incubated with 3 mL of Tris-HCl 100 mM, pH (8.0), 0.5 mL of 100 mM $CaCl_2$ and 5 mL distilled water with stirring at 37°C for 10 min. One mL of enzyme solution was added to give a final volume of 10.5 mL. After 20 min, the reaction was stopped by adding 20 mL of acetone/ethanol solution (1:1). The amount of free fatty acid was titrated with 0.01 N NaOH solution to pH 10. Blank samples were treated similarly. One unit of extracellular lipase activity (U) was defined as the amount of enzyme necessary to release 1 µmol of fatty acid per minute under the assay conditions.

Protein determination

The protein content of the crude enzyme extract was determined by the Lowry *et al*. method (1951) using bovine serum albumin as the standard protein.

Specific activity determination

The specific activity was calculated as the ratio of lipase activity (U mL^{-1}) and protein content (mg g^{-1}) expressed as unit activity per mg of total proteins.

Cell growth determination

Cell concentration was determined by measuring the absorbance at 400 nm using a standard curve of absorbance against dry cell weight. The specific growth rate of the *P.fluorescens* (µ) is also defined as Eq. (1):

$$\mu = (1/X)\, dX/dt \quad \ldots(1)$$

It was determined from the slope of $\ln X$ versus time plot at the exponential growth region.

Partial characterization of crude lipase

The optimum pH of the crude lipase activity was investigated with incubation at different pH values from 5 to 10 for 20 min. The following buffers were used at 100 mM concentration: citrate buffer (pH 5.0), phosphate buffer (pH 6.0 and 7.0), Tris-HCl buffer (pH 8.0, 8.5 and 9.0) and glycine-NaOH buffer (pH 10). The residual activity at various pH values was calculated by taking the activity at pH 8.0 at 37°C as 100%. The lipase activity of each sample in each buffer was assayed under standard titrimetric assay conditions. The optimum temperature for lipase activity was determined by incubating the assay mixture over different temperatures (25 to 60 °C) at pH 8.0 for 20 min. Residual activity was calculated by taking the activity at 50°C as 100%. The lipase activity of each sample at the desired temperature was measured under standard titrimetric assay conditions (Rosu *et al*. 1997).

RESULTS AND DISCUSSION

Influence of initial pH on lipase production

The initial pH of the culture medium is one of the most important environmental parameters affecting microbial cell growth and enzyme production (Maia *et al*., 2001). The effect of initial pH on the lipase production from *P. fluorescens* was investigated for pH values from pH 5.0 to 9.0 as shown in Fig. a.1. The enzyme activity increased during logarithmic phase, and the highest lipase activity was obtained at pH 6.0 as 0.92 U mL^{-1} (Fig. b.1). Makhzoum *et al*. (1995) and Rajmohan *et al*. (2002) also reported lipase production during the logarithmic phase with different *P. fluorescens* strains. Many other researchers have also demonstrated the extracellular secretion of lipase and proteinase with psychrophilic bacteria during the logarithmic growth phase (Juffs *et al*. 1968; Adams *et al*. 1975; Stead, 1987).

Effect of carbon sources on lipase production

Studies on extracellular lipase productions with different microorganisms claim that both sugar and oils enhance lipase production (Maia *et al*. 2001; Ramani *et al*. 2010; Rodriguez *et al*. 2006). In this work, different triglycerides (1% v/v), olive, corn soybean, sunflower and tributyrin, and a carbohydrate, glucose (0.1% w/v), were used as carbon sources. For each carbon source, the changes in the medium properties depending on maximum lipase activity of all samples as assayed

Table 1. Effect of different carbon sources on fermentation medium properties

Carbon source	Lipase activity (U mL^{-1})	Biomass (g cell dry wt. L^{-1})	μ (h^{-1})	Total protein concentration (mg mL^{-1})	Specific activity (U mg^{-1})	pH
Glucose	0.80	0.86	0.621	0.35	2.29	6.80
Olive oil	0.92	0.72	0.015	0.38	2.41	7.16
Corn oil	1.47	1.69	0.013	0.26	5.61	5.85
Soybean oil	1.12	0.67	0.012	0.20	5.61	6.87
Sunflower oil	1.10	1.01	0.021	0.23	4.84	7.17
Tributyrin	1.42	0.76	0.018	0.35	4.08	6.82

Table 2. Effect of different nitrogen sources on fermentation medium properties

Nitrogen source	Lipase activity (U mL^{-1})	Biomass (g cell dry wt. L^{-1})	μ (h^{-1})	Total protein concentration (mg mL^{-1})	Specific activity (U mg^{-1})	pH
Yeast extract	1.49	1.42	0.025	0.44	3.37	7.25
Peptone	2.50	2.96	0.029	0.23	10.74	6.57
$(NH_4)_2SO_4$	0.75	0.69	0.014	0.06	11.94	3.63
$(NH_4)H_2PO_4$	0.87	0.68	0.022	0.05	15.72	5.91

