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ISOLATION, PRODUCTION AND PURIFICATION OF LIPASE FROM ASPERGILLUS NIGER USING GINGELLY OIL CAKE AS SUBSTRATE

Kamatham Sushma and V. Judia Harriet Sumathy†

ABSTRACT

The interest in lipase has grown over the last few years due to their excellent catalytic properties and their diverse industrial applications, for example, they are the major industrial enzymes extensively used in pharmaceuticals, textiles, food, medical, and chemical industries obtaining bioactive molecules in the pharmaceutical industry and pure optical compounds in chemical synthesis processes, as well as modifications of fats and lipids by hydrolysis and esterification reactions. Lipases occur widely in bacteria, yeasts and fungi. Fungi are broadly recognized as most of the lipase research focuses on the production of extracellular lipases through a wide variety of microorganisms. *Aspergillus niger* is among the most well known lipase producers. *Aspergillus niger* is considered to be a safe production organism. They are highly aerobic organisms and are found in almost all oxygen-rich environments, where they commonly grow as moulds on the surface of a substrate, as a result of the high oxygen tension. Recently cheap agricultural byproducts like gingelly oil cake and olive oil cake have been gaining a great interest as substrates in solid state fermentation for fungi. High lipase productions were obtained by cultivation of *Rhizopus* sp, and *Aspergillus* sp among which the latter seems to be the best lipase sources and hence are heavily employed in food industry.

KEYWORDS: Lipase, *Aspergillus Niger*, Aerobic, Gingelly oil cake and Food Industry

INTRODUCTION

Lipases from micro organisms serve important roles in human practices as ancient as yogurt and cheese fermentation. However, lipases are also being exploited as cheap and versatile catalysts to degrade lipids in more modern applications and even as Biocatalyst in alternative energy strategies to convert vegetable oil into fuel [1]. Lipase (E.C. 3.1.1.3) hydrolyses triglycerides to fatty acid and glycerol, and under certain conditions, catalyses the reverse reaction forming glycerides from glycerol and fatty acids. Some lipases are also able to catalyze both transesterification and enantioselective hydrolysis reactions [2]. Lipases find use in a variety of biotechnological fields such as food and dairy (cheese ripening, flavour development, EMC technology), detergent, pharmaceutical (naproxen, ibuprofen), agrochemical (insecticide, pesticide) and oleochemical (fat and oil hydrolysis, bio-surfactant synthesis) industries [3]. Lipases can be further exploited in many newer areas where they can serve as potential biocatalysts in alternative energy strategies to convert vegetable oil into fuel. *Aspergillus* is a member of the phylum Ascomycota. There are over 185 known species, about 20 of which are known to be harmful to humans and other animals. The most infamous species of this genus is *Aspergillus flavus*, which produces aflatoxin. Aflatoxin is a contaminant of nuts and grains. It is both a toxin and a carcinogen. Gingelly oil is sometimes found to be strong smelting by some, yet as a good source of vitamins E and B complex and minerals such as Calcium, Magnesium and Phosphorus. This Gingelly oil further contains protein as well as lecithin. In folk medicine it has also been used to help fade blemishes. The Sesame herb is grown for its seeds and was used by the Chinese 5000 years ago [4]. Coconut cake is a potent substrate used for the production of lipase by *Candida rugosa* in solid state fermentation. High lipase productions were obtained by cultivation of *Rhizopus* sp, *Aspergillus* sp [5]. Recently cheap agricultural by products like gingelly oil cake and olive oil cake have been gaining a great interest as substrates in solid state fermentation for fungi. Mixed solid substrate fermentation, a novel process for enhanced lipase production by *Candida rugosa* has also evolved (Figure 1 & 2).

