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OPTIMIZATION OF LIPASE PRODUCTION FROM *BACILLUS* SP.

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Abstract

Lipases are a class of enzymes, which catalyze the hydrolysis of long chain triglycerides. Microbial lipases are currently receiving much attention with the rapid development of enzyme technology. Lipases have industrial potential in the chemical, pharmaceutical, medical, cosmetic and leather industries, paper manufacturing industry, biosurfactant synthesis, mutation and agrochemicals. *Bacillus subtilis* strain was isolated from soil. This strain was tested for the production of extracellular enzyme "Lipase" by batch culturing in shake flask. The growth conditions were optimized for the maximum production of enzyme. Parameters such as temperature, incubation period, pH, carbon source, nitrogen source and lipids to be used were studied. Maximum lipase production was found in 48 hour old culture filtrate at 37°C, pH 8.0. Lipase activity was measured spectrophotometrically by using *p*-nitrophenyl laurate as substrate. Among all the carbon sources, fructose, salicin and glycerol gave the maximum activity and among all the nitrogen sources yeast extract gave maximum production/ activity. Tween (20 & 80) does not stimulate the growth much but assisted in enzyme synthesis.

Introduction

Lipases (glycerol ester hydrolases, E. C. 3. 1. 1. 3) are hydrolases acting on the carboxylic ester bonds present in acylglycerols to liberate organic acids and glycerol. Microbial lipases are usually extracellular enzymes, which are produced by various fungi, actinomycetes, yeasts and bacteria (Hou and Johnston, 1992). In recent years there has been a great demand for thermostable enzymes in industrial fields (Sugihara et al., 1992). Lipases are prepared commercially as digestive enzymes, for the dairy industry to modify odour and taste, for the detergent industry, production of fatty acids and glycerol via hydrolysis of oil and fats, leather industry, etc.

The pH dependencies of activity and stability may depend on culture conditions. If the organisms are grown at alkaline pH, the lipase produced may have an alkaline pH optimum. When screening bacteria for lipase production both culture pH and assay pH are important parameters. The stability depends upon the presence of substrate (Andersson et al., 1979). There are a variety of conditions that have been described, which stimulate or repress the production of lipases by bacteria. Carbon and nitrogen sources are the fundamental substances for the growth of the bacteria. Normally they are supplied from natural sources (Pimental et al., 1994).

It was found by Lesuisse et al. (1993) that the lipase from *Bacillus subtilis* 168 was active towards *p*- nitrophenyl esters. The stimulation of lipase production by lipids in the growth medium has frequently been observed, but no systematic investigation of the effects of lipids on the lipase production has been formed.

During processing of hides and skins, one important step is the removal of residual fats and protein debris that are associated with the hide and the hair. Such removal by chemical processes, such as liming, is not efficient. It has now become common practice to utilize a mixture of lipases and proteases for this purpose (known in the technical jargon as the bating process) (Posorske, 1984).

We report here the optimum conditions for the production of extracellular alkaline lipase from *Bacillus* sp., which has also been reported as the most probable

source of protease production. This property makes this strain very useful for the use in tanning industry in the process of bating.

Materials and methods

Microorganisms: Four strains of Bacillus subtilis were isolated from soil and wastes of tanneries. These were identified on the basis of morphological and biochemical tests.

Qualitative test: The ability of four of Bacillus subtilis to produce lipase was tested. Medium (Sierra, 1957) containing Tween 80 as lipid substrate, at pH 8.0 was used for qualitative test of lipases. The lipolytic activity of each bacterial strain was determined by measuring the diameter of hydrolytic zones around each colony. The strain with the largest zone of hydrolysis was used for further study.

Growth and media: *B. subtilis* was grown aerobically in liquid medium containing/L; yeast extract, 2.4%; casein hydrolysate, 1.2%; KH_2PO_4, 0.17M; K_2HPO_4, 0.72M; $MgSO_4$, 0.25g; NaCl, 0.5g; Glycerol, 0.4%; $CaCl_2$, 0.02g; olive oil, 1%. All the optimization studies were carried out on this media.

Separation of lipase: The samples were taken after every 24 hours for 72 hours and the culture broth was centrifuged at 10, 000 rpm. The supernatant or the crude enzyme extract was filtered and stored at –20°C till the assay for lipolytic activity is performed.

Total viable counts of bacteria: *Total viable counts of bacteria were taken from the cultures of 24, 48 and 72 hours to check the growth (Sharpley, 1960).*

Enzyme Assay: Lipase was estimated by using a colorimetric assay system with p-nitrophenyl laurate (pNPL) as substrate according to the method of Lesuisse et al., (1993).

