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Optimization of Lipase Production by a *Rhizopus* MR12 in Shake Culture

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Abstract: *Rhizopus* sp. a mould of mucor family, excrete lipase when cultured on lipolytic media. The *Rhizopus* sp. produced a larger clear zone on tributyrin agar medium suggesting its esterase activity. It was further investigated in liquid medium in order to optimize the lipase production conditions under shake culture. Lipase production was found to be maximum with medium containing maltose (1%) and peptone (5%) as carbon and nitrogen sources, respectively with *Rhizopus* sp. The enzyme production was profoundly influenced by initial pH of the medium and optimum value of this parameter was found to be 6.0. Maximum enzyme production was obtained at 30°C with a shaking rate of 200 rpm. Ca^{2+} was found to stimulate lipase production, while it was strongly inhabited by Hg^{2+}. Lipase production was increased about 23.7% under optimized cultivation conditions over olive oil-peptone medium.

Key words: Lipase activity, *Rhizopus*, optimum condition

INTRODUCTION

Lipases (Triacylglycerol hydrolase, EC 3.1.1.3) are ubiquitous among microbes, plants and animals which catalyze the hydrolysis of ester linkages of triglycerides to form glycerol and fatty acids. Traditionally, these microbial lipases have been used in the food and detergent industry for the ripening of the cheese and as laundry detergent additives. Recently these enzymes have become of interest to the chemical, pharmaceutical and leather industries (Gulati *et al*., 2005; Gunstone, 1999) because of their ability to hydrolyze ester bonds, trans-esterify triglycerides, resolve racemic mixtures (Muralidhar *et al*., 2001; Reetz, 2002) and synthesize a variety of stereo-specific esters, sugar esters, thioesters and amides (Singh *et al*., 2003; Dellamora-Otiz, 1997).

Because of huge variation in applications, it has been renewed interest in the development of sources of lipase. Choo *et al*. (1998) reported that though numerous species of bacteria, yeasts and moulds produce lipase with different enzymological properties and specificities, moulds are known to be more potent lipase producer. These microorganisms produce lipase both by solid substrate and submersed fermentation (He *et al*., 2004). The best results in the production of lipase were obtained in the present of carbon and nitrogen sources (Fadiloğlu and Erkmen, 2002). Alonso *et al*. (2005) studied the lipase production by a brazilian wild srain of *Yarrowia lipolytoca* at different stirring speeds and air flow rates. They detected maximum lipase activity at 200 rpm.

Factor affecting microbial extracellular lipase production have been widely studied in bacteria (Lawrence *et al*., 1967; Mates and Sudakevitz, 1973), moulds (Chander *et al*., 1980; Chopra and Chander, 1983) and yeasts (Ota *et al*., 1968). Recently Kshmiri *et al*. (2006) cultivated a new strain *Trichoderma viride* for lipase production in shake flasks at 30±1°C and highest specific growth rate was observed between 6 and 18 h, though maximum fungal biomass was present at 13.6 g L^{-1} at 60 h. Few studies have been conducted with *Rhizopus* to examine the control of lipase production and the growth of the fungus. This study was, therefore, initiated to investigate the effect of different growth conditions supplemented with various nitrogen and carbon sources on the lipase production by *Rhizopus* MR12.

MATERIALS AND METHODS

The *Rhizopus* sp. was isolated from decomposed of mushroom culture. The organism was grown on Potato Dextrose Agar (PDA) plates or slants at 30°C and pH 5.5 for 2-3 days and stored at 4°C, with sub culturing every

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3 or 4 weeks interval. All experiments were conducted in the laboratory of the Department of Microbiology, University of Dhaka, Bangladesh.

Media and shake flask culture condition: The basal medium contained the nitrogen source 5%, $(NH_4)_2SO_4$, 0.1%; K_2HPO_4, 0.1% and different concentration of carbon sources (Maltose, lactose, xylose, glucose and glycerol) in 100 mL distilled water. The initial pH of the medium was adjusted to pH 5.5.

A culture medium of 50 mL (in baffled-Erlenmeyer flask) was inoculated with the fungal mycelia from 23 cm^2 area if a PDA plate containing a mat like mycelia of 2-3 days growth of *Rhizopus* MR12. For each treatment at least two replica flasks ware made. The flasks were then shaken at 30°C on an orbital shaker (Gallenkamp, Germany) at 150 rpm for 5 days. The culture filtrate was assayed for lipase activity and other compounds.

