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Original Article

Production and Optimization of Lipase from *Bacillus Subtilis*

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Abstract

In th is st udy, the l ipolytic *Bacillus subtilis* w as iso lated fr om the oi l m ill w aste by E nrichment techniques. The iso lated c olonies w ere sc reened on O live oi l m edium, colonies w hich pr oduce t he maximum zo ne of t he particular organisms w as used for further op timization s tudies. A mong the 5 *Bacillus subtilis* isolates a single isolates was the subjected to submerged fermentation medium and the enzyme characteristics were studied with respect to substrate, temperature and pH. The production of lipase is significantly influenced by carbon sources such as Olive oil, Castor oil, Gingelly oil, Palm oil, and S unflower oi l a t d ifferent temperature r ange. The m aximum l ipase ac tivity w as re ached by t he *Bacillus* `at 37°C and pH 7 where its production reached upto 0.01033 and 0.01066 µg/ml/min. Among the different substrate the maximum activity was observed in Gingelly oil (0.01066 µg/ml/min) at pH7 and temperature 37° C. D egradation of o il w astage by the c rude e nzyme ex tract a nd ba cterial suspension wer e com pared. The cru de en zyme e xtract li berate mor e free fa tty a cid (10.95 64 %) compared t o *Bacillus* isolates (8.42 40%). From the st udy i t w as co ncluded t hat th e commercially important enzyme can be p roduced by submerged fermentation techniques using frequently available edible oil sources it can be used for the biodegradation of oil effluents.

Keywords: *Bacillus subtilis*, Lipase,pH, Temparature, substrate

Introduction

Lipase (Tri acryl glycerol acylhydrolase E.C.3.1.1.3) is a class of hydrolases catalyzes the hydrolysis of tr iglycerides to m onoglycerides, diglycerides, free fatt y a cids a nd glycerol. But these re actions ar e re versible. I t p osses characteristic properties like substrate specificity, stereo sp ecificity a nd th e a bility t o ca talyze heterogeneous reactions at the interface of water soluble and water insoluble s ystems (Shah *et al.,* 2007). Lipase enzymes have been found in many species of a nimals, p lants, an d mi croorganisms. In partic ular li pases from fu ngi ar e i mportant in industrial used for biotechnological application in dairy i ndustry, oi l processing, an d pr oduction of surfactants (Bapiraju *et al.,* 2004). Microbial lipases are preferred potent sources due to several industrial pot ential. The world ma rket for li pase has bee n e stimated at a pproximately U S$ 2 0 million o f t he i ndustrial enz ymes market (Rahman, *et al*., 2005).

Environmental pollution is one of the major pro blems faci ng i ndustrialized world today. The need to remediate these sites has led to the de velopment of ne w t echnologies tha t emphasize on the detoxification and destr uction of the contaminants rather than the con ventional approach of di sposal. Bioremediation, the use of microorganisms or microbial process to detoxify and degrade env ironmental contaminants is among these ne w tec hnologies. D ifferent microbes pro ducing li pase are used for the remediation proc ess (Chuks Ugochukw, *et al*., 2008).

The ex ponential increase i n the application of lipase in various fields in the last few dec ades demands e xtension in both qualitative i mprovement an d q uantitative enhancement. Quantitative enhancement requires medium optimization w ith pH, Temperature and different m edium c omposition. The h igh cost of

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lipase, however, makes enzymatically driven process economically unattractive. The use of medium optimization is a possible solution to this problem because the enzyme can be recovered with easy and quality so this simple method provides a reliable and efficient technology (Bapiraju *et al*., 2004).

The aim of research is to assess the relative abilities of microbes isolated from crude oil polluted soil to degraded crude oil hydrocarbons, to assay and possibly determine some kinetic parameters of crude lipase extract from the microbial isolates.