One unit of activity is the amount of enzyme which released one nmol of *p*-nitrophenol per s under the assay conditions (37°C, pH 8.0).

Investigation of optimal culture conditions for lipase production

Effect of incubation period: Lipase production was carried out for 72 hours and the samples were collected after every 24 hours to check the production of lipase and growth.

Effect of pH and temperature on enzyme production: The enzyme production and the growth of *Bacillus sp.* was observed on media with pH 5, 6, 7, 8 and 9 and temperatures 30, 37 and 45°C.

Effect of various carbon and nitrogen sources: *Bacillus* sp. was cultured in medium containing different carbon and nitrogen sources (1% w/v) to find the conditions leading to the highest production of the enzyme. The pH and incubation temperature was kept at 8 and 37°C respectively.

Effect of lipids on enzyme production: The production of lipase was carried out at different lipids (Tween 80, 20 and olive oil) (1% v/v) in the production media with pH 8 and 37°C.

Results and discussion

Biocatalysts are chosen in the industry as a good alternative for inorganic catalysts where specific products are obtained (Sarkar et al., 1998). Lipases are the enzymes that hydrolyze oils and fats to glycerols and free fatty acids, carbon energy sources that can easily be utilized by many organisms. Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. Most of the well studied microbial lipases are inducible extracellular enzymes, synthesized within the cell and exported to its external surface or environment (Ota et al., 1982). So the biosynthesis of lipases was done by submerged culture fermentation on shake flask. Investigation of optimal cultural conditions i.e., incubation time, pH, temperature, carbon and nitrogen source and lipids, was done for maximum biosynthesis of lipolytic enzyme from *Bacillus subtilis* on shake flask culture. Lipolytic activity was assayed in the culture supernatant by using on *p*- nitrophenyl laurate as substrate.

Effect of incubation period: The culture supernatant obtained after 48 hours of incubation showed the maximum lipase activity (43.4 U/ml) as shown in Table I. Sarkar et al., (1998) have found that Pseudomonas strain isolated from soil gave the maximum yield of lipase after 72 hours of incubation. Handelsman and Shoham, (1994) showed that the production of lipase continued during the stationary phase for over 24 hours.

Effect of pH and temperature: As the pH plays an important role in all the biological processes, so lipase production was tested within a broad range of pH i.e. 5, 6, 7, 8 and 9. The study of the effect of different pH reveals that the enzyme secretion is greatly influenced by varying pH of the environment. The maximum enzyme was produced at pH 8 (48.5 U/ml) then followed by pH 9 (42.5 U/ml) and minimum production at pH 5 (Fig. I). This finding is supported by most of the investigators (Shabtai and Daya- Mishne, 1992; Falk et al., 1991). Although exceptions can be expected e.g. the optimal pH 6.5, temperature 30 degrees C, agitation 180 r/min and time 60 h was required for the maximum lipase production in case of Candida rugosa (Song et al., 2001). The variation in the enzyme production at different pH value could be due to the strain specificity.

In our study the effect of temperature on the activity of lipase reveals that the optimum temperature was 37°C (43.4 U/ml) (Fig. II). Same results have been observed in case of different strain of *Bacillus* sp. by Tsai et al., (1988).
An efficient lipid-degrading thermophilic aerobic bacterium was isolated from an icelandic hot spring and classified as *Bacillus thermoleovorans* IHI-91 that produces maximum lipolytic activity at 65 degrees C and pH 6.0 (Markossian et al., 2000).

Effect of carbon source: There are two main functions of carbon compounds in the living systems, i.e. to provide raw material for the structure and energy production. Each microorganism requires a different carbon source to produce lipase at its maximum level. The influence of different carbon sources on lipase synthesis was

therefore investigated by their addition to the growth medium. To select the most potent source for lipase production a variety of carbohydrates were used. Salicin and fructose are the two sugars that gave maximum lipase activity, 42.5 U/ml and 42 U/ml respectively (Fig. III), ph 8, 48 hrs of incubation at 37°C, similar to the results of Mates and Sudakevitz (1973). Song et al. (2001) found olive oil as the best among all the carbon sources used in case of *Candida rugosa* lipase production. Kamini et al., (2000) have reported that the optimum carbon sources and inducers in case of *Cryptococcus* sp. S-2 that enhanced lipase activity were sardine oil, soy bean oil and triolein. The effect of different carbon sources on the lipase production from a Brazilian strain of *Fusarium solani* FSI has been investigated by Maia et al., (2001) and it was shown that lipase activity was highest for sesame oil.
Lipase from Bacillus sp. was found to be inducible and its yield was significantly affected by the type of carbon source used.