Figure 1. Sesame Oil Cakes in Powdered Form

Post Graduate and Research Department of Biotechnology, Women's Christian College, College Road, Chennai
†Corresponding author: info@wcc.edu.in; sbsj@rediffmail.com

Figure 2. Coconut Oil Cakes in Powdered Form

Microorganisms are able to grow and transform vegetable oils due to the excretion of lipase, which renders the lipid substrates available to cells. The agro-industrial wastes also can provide alternative substrates for industry and their utilization in this manner may help solve pollution problems. Many agro-industrial residues can be used as potential substrates for production of enzymes [6]. The main objective of this work was the isolation and screening of microorganisms which has the potentiality to produce lipases. Among 24 fungi, five were selected as good lipase producers using tributyrin on agar plates and solid state fermentation of soybean bran. Two of them were isolated from soil samples, another two from soybean bran, and one from dairy products. These fungi were identified by micro-cultivation technique as from *Penicillium* and *Aspergillus* genera. Through random amplified polymorphic DNA technique, the most promising strains could be genetically discriminated, selecting two fungi as good lipase producers but genetically different. One isolated from soybean bran could hydrolyze efficiently triglycerides with fatty acids with different chain length. Another isolated from dairy products was only effective to hydrolyze triglycerides with long-chain fatty acids. Two distinct groups could be verified by means of this technique, comprising the most productive strains and the lowest or non - productive ones in terms of hydrolytic activity.

Thus this study was focused on isolating and Identifying *Aspergillus niger* from oil contaminated soil and later isolate and purify Lipase enzyme from it. The Enzymes were later characterized by determining their optimum pH, Temperature and Protein Assay. Enzyme immobilization was done by gel entrapment method and thus the application of the enzyme was determined.

MATERIALS AND METHOD

Sample Collection

The soil sample was collected from Tondiarpet petrol bunk in Chennai and was diluted using sterile saline solution. The diluted samples were spread on Potato Dextrose Agar plates.

Potato Dextrose Agar Composition

- Beef infusion - 300g/L
- Casein acid - 17.5g/L
- Starch - 1.5g/L
- Agar - 17g/L
- Distilled water - 1000ml

The plates were incubated at 37°C for 24 hours. Colonies appeared. The isolated colonies were further purified by swab plate method using sterile potato dextrose agar medium.

Lactophenol Cotton Blue Staining

- Phenol crystals
- Lactic acid
- Glycerol
- Cotton blue

NOTE: Dissolve the phenol crystals in the other ingredients by heating the mixture gently under a hot water tap.

Substrate

- Coconut oil cake
- Gingelly oil cake

Different oil cakes were used as substrate and their biotechnological applications were identified. They were procured from a local market of Tondiarpet, Chennai, India and were dried at room temperature to reduce the moisture content and ground to the desired size.

Media Preparation

10 gm of desired oil cake was suspended in 90 ml of minimal media in a 250 ml flask. It was then autoclaved at 15 lbs pressure, 120°C for 20 minutes. It was cooled before using.

Minimal Media Composition

- Sodium dihydrogen phosphate - 12g/L
- Potassium dihydrogen phosphate - 2g/L
- Magnesium sulphate - 0.3g/L
- Calcium chloride - 0.25g/L
- Ammonium sulphate - 1g/L
- Oil cake - 2g/L
- pH - 6

Lipase Production in Submerged Filteration

The culture was grown in 100 ml Erlenmeyer flasks containing 20 ml of mineral medium. The above contents were sterilized by autoclaving at 121°C for 15 min. After cooling, the sterilized medium was inoculated with spores (10^7/ml) from a 7 day old culture. The flasks were incubated at 30°C in a rotating shaker at 100 rpm for 8 days.

Enzyme Extraction

The mycelium was harvested by filtration under vacuum and later centrifuged at 12000 rpm for 5 min. The clarified supernatant was used as a source of extracellular enzyme.

Lipase Assay

Isolated *Aspergillus niger* was assayed for extra cellular lipase production using titrimetric method. One unit of lipase activity was defined as the amount of enzyme releasing one mole of free fatty acid in one minute under standard assay condition.