Enzyme assay: Lipase activity was assayed by titrating fatty acid liberated from olive oil due to lipase activity with 0.05 M NaOH as describe by Ota *et al.* (1968). One unit of lipase activity was defined as the amount of lipase which liberated 1 μmol of fatty acid per minute at 30°C.

Optimization of cultural conditions for lipase production: Lipase production was optimized by altering various physico-chemical and cultural conditions observing the effect after 8 h of incubation at different temperature.

- The effect of incubation period on lipase production from *Rhizopus* sp. was studied measuring the enzyme activity, growth and extra cellular protein at different incubation period (8, 16, 24, 32, 40, 48, 56 and 72 h).
- The effect of various supplements on lipase production was studied by adding different carbon sources (glucose, maltose, lactose, sorbitol, xylose, glycerol at an amount of 1% and olive oil at an amount of 2%), nitrogen sources (peptone, yeast extract, meat extract, urea, casein, corn steep liquor at an amount of 5%), metal ions ($CaCl_2$, $ZnCl_2$, $MgCl_2$, $FeCl_2$, $BaCl_2$, $HgCl_2$, con. 1 mM) in the basal medium.
- Since the lipase production was maximum in the maltose (carbon source) and peptone (nitrogen source), different concentration of maltose (0.5, 1, 2%) and peptone (1, 3, 5%) were used to determine optimum concentration of them.
- The effect of incubation temperature on the production of lipase was observed at different temperature (25, 28, 30, 33, 35, 40°C) with 1% maltose as the carbon source and 5% peptone as the nitrogen source.
- The effect of initial pH of the culture was investigated using shake flask cultivation with 1% maltose as the carbon source and 5% peptone as the nitrogen source. The initial pH of the culture medium was adjusted to (3.0, 4.0, 5.0, 5.5, 6.0, 7.0, 8.0 and 9.0) with 1 M HCl and 1 M NaOH.
- The effect of shaking rates was carried out at different values (120, 140, 160, 180 and 200 rpm).

Others analysis: Soluble protein in the culture filtrate was analyzed by the method of Bradford (1976). Total Dry Mass (TDM) in the filtrate medium was determined by the method of Hoq *et al.* (1984).

RESULTS AND DISCUSSION

The optimum incubation period for lipase production (Fig. 1a), growth and extracellular protein section was found to be 30 to 32 h (Fig. 1b). The kinetics of growth and protein secretion was correlated. The production of

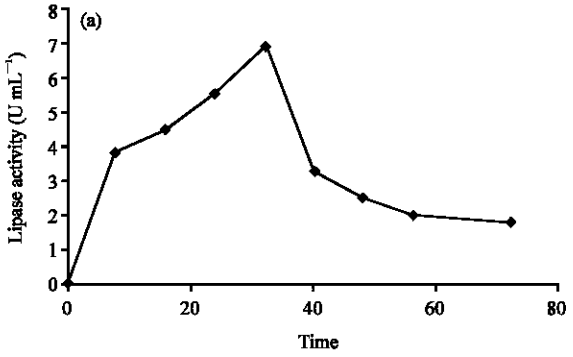

Fig. 1a: Effect of incubation period on the production of lipase by *Rhizopus* sp.

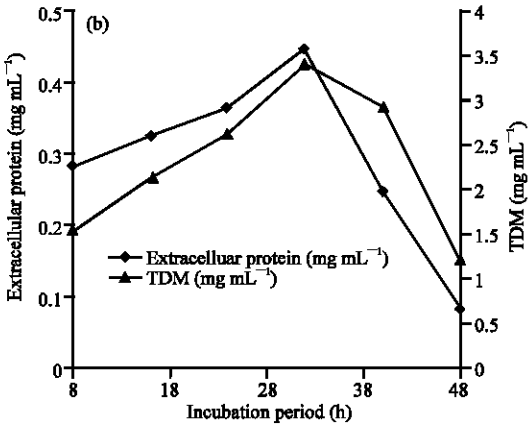

Fig. 1b: Effect of incubation period on the production of extracellular protein and growth (Total Dry Mass-TDM) by *Rhizopus* sp.