Effect of nitrogen source: Different sources of nitrogen were tested in order to determine their influence on the synthesis of lipase. Yeast extract produced maximum enzyme activity (44 U/ ml) (Fig. IV) among all nitrogen sources studied, followed by peptone, tryptone and beef extract.

A strain of *Penicillium citrinum* has been found to produce lipase when the microorganism was cultured in the simple medium (1.0% olive oil and 0.5% yeast extract), using olive oil in as carbon source in the inocula, the enzyme extracted showed maximum activity. When yeast extract was replaced by ammonium sulfate, no activity was detected by Pimentel et al. (1994).

Song et al., (2001) found that *Candida rugosa* produces optimum lipase when NH_4NO_3 is used as the nitrogen source. Whereas, the lipolytic enzyme from *Bacillus thermoleovorans* IHI-91 have been shown to secrete a high level of lipase on yeast extract (Markossian et al., 2000).

Effect of lipids: Espinosa et al. (1990) suggested a double effect of Tweens that can serve as both inducers because of their chemical nature similar to that of some natural substrates, as well as the surfactants, stimulating the enzyme release. Song et al. (2001) found that the surfactants could be helpful to the lipase production, GPE in case of *Candida rugosa.*

The induction of production of lipolytic activities by Tween 80 has been reported for *Bacillus* sp. CM7 (Emanuilova et al., 1993). In the present study it was found that maximum lipase levels were obtained when Tween 80 is used as a source of lipid.

In the present investigations Tween 80 appeared to be the best inducer (with lipase activity 36 U/ ml) (Fig. V) followed by olive oil and Tween 20. This result is supported by the observations of Sidhu et al. (1998) in case of *Bacillus* sp. Tween 80 has been shown to increase the lipolytic activity of other microorganisms such as *Rhizopus delemar* (Espinosa, et al., 1990) and *Bacillus stearothermophilus* (Gowland, et al., 1987). However, the study by Mates and Sudakevitz, (1973) shows that the Tween substrate did not affect growth and lipase production was inhibited in case of *Staphylococcus aureus.*

Lipase has been of industrial interest in the recent years. Keeping in view the industrial importance of *B. subtilis* an attempt was made to investigate different optimal culture conditions for the maximum production of lipase. The present study suggests that this strain can be used for the large scale production of lipase which

help in accelerating certain biological processes will help revealing more properties for its possible use at industrial scale.

Table I: Effect of incubation period on lipase production shown by *B. subtilis* and total bacterial counts at 37°C, pH 8.0 and 150rpm.

Incubation period	Lipolytic activity (U/ml)	CFU/ml
24	25	$1.5X10^8$
48	43.4	$2.0X10^7$
72	20	$3.4X10^5$

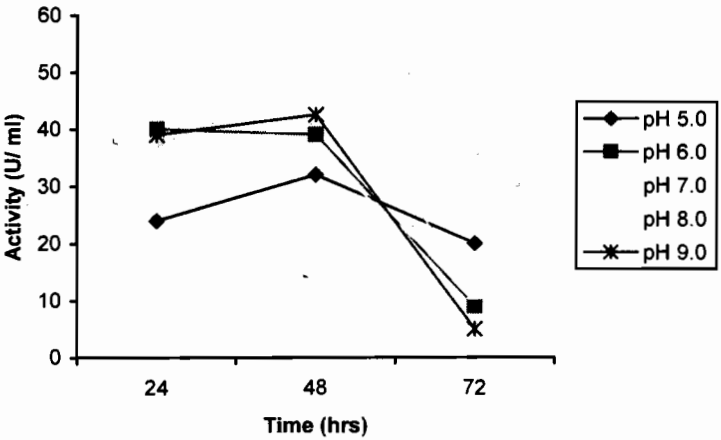

Figure 1: Effect of pH on lipase activity at 37°C, 150rpm.

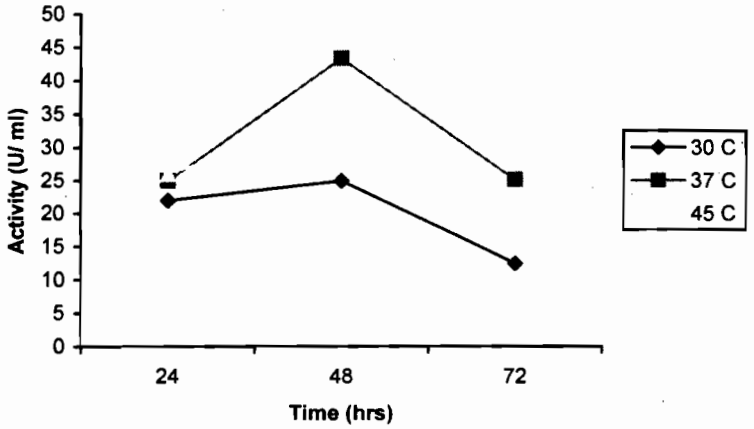

Figure 2: Effect of temperature on lipase activity at pH 8, 150rpm.