Reagents

- Gingelly oil
- Phosphate buffer, pH 7.0 – Mix 100 ml of M/3 disodium hydrogen phosphate (47.3 g disodium hydrogen phosphate/ L
- Ethanol
- Sodium hydroxide, 0.05N
- Phenolphthalein, 1% solution in ethanol

Procedure

- 3ml of buffer was taken in a 100 conical flask,
- To this 1ml of enzyme (lipase) was added, followed by 2ml of substrate (gingelly oil).
- It was shaken well and incubated at 37°C for 24 hours.
- After 24 hours of incubation, 3ml of 95% ethanol was added into it, followed by 2 drops of phenolphthalein.
- Then it was titrated with 0.5N sodium hydroxide to a pale pink colour.
- The above step was repeated to get the concurrent values.

Lipase activity can be calculated by using the formula:

LIPASE ACTIVITY =

$$\frac{\text{Volume of alkali consumed} \times \text{Normality of sodium hydroxide}}{\text{Time of incubation} \times \text{Volume on enzyme solution}}$$

Effect of pH on lipase activity

3ml of phosphate buffer of varying pH was added i.e. 5.6, 5.8, 6.0, 6.2 and 6.4 in five different conical flasks. To this 1ml of enzyme (lipase) was added in each flask, followed by 2ml of substrate (gingelly oil). After incubation of 24 hours, this was titrated with 0.5N sodium hydroxide to a pale pink colour. Each set of sample was titrated thrice to get the concurrent values.

Effect of temperature on lipase activity

3ml of phosphate buffer of pH 7 was taken in 5 set of conical flasks. To this 1ml of enzyme (lipase) was added in each flask, followed by 2ml of substrate (gingelly oil). Then these were incubated at different temperatures i.e. 25°C, 35°C, 45°C, 55°C, and 65°C for 24 hours. After incubation, it was titrated with 0.5N sodium hydroxide to a pale pink colour. Each set of sample was titrated thrice to get the concurrent values.

Drop Collapse Assay

This assay relies on the destabilization of liquid droplets by lipases. The stability of drops is dependent on lipase concentration and correlates with surface and interfacial tension. This assay is rapid and easy to carry out. A drop of lipase was added on an oil coated solid surface.
Positive Result- If the liquid contains lipase, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced.
Negative result- If the liquid does not contain lipase, the polar water molecules are repelled from the hydrophobic surface and drops remain stable.

Salting out of proteins has been employed for many years and has fulfilled the dual purpose of both purification and concentration of specified proteins. The salt most commonly used is ammonium sulphate, because of its solubility, lack of toxicity to most enzymes, cheapness and in some cases its stabilizing effect on enzymes. The concentration of salt needed to precipitate an enzyme will vary with concentration of enzyme. Fractionation of protein mixtures by the stepwise increase in the ionic strength can be very effective way of partly purification of enzymes. Thus the enzyme was purified by ammonium sulphate precipitation method.

Dialysis Tubing is a type of semi or partially permeable membrane tubing made from regenerated cellulose or cellophane. It is used for diffusion, or more accurately osmosis. Only those molecules that are small enough to fit through the membrane pores are able to move through the membrane and each equilibrium with the entire volume of the solution in the system. Once equilibrium is reached, there is no further net movement of the substance because molecules will be moving through the pores in to and out of the dialysis unit at the same rate. By contrast, large molecules that can't pass through the membrane pores will remain on the same side of the membrane as they were when dialysis was initiated. To remove additional unwanted substance, it is necessary to replace the dialysis buffer so that new concentration gradient can be established. Once the buffer is changed, movement of the particles from high (inside the membrane) to low (outside the membrane) concentration will resume until equilibrium once again reached. With each change of dialysis buffer, substances inside the membrane are further purified by a factor equal to the volume difference of two compartments. For example, if one is dialyzing 1 ml of sample against 200ml of dialysis buffer, the concentration of the dialyzable substances at equilibrium will be diluted

200 less than at the start. Each new exchange against 200 ml of the new dialysis buffer will dilute the sample 200 times more. For example, if the three exchanges of 200 ml, the sample will be diluted 200 X 200X 200 or 800 times assuming complete equilibrium was reached each time before the buffer was change. Thus the enzyme was also purified using dialysis method.

Estimation of Protein was done following the standard Lowry's Method. SDS Page was run and the Enzyme Immobilization was carried out following Gel (Sodium Alginate) Entrapment method.