Table 1: Effect of various carbon and nitrogen sources on the production of lipase and extracellular protein by *Rhizopus* sp.

Sources	Substrate added to base medium	Lipase productivity (U h^{-1} L^{-1})	Extracellular protein (mg mL^{-1})	TDM (mg mL^{-1})
Carbon sources	Basal	121.875	0.156	2.1
	Maltose	614.375	0.657	15.8
	Lactose	527.812	0.256	12.8
	Xylose	481.250	0.190	7.94
	Glucose	462.500	0.231	10.6
	Glycerol	400.000	0.238	8.96
	Olive oil	193.750	0.429	6.06
	Sorbitol	195.312	0.224	5.6
Nitrogen sources	Basal	190.625	0.325	5.2
	Peptone	567.500	0.597	18.8
	Meat extract	318.750	0.329	4.2
	Yeast extract	512.500	0.412	18.74
	Casein	325.000	0.442	18.2
	Urea	-ve	0.020	3.6
	Corn steep liquor	-ve	-ve	-ve

enzyme was found to be growth associated as the lipase activity increased with the increase in growth. This type of shorter incubation period is unique as of the previous reports indicated that optimum incubation period for lipase production by most fungi was around 72 h or longer (Samad *et al*., 1990; Akhter *et al*., 1980).

The effect of carbon sources on the production of lipase by *Rhizopus* was tested by using glucose, maltose, lactose, sorbitol, xylose, glycerol at an amount of 1% and olive oil at an amount of 2% as depicted in Table 1. In the present study, lipase productivity, production of soluble protein and biomass production was observed maximum with maltose followed by lactose. This finding agreed with Nakashima *et al.* (1988), who observed similar effect with *Rhizopus chinensis*. Lipase production in the media on olive oil was much lower than in media containing carbohydrates. This decrease of lipase production is comparable with that reported by Chander *et al.* (1980) who concluded that butter oil and olive oil inhibited lipase activity by 53 and 63%, respectively, similar observation were made both Lawrence *et al.* (1967) and Eittenmiller *et al.* (1970). As maltose supported high enzyme production than olive oil or fat, so mass production in water soluble media will be advantageous, because keeping the lipid substrate under emulsified condition needs vigorous agitation as opposed to soluble substrates. Olive oil was found to decrease lipase production similarly might be due to the inhibition by fatty acid concentration, which was liberated during the hydrolysis of triglycerides (Omer *et al*., 1987).

Among the nitrogen sources tested the maximum lipase (567.5 U h^{-1} L^{-1}) and extracellular protein (0.597 mg mL^{-1}) production and growth (18.8 g mL^{-1}) was obtained on peptone (Table 1). Urea and ammonium sulphate failed to support lipase production by the fungi as they did with *R. oligosporus* (Nahas, 1988) and *Acremonium stricum* (Okele and Okolo, 1990) respectively. These indicate that organic nitrogen sources

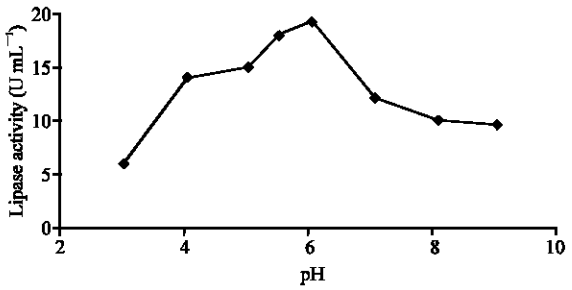

Fig. 2: Effect of pH on the lipase activity

were preferred to inorganic ones for lipase production. In the present study corn steep liquor was failed to support both growth and synthesis of the enzyme, although it was a good nitrogen sources for lipase production by *Humicola lanuginosa* (Omar *et al*., 1987). So from these observations it can be stated that the lipase production was highly substrate specific.

The pH dependency of the culture played an important role for both growth of the fungi and the production of enzymes. The optimum pH for lipase production by *Rhizopus* sp. was found to be 6.0 (Fig. 2). During the growth of *Rhizopus* the pH was found to fluctuate in a narrow range. The change in medium pH may be compounds in the culture conditions employed.