Materials and Methods

Sample Collection and Enrichment

Soil samples were taken from the oil mill surrounding area in Namakkal. Totally 15 samples (5 g each) were collected and transported in plastic bags to the laboratory. One gram of sample was suspended in 10ml of sterile normal saline. After shaking, 5ml of the suspension was transferred in to conical flask containing 50 ml of enrichment medium (Olive oil 0.5%, yeast extract 0.1%, Nacl 0.2%, $MgSo_4$ 0.04%, $CaCl_2,2H_2O$ 0.05%, KH_2PO_4 0.03 %, K_2HPO_4 0.03%, $(NH_4)_2SO_4$ 0.05%, pH 7). The culture was incubated at 37ºC under shaking (150rpm) condition for 48 hours. The soil samples were aseptically subjected to serial dilutions and plated on nutrient agar and incubate the plate at 37º C for 24 hrs. After incubation, bacterial species were isolated and subjected to morphological, cultural and biochemical examination by Cheesbrough, (2004) method.

Screening of Lipase Producing Isolates

The isolated bacteria were subjected to screening. The plate medium containing (gm/L): beef extract 3.0, peptone 5.0, sodium chloride 5.0, agar 15.0, calcium chloride 0.05 and glycerol tributyrate 0.2 mL, after incubation for 24 h observe clear zone around colonies. Which showed maximum activity was selected and maintained on tributyrin agar slant for further studies (Chatravedi *et al*., 2010).

Production of Lipase

The production media was prepared in 100ml of Erlenmeyer flask containing 50ml of nutrient broth and 1% olive oil as substrate. The medium was sterilized autoclaving at 121ºc for 20 minutes and then it was inoculated with 1ml of bacterial suspension and incubated at 37ºc for 48 hours.

Production of Lipase with Different Parameters

The activity of lipase producing *Bacillus subtilis* was assayed with different substrates, olive oil, castor oil, gingelly oil, palm oil, and sun flower oil at different temperature and pH.

Enzyme Extraction

From the production media, 5ml of the sample was taken and centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected separately and the enzyme mixture was stored at 4ºC for further studies.

Determination of Enzyme Activity by Emulsified Free System Method (Sadasivam and Manikam, 1996)

250 ml of Erlenmayer flask containing 2ml of 0.1*M* of phosphorus buffer, 1 ml of olive oil and 1ml of crude enzyme extract was incubated at 40ºc for 30 minutes. The reaction was stopped by the addition of 5ml of ethanol and it was titrated against 0.1*N* NaOH using phenolphthalein as indicator. The appearance of pale pink color was the end point. The same method was used for all the samples to determine the lipase activity.

Lipase activity was calculated using the following formula

$$\text{Lipase activity } (\mu g/ml/min) = \frac{\text{Volume of alkali consumed X Normality of NaOH}}{\text{Time of incubation X Volume of enzyme solution}}$$

Degratation of Crude oil on Oil Effluents

10g of oil mill waste was taken in 3 different conical flask labeled as control, enzyme extract with oil mill waste and bacterial suspension with oil mill waste. All the flasks were incubated at 37 ºC for 1 hour. The oil in each sample was extracted using soxhlet apparatus. To these samples 50ml of spirit, 2-4 drops of 0.1N NaOH and 2 drops of phenolphthalein indicator was added. The extracted oil from waste material was titrated against 0.1N NaOH in the burette. Appearance of pale pink colour is the end point (Sadasivam and Manikam,1996).

$$\text{\% of free fatty acid in sample} = \frac{\text{Volume of alkali consume X Constant Value (0.02825)}}{\text{Sample weight}} \times 100$$

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Results

The bacterial st rain was isol ated and identified a s *Bacillus subtilis* base d on t he morphological character a nd b iochemical characters. A totally 5 isolates were obtained out of 15 samples. By performing qualitative assay of *Bacillus subtilis* and tributyrin were used for the screening of l ipase prod ucers. The pre sence of clear zone w as in dicated a s li pase producers. Among tha t fi ve isolates, the single iso late producing colonies exhibit the maximum zone of clearance (1 6mm) t his iso lates was use d for further st udies (P late-1). The result s were represented in table-1.