RESULTS AND DISCUSSION

The soil sample collected from oil contaminated soil was diluted and plated on Potato Dextrose Agar. On microscopic examination, various fungal colonies were observed. Lactophenol cotton blue staining was performed to the colonies on PDA, and *Aspergillus niger* was isolated based on colony morphology microscopic examination (Figure 3)

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Figure 3. Microscopic View of *Aspergillus Niger*-Lactophenol Cotton Blue

Assay for Enzyme Production

For the production of enzyme the organism was inoculated in minimal media containing gingelly oil cake. After 8 days of incubation, the medium filtrate was assayed for the lipase activity by titrimetric method (Figure 4 & Table 1).

Figure 4. Filtration of the Enzyme (Lipase) Using Millipore Filter

Table 1. Activity of Lipase

S. No.	Burette Reading		Volume of NaoH
	Initial	Final	
1	0	14.4	14.4
2	14.4	29.1	14.7
3	29.1	43.5	14.4

LIPASE ACTIVITY=

$$\frac{\text{Volume of alkali consumed} \times \text{Normality of sodium hydroxide}}{\text{Time of incubation} \times \text{Volume on enzyme solution}}$$

Volume of alkali consume – 14.4 ml
Sodium hydroxide – 0.5N
Time of incubation – 24 hours (3600 sec)
Volume of enzyme solution -1 ml

$$\text{Lipase activity} = \frac{14.4 \times 0.5}{3600 \times 1}$$

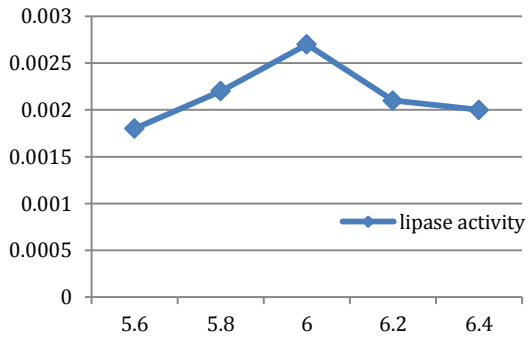
$$= 0.002\ \mu g/ml/min.$$

Effect Of pH on enzyme activity

The optimum pH at which the enzyme is stable was determined using phosphate buffers of varying pH(5.6, 5.8, 6.0, 6.2, 6.4). the optimum activity was observed at pH 6.0. When the graph was plotted the peak value (maximum activity) was observed at pH 6 (Table 2 & Graph 2).

Table 2. Effect of pH on Enzyme Activity

Sr. No	pH	Lipase Activity
1.	5.6	0.0018
2.	5.8	0.0022
3.	6.0	0.0027
4.	6.2	0.0021
5.	6.4	0.002



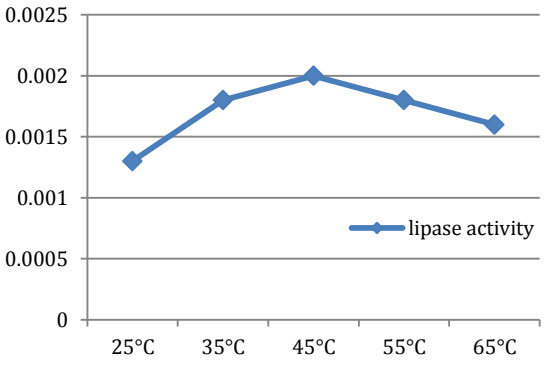
Graph 2. Effect of pH on enzyme activity

Effect of Temperature on Enzyme

The stability of the enzyme was examined at different temperatures (25, 35, 45, 55, and 65) in phosphate buffer at constant pH. When the graph was plotted the value (maximum activity) was observed at 45°C (Table 3 & Graph 3).

Table 3. Effect of Temperature on Enzyme Activity

S.No	Temperature	Lipase Activity
1.	25°C	0.0013
2.	35°C	0.0018
3.	45°C	0.002
4.	55°C	0.0018
5.	65°C	0.0016



Graph 3. Effect of Temperature on Enzyme Activity

The drops collapse assay indicated the presence of enzyme.