Incubation temperature has been found to be significant controlling factor for enzyme production. The optimum incubation temperature for growth and lipase production by the fungi was found to be 30°C (Fig. 3). Most of the industrial application of microbial lipases for the hydrolysis of glycerides, interesterification of fatty acid moieties and so on, basically involved the use of mesophilic enzymes. Lipases of various species *Rhizopus* have been purified and characterized, including *Rhizopus delemar* (Iwai and Tsuijisaka, 1974), *R. chinensis* (Nakashima *et al*., 1988), *R. arrhizus* (Benzonana, 1974) and *R. rhizopodifornis* (Samad *et al*., 1990). However with

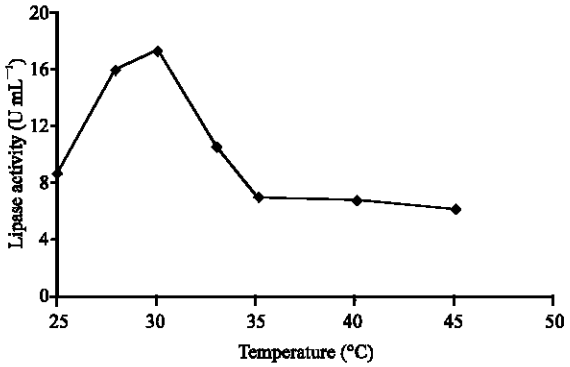

Fig. 3: Effect of temperature on the production of lipase by *Rhizopus* sp.

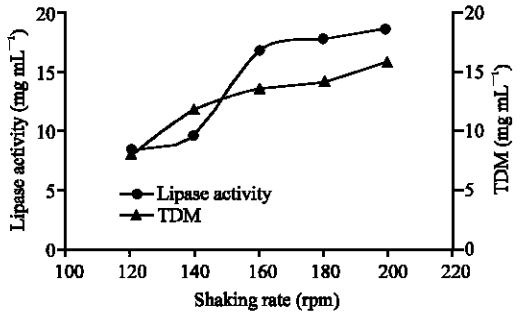

Fig. 4: Effect of agitation rates on growth and lipase production

the exception of *R. rhizopodiformis* (Nahas, 1988; Razak *et al*., 1989) reported that most of the *Rhizopus* species are mesophilic fungi.

Usually shaking rate i.e., agitation/aeration had profound influenced on the lipase production by aerobic microorganisms as increase in shaking rate increase the availability of dissolved oxygen. In addition shaking may also create condition of higher availability of the carbon sources to microorganisms (Nahas, 1988). In the present study, enzyme production was increased with increase in shaking rates (Fig. 4). This type of result was observed in case of mesophilic *R. oryzae* (Razak *et al*., 1989), *A. oryzae* (Ohnishi *et al*., 1994). But incase of *R. oligosporus* static incubation was required for maximum lipase production. This may be due to the denaturing effect of stress on the enzyme protein. The presence of Ca^{2+} in the culture medium was shown to stimulate lipase activity. Other ions such as Fe^{3+}, Zn^{2+} slightly increased lipase production while Hg^{2+}, Ba^{2+} and Mg^{2+} decreased the lipase production (Fig. 5) as happened with *Humicola lanuginosa* on sorbitol corn steep liquor (Omar *et al*., 1987). Metal ions generally form complexes with ionized fatty acids, changing their solubility and behavior at the interface. Lipase production was maximum in maltose and peptone and their optimum concentration as shown in Fig. 6 and 7.

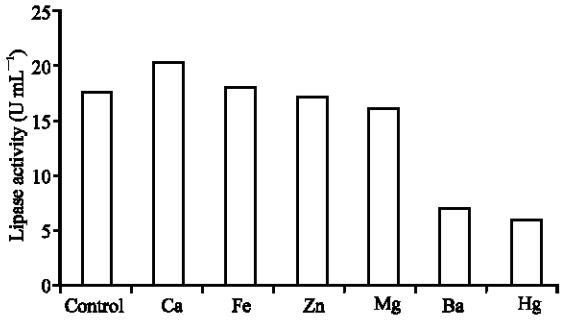

Fig. 5: Effect of metal ions on the production of lipase by *Rhizopus* sp.