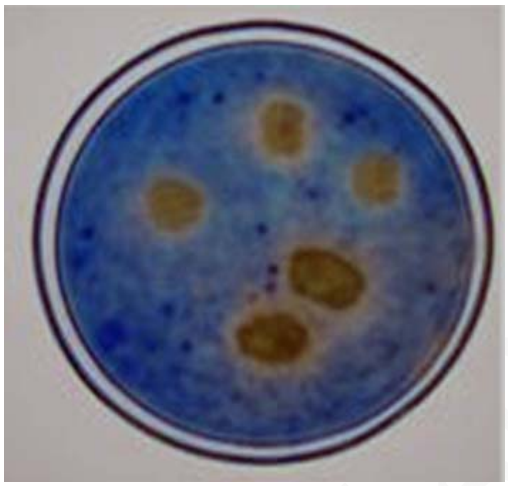

Plate-1: Lipase Production *of Bacillus* sp

Table - 1: Zone formation of *Bacillus* sp

S.No	Isolates	Zone Of Clearance In Diameter
1.	Bacterial isolate-1	9
2.	Bacterial isolate-2	6
3.	Bacterial isolate-3	16
4.	Bacterial isolate-4	12
5.	Bacterial isolate-5	8

Lipase Production with different Parameters

Microbial e nzyme prod uction w as checked with va rious factors l ike tem perature, pH, and the nature of substrates. The v arious results that were obtained during the optimization of these process parameters were as follows:

Effect of different substrates

The best sub strate f or t he lipase production was found to be the gingelly oil where its productivity reached upto 0.01066 µg/ml/min. The Palm oil and Sunflower oil were found to be the ne xt m ost subs trates where its pro duction reached u pto 0. 01033 a nd 0 .01 µg /ml/min. The maximum activity wa s ob served a t 37 °C an d pH 7. The results were seen in figure-1.

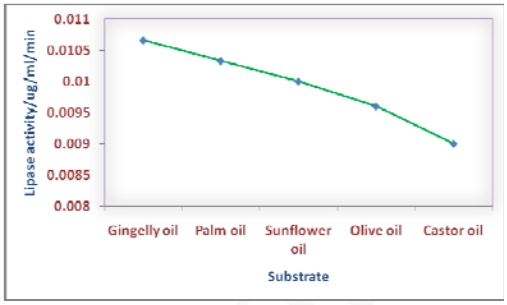

Fig.1: Effect of di fferent substrate o n Lipase production of *Bacillus* sp

Effect of Temperature

The production o f lip ase wa s greatly induced by the temperature. The maximum lipase activity wa s re ached by the *Bacillus species* at 37°C and pH 7 where its production reached upto 0.01033 a nd 0.01066 µ g/ml/min. The ot her temperatures suc h as a t 2 7°C, 47°C a nd 57°C were also seen to induce the less amount of lipase production. The results were seen in figure -2.

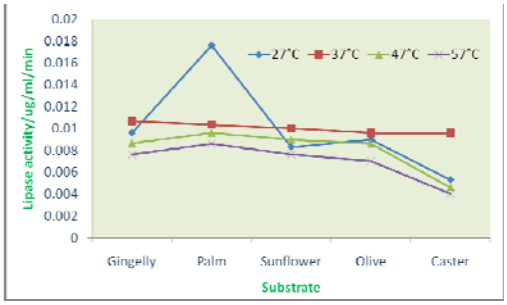

Fig.2: Effect of different Temperature on Lipase production of *Bacillus* sp

Effect of pH:

The organisms a t various p H such a s 4,5,6,7 and 8 showed different lipase activity on different substrates at 37°C. The maximum lipase activity w as obs erved a t pH 7, and it w as calculated as 0 .01066 µg/ml/min in Gingelly oil. Other substrates such as Palm oil, S unflower oil, Olive oil and Castor oil were also seen to induce the less amount of lipase production. The results were seen in figure -3.

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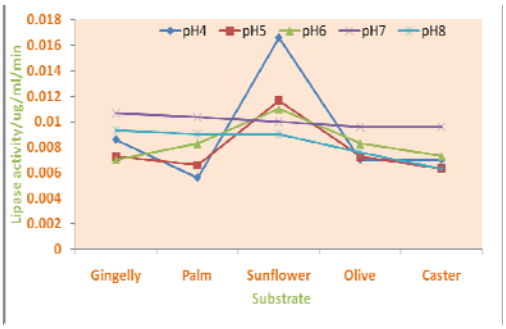

Fig.3: Effect of different pH on Lipase production of *Bacillus* sp

Activity of Lipase on Oil Effluents

The extracted lipase enzyme has the ability to liberate free fatty acid. Compared to control (6.72%), the test samples with crude enzyme release free fatty acid in higher level. It was found to be 10.85%. Compare to test sample with crude enzyme, the bacterial suspension with sample release free fatty acid in lower level. It was found to be 8.41% so it was conclude as the maximum degradation take place only in crude enzyme extract comparatively others. The results were observed in table 2.