Table 3. Effect of the metal ions addition in different concentration to fermentation medium properties

	Concentration (%)	Lipase activity (U mL^{-1aw})	Biomass (g cell dry wt. L^{-1})	Total protein concentration (mg mL^{-1})	Specific activity (U mg^{-1})	pH
Na^+	0.01	3.10	0.92	0.25	12.40	6.56
	0.02	3.00	1.00	0.24	12.50	6.54
	0.03	2.90	0.93	0.28	10.35	6.54
Ca^{2+}	0.01	3.30	2.57	0.27	12.22	6.69
	0.02	3.10	1.74	0.25	12.40	6.65
	0.03	3.02	2.14	0.26	11.61	6.42

at different time intervals up to 96 hours are displayed in Table 1. The highest lipase activity and biomass (1.42 U mL^{-1} and 1.69 g cell dry wt. L^{-1}, respectively) occurred in the fermentation medium containing corn oil. In the presence of other carbon sources, especially glucose, there was a reduction in lipase production. Some other studies have also reported that glucose has a repressive effect on lipase production (Kiran *et al*. 2008; Narasaki 1968, Mates and Sudakevi, 1973). This can be observed frequently in the fermentation processes using easily utilizable carbon sources such as glucose. There are also some studies indicating that glucose totally inhibits the production of lipase enzyme (Makhzoum *et al.* 1995; Lima *et al.* 2003; Eltawell *et al*. 2005). With the rapid utilization of glucose, a high specific grow rate of *P. fluorescens* was obtained (0.62 h^{-1}). Fermentation medium containing corn oil resulted in the lowest pH value compared with other media. This may be due to the fatty acids remaining unused in the medium by the microorganisms.

Effect of nitrogen sources on lipase production

Inorganic and organic nitrogen sources play an important role in regulating the synthesis of hydrolytic enzymes. Inorganic nitrogen sources are consumed rapidly and may cause repression of enzyme synthesis because of the formation of ammonium repressible entities, whereas organic nitrogen sources can provide amino acids, and many cell growth factors, which are essential for cell metabolism and protein synthesis (Iftikhar *et al*. 2008). The influence of organic nitrogen sources (yeast extract and peptone) and inorganic nitrogen sources ($(NH_4)_2SO_4$ and $(NH_4)H_2PO_4$) at a 0.4% (w/v) concentration on enzyme production in a medium with corn oil as the carbon source was is shown in Table 2. Peptone was determined to be the best nitrogen source for lipase production (2.50 U mL^{-1}). In the presence of inorganic nitrogen sources, there was a reduction in both the biomass and the lipase activity. Some researchers have also observed that organic nitrogen sources provide the best results (Al-Saleh and Zahran, 1999; Sekhon *et al*. 2006; Rahman *et al*. 2006; Babu and Rao, 2007). In contrast, some other researchers, such as Kanwar *et al*. (2002) and Rathi *et al*. (2001), have obtained high lipase activities with inorganic nitrogen sources. Kumar *et al*. (2005) reported that strain differences and synergistic effects with other

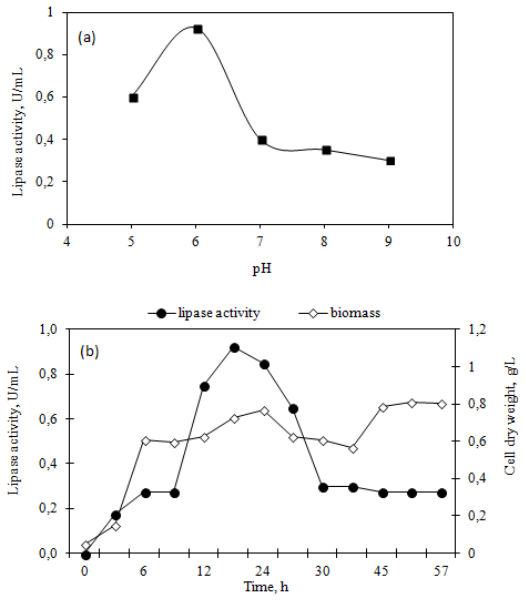

Fig. 1(a). Effect of pH on lipase production by *P. fluorescens*; b) Lipase activity and cell dry weight by time at pH 6