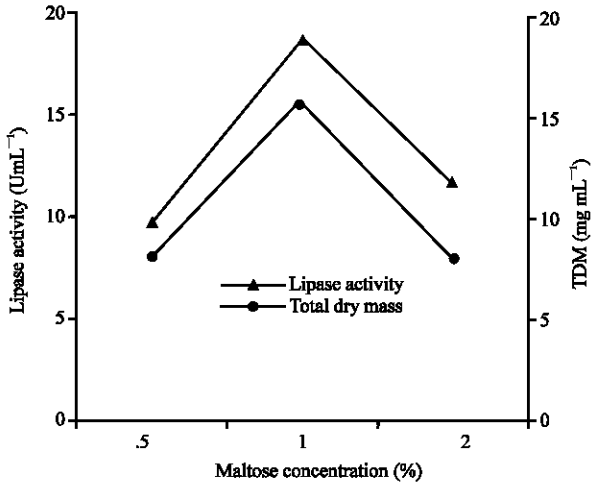

Fig. 6: Effect of different concentration of maltose on the growth and production of lipase by *Rhizopus* sp.

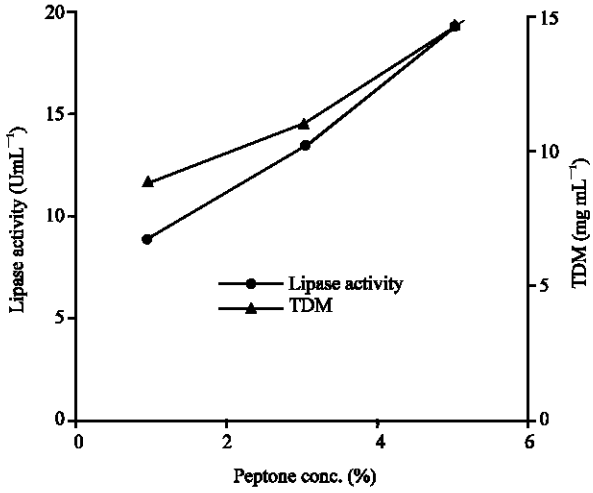

Fig. 7: Effect of different concentration of peptone on the growth and production of lipase by *Rhizopus* sp.

With the optimized medium (1% maltose and 5% peptone with $CaCl_2$) maximum amount of lipase production

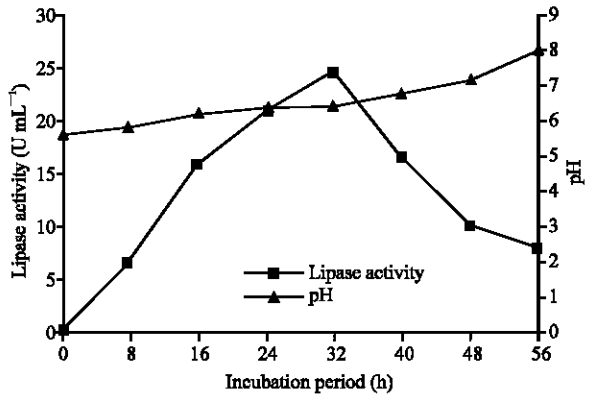

Fig. 8: Lipase production and change of pH in the culture under optimized cultivation conditions (30°C)

(24.32 U mL^{-1}) was possible within 32 h cultivation at 30°C with the pH maintained within 6.0-7.0 (Fig. 8).

The results of the present study revealed that the maximum amount of enzyme is produced after 30 to 32 h of incubation period at 30°C. This shorter incubation period and ambient temperature of lipase production offers biotechnological exploitation of this organism. Purified lipase of this fungus can be used in tanneries and detergent as cleansing aid in our industries.

CONCLUSIONS

Modification and improvement of lipase production by *Rhizopus* sp. will lead to new and useful applications for this organism. More over it was observed that enzyme production was highly growth associated. Hence further study relating to increase in growth rate may be helpful to increase the enzyme production. It was also observed that cultivation conditions greatly affected lipase production and optimization of the cultivation factors improved lipase production. Thus optimized conditions for maximum enzyme production in the shake culture study will be useful for lipase production in controlled conditions of bioreactor.

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