Discussion

Lipases are extremely versatile enzyme, showing many interesting properties of industrial applications. Microbial lipases are high in demand due to their specificity of reaction, stereo specificity and less energy consumption than conventional method. In the recent years, the interest on lipase has grown significantly. The development of technologies using lipase for synthesis of novel components will result in this expansion in to new area and increasing number of industrial application (Bjorling *et al*., 1991). In the present studies, the lipase producing organism where isolated form oil mill waste and identified as *Bacillus subtilis*. Among the three isolates the most potent in secreting lipase was screened as isolate '3'.

Effect of Different Substrates

The production of lipase is significantly influenced by carbon sources such as Gingelly oil, Palm oil, Sunflower oil, Olive oil and Caster oil, temperature and pH were tested. Among five different substrates the Gingelly oil showed the maximum activity (0.0106 µg/ml/min), compare to other substrate. This result was not accordance with the report of Selva Mohan (2008), who reported that maximum lipase activity was observed in Olive oil and it activity was screened (0.0039 µg/ml/min). Lakshmi *et al.*, (1999) reported that the production of lipase was high in medium added with vegetable oil than the medium added with glucose. In contradiction Banerjee *et al.*, (1985) reported that some microorganisms showed higher activities when grown in medium containing glucose, Novotny *et al.*, 1988 reported that Olive oil in combination with glucose increased lipase activity and in most cases and also the presents of Olive oil, together with glucose or glycerol in the medium significantly decreased both lipase and esterase levels. They also further inferred that, if Olive oil was used as the only carbon sources for growth, the enzyme activities of *Candida guilliermondii* showed a four to five fold increases. As reported by Nakashima *et al*, 1998 the presents of Olive oil as growth medium greatly enhanced in the lipase activity of *Bacillus* strain 5 (B3) in the present study. Fadinoglu *et al*.,(1999) reported that Olive oil combination with other nitrogen sources enhanced the lipase production, but the presence of the carbon sources in the Olive oil significantly ($P < 0.01$) decreases the lipase activity and biomass content. They also reported that organic nitrogen sources were found to be increase lipase synthesis by *Candida rugosa* grown in the presences of Olive oil.

Effect of Different Temperature

Rohit *et al.*,(2001) reported that the lipase production was more in vegetable oil, Olive oil; Soy oil, Gingelly oil and Sunflower oil were used as the carbon sources. In the presence study the influent of medium temperature indicated with lipase production by the isolated organism was highest 0.01033 to 0.01066 µg/ml/min at 37° C when compare to other degrees. In the argument to the presence result Selva Mohan *et al.*, 2008 found at optimum temperature for lipase production by bacillus sps at 37 °C. Similarly Walavalkar and Bapal, 2002 have reported that, the lipase activity of *Staphylococcus spp.* was high at 37°C.

Effect of Different pH

The pH of the culture medium place a critical role for the optimal physiological performances of the bacterial cell and the transport of various nutrient components across the cell membrane aiming at maximizing the enzyme yields. Concerning lipase production in this study it has been found that the yield was reached its maximum at pH7, compare to the

other pH ranges. The result was in consistence with earlier report (Achamman *et al*., 2003). They interred that the lipase activity of *Bacillus sps* was maximum at pH 7. The study Mannuel Ferrer *et al.*, 2000 reported that the optimum pH ranges for lipase production at pH7 by *Penicillium chrysogenum.*

Activity of Lipase on Oil Effluents

Degradation of crude oil by the isolates in the enzyme extract was studied. The cured oil with enzyme extract showed the maximum degradation when compare to cured oil with bacterial isolates. This study suggested that when the catalytic agent was in close proximity with the substrate the rate of reaction will be higher. Since the same enzyme catalyze the initial degradation of both the growth and non growth substrates, competition for the enzyme can occur, reducing the rate of growth substrate degradation (Subhas and Robert, 1998). The results have also shown that the reduction in the rate of non growth substrate degradation can occur in the presents of growth substrate (Gill and Robothan 1989).

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