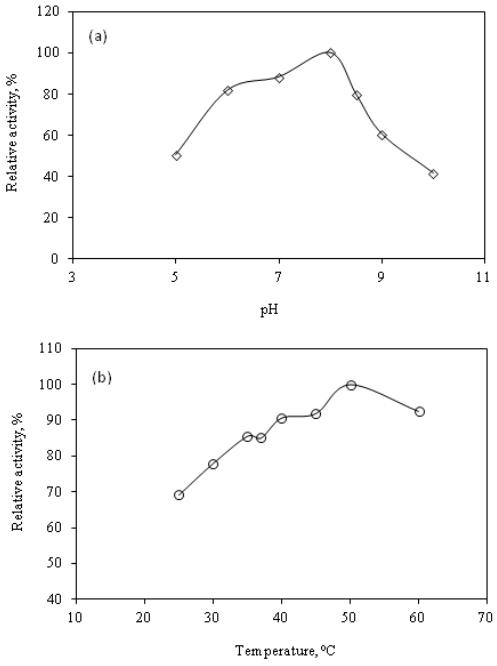

Fig. 2. Effect of (a) pH and (b) temperature on crude lipase activity.

factors present in the medium might be responsible for differences in the obtained results. In agreement with these studies, in the present study, it was observed that the pH of the medium containing ammonium sulfate decreased. This could be due to the acidic characteristics of ammonium sulfate being used as a nitrogen source. Because the total protein concentration was low in the medium containing ammonium di hydrogen phosphate, a high specific activity (15.72 U mg^{-1}) was obtained.

Effect of the addition of metal ions on lipase production

Metal ions may have stimulatory or inhibitory effects in enzyme synthesis. To determine the effect of Ca^{2+} and Na^{+} ions on lipase production, their chlorides ($CaCl_2.2H_2O$ and NaCl, respectively) at different concentrations were added to basal media containing 1% corn oil and 0.4% peptone (Table 3). When the culture media were supplemented with metal ions, the enzyme production was much higher than in the same media without metal ions (3.30 U mL^{-1} and 3.10 U mL^{-1} for Ca^{2+} and Na^{+} ions in 0.01% concentration, respectively). The importance of metal ions for lipase production has been previously demonstrated in several bacterial species. Sharma *et al*. (2002) reported stimulation of lipase production from *Bacillus* sp. RSJ1 in the presence of calcium chloride. Similarly, Lin *et al*. (2006) demonstrated a stimulatory effect on the lipase production by *Antrodia cinnamomea* with the addition of NaCl to the fermentation medium. McKellar (1989) studied the synthesis of extracellular enzymes by psychrotrophs and noted that calcium is one of the most important ions involved in this synthesis. Our study also suggests that calcium is one of the effective ions in enhancing lipase production by *P. fluorescens*.

Effect of temperature and pH on crude lipase activity

The effect of pH on crude lipase activity was examined in the pH range of 5-10 at 37°C and the optimum pH value was determined to be 8.0 (Fig. a.1). The lipase activity at pH 6.0, 7.0 and 8.5 was approximately 81, 88 and 79% of the optimum activity observed at pH 8.0, respectively. This alkaline pH value indicates shows that lipase is suitable for adsorption to an oil–water interface and helps lipase to open barriers closing active sites to reduce the activation energy of hydrolysis (Sharma, 2009). Lipases active at alkaline pH are very crucial in some industries for running bioprocesses, for their use to concentrate target polyunsaturated fatty acids, and other biotechnological applications (Chakraborty and Paulraj, 2009).

High crude lipase activity was observed in the temperature range 35-60°C, with maximal activity at 50°C (Fig. b.2). In addition, 92% of the maximum activity was preserved at 60°C. Protection of the activity of lipase at thermophilic temperatures is important for some industries that require processing at high temperatures. Some other researchers have also reported some optimal pH and temperature values or ranges for crude or purified lipase using different *P. fluorescens* strains such as *P. fluorescens* P21, 7.0 and 20-60°C (Cadirci and Yasa, 2010); *P. fluorescens* HU 380, 8.5 and 45°C (Kojima and Shimizu, 2003); *P. fluorescens* MC50, 8.0-9.0 and 30-40°C (Bozoglu *et al*., 1984) and *P. fluorescens* NS2W, 9.0, 55°C (Kulkarni and Gadre, 2002).

DISCUSSION

Pseudomonas lipases play an important role in industrial applications. The production of lipase is generally practiced using the submerged fermentation method. In this study, the effect of medium components on the production of lipase with *P. fluorescens* using submerged fermentation was investigated. The highest enzyme activity was obtained as 2.50 U mL^{-1} in medium containing 1% corn oil as the carbon source and 0.4% peptone as nitrogen source with an initial pH of 6.0. A stimulatory effect on lipase production as a result of the addition of Na^{+} and especially Ca^{2+} ions to fermentation medium was observed. The crude lipase displayed high activities at an alkaline pH range of 6.0-8.5 and in a wide range of temperatures (35-60°C), with optimum activity at pH 8.0 and 50°C.

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