Purification of Lipase

Ammonium sulphate precipitation followed by dialysis with Tris buffer was carried out with supernatant of the extract for the purification of the enzyme. The purified enzyme obtained was analyzed by SDS-PAGE. Sodium dodecyl sulphate poly acrlyamide gel electrophoresis was

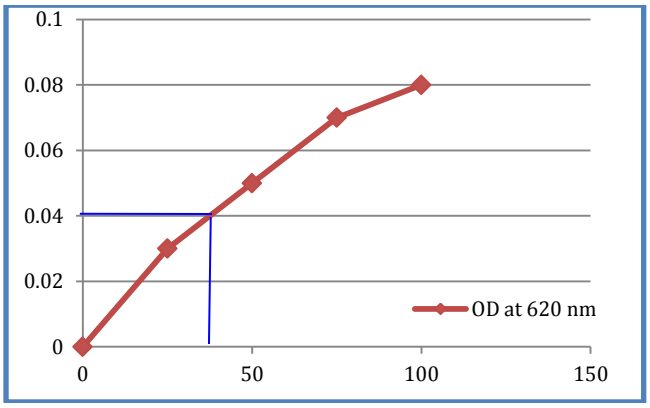
performed with (12% separating gel) and distinct protein band was obtained.

Protein determination (L*owry et.al.* 1951)

The amount of protein was estimated with bovine serum albumin (concentration of 50 mg per 50 ml) as standard (Table 4 & Graph 4).

Table 4. Estimation of Protein by Lowry's Method

S. No	Reagents	B	S_1	S_2	S_3	S_4	S_5	U_1	U_2
1.	Volume of protein (ml)	-	0.5	1.0	1.5	2.0	2.5	-	-
2.	Concentration of protein (µg)	-	25	50	75	100	125	-	-
3.	Volume of unknown (ml)	-	-	-	-	-	-	0.5	1.0
4.	Volume of water (ml)	2.5	2.0	1.5	1.0	0.5	-	2.0	1.5
5.	Volume of alkaline copper tartarate (ml)	5	5	5	5	5	5	5	5
	Incubated for 10 minutes at room temperature.								
6.	Volumes of Folin's reagent (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Incubated for 20 minutes at room temperature and read at 620 nm.								
7.	Optical density at 620 nm	0.00	0.03	0.05	0.06	0.08	0.09	0.04	0.08



Graph 4. Estimation of Protein by Lowry's Method

Within the standard value, unknown were plotted and thus the amount of protein in the purified enzyme was found to be 9.5µg/ml.

Immobilization of Enzyme by Gel Entrapment

Enzyme was successfully immobilized by gel entrapment via Sodium Alginate. This is an efficient method and it can be used for further large application. The immobilized enzyme activity was assayed by titrimetric method and the activity was found to be same as that of crude enzyme.

SUMMARY AND CONCLUSION

Lipases have a number of practical applications in industries such as detergent, tanning, dairy, tannery, brewing, fat and oil hydrolysis, etc. Lipases find major applications in the detergent industry as they have high temperature and pH optima and are able to withstand harsh conditions. The objective of the present work was to isolate and characterize lipase producing organism from soil. The isolated organism was identified to be *Aspergillus niger* through Lactophenol cotton blue staining. The culture was grown on minimal media with gingelly oil cake as substrate which was assayed by titrimetric method. Drop collapse assay was also performed to check the presence of lipase. The enzyme was precipitated by Ammonium sulphate precipitation followed by dialysis. After isolation, an assay for lipase was done and the activity of lipase was obtained. SDS-PAGE was run to ascertain the molecular weight. Optimization studies were also carried out with respect to pH, temperature. The above study can further be improved by causing for hyper production of the enzyme through mutation of the organism. Immobilization of the enzyme can also be done and the activity of immobilized and free enzyme can be compared. Such studies are being contemplated for the future. An immobilized counterpart will have a better recycling value and added efficiency, which would be beneficial on an industrial scale. Thus this study has proved that the optimization of growth parameters in a suitable medium has significant effect on improved activity of the enzyme. This is one of the prime objectives of industrial microbiology for large production of valuable metabolites, which can be balanced nutrient supply.

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