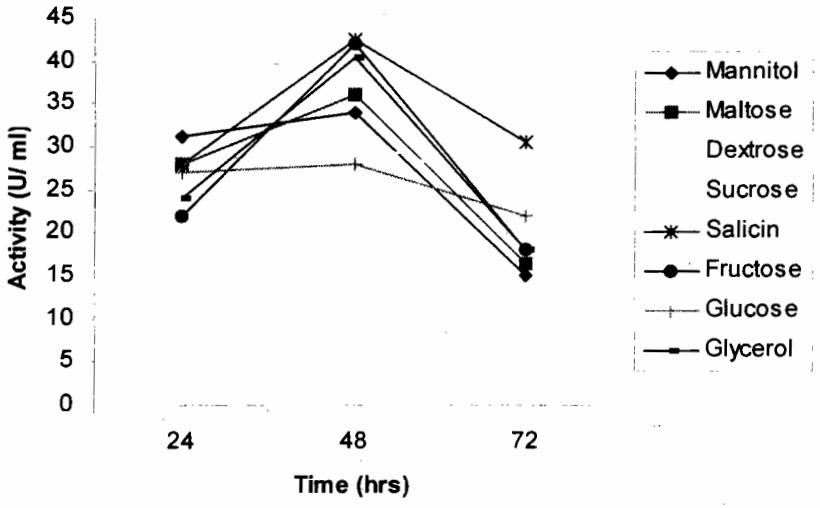

Figure 3: Effect of carbon source on the lipase activity at 37°C, pH 8, 150rpm.

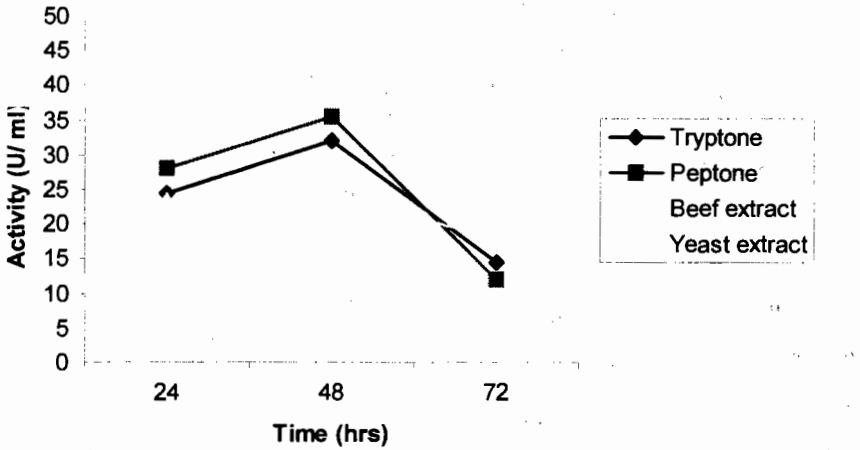

Figure 4: Effect of nitrogen source on the lipase activity at 37°C, pH 8, 150rpm.

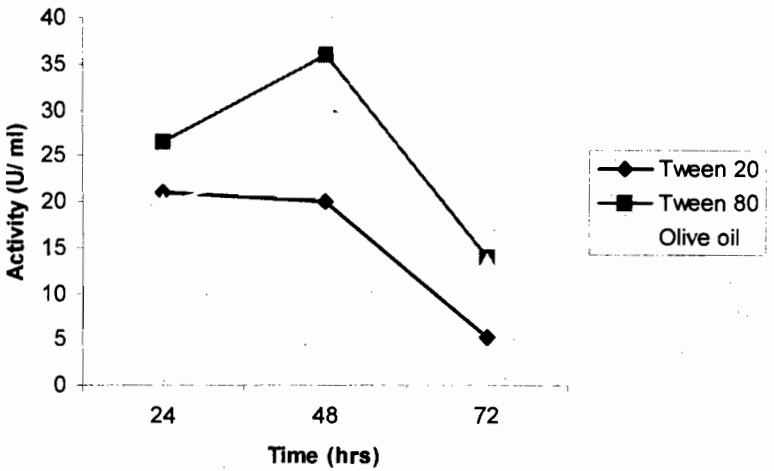

Figure 5: Effect of lipid on the lipase activity at 37°C, pH 8, 150rpm.

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