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Production, Characterization and Partial Purification of Extracellular Lipase from *Aspergillus* Sp.,

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Abstract
Lipase are produced extracellularly by the fungal strain *Aspergillus sp.,* were isolated from food effluent by enrichment culture technique. The fungal organism was then subjected to liquid fermentation and characterize the enzyme activity. Tween 80 was used as the substrate with Victoria blue as the indicator lipolytic activity was determined by the formation of a zone of intensification of the indicator colour after 24 hours. *Aspergillus* sp was subjected to liquid fermentation in optimum pH, temperature, different substrate, carbon source and the nitrogen source and the highest lipase activity was recorded. The enzyme activity was optimal growth 72 hours of incubation time, with an activity. Lipase fermentation using tween 80 as a substrate was found to be more effective in lipase production. The optimum pH for *aspergillus* sp., is 7.0 and temperature 37°C enhanced the lipase activity. Sucrose and urea +beef extract in the medium enhanced the lipase activity. The extra cellular protein pattern of *aspergillus* sp. was revealed by SDS-PAGE and NATIVE PAGE.

Keywords
Lipase, Olive oil, Enzyme production, *aspergillus* sp., Tween 80.

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INTRODUCTION

Lipase is produced by various microbes, bacteria, fungi, yeast, mammals and plants in large amounts. Aspergillus is afilamentous, cosmopolitan and ubiquitous fungus found in nature and commonly isolated from soil, plant debris, decayed food materials and indoor air environment while a telemorphic state has been described only for some of the aspergillus sp., that could grow at up to 50 c and produced boyh extra and intra cellular lipase in liquid medium. Lipse from aspergillus sp has found to catalyze the hydrolysis both short and long fatty acids from triglycerides(Iwai et al.,) Extracellular lipase produced by microorganisms is being investigated for its potential application in

various industrial processes like detergens, oils, fatty acids and diary coupled with enormous therapeutic uses.

MATERIALS AND METHODS:

In the presence study, the *Aspergillus sp.,* was used to production of extracellular lipase by fermentation by using various substrates. The *Aspergillus sp.* were identified from food sample from food effluent Vennandur, Namakkal.

Sample Collection

Contaminated food samples were aseptically collected in sterile petridish. The collected samples were aseptically transferred and processed with in 1hour.

Sample Processing

The Collected sample was aseptically transferred to the laboratory, serialy diluted and spread on the sterile Czapek Dox Agar plates. Then the plates were incubated at room temperature for 3 days. The test organisms isolated from the food effluent was used for the production of lipase enzyme.

Media and culture conditions

The Aspergillus sp., streaked on Czapek Dox Agar medium containing Yeast extract 5g/l, Sodium nitrate 3g/l, di-potassium hydrogen phosphate 1g/l, potassium chloride 0.5g/l, Magnesium sulphate 0.5g/l, Ferrous sulphate-0.01g/l, Sucrose -30g/l, Agar 15g/l, pH-7.3 and grown at 25°C.After grown the slants were stored at 4°C, the cells were sub cultured twice a month.

Preparation of Victoria Blue Agar Plate

The Victoria blue agar plate medium used for the following composition Tween 80- 2ml, Victoria blue - 0.01 g, agar -2.5g and distilled water 100ml.The medium was sterilized and poured into sterilized pertiplates and allowed to solidify. The obtained culture filtrates were spotted onto wells of agar plates containing Tween 80. The formation of opalescence surrounding the fungal colony was recorded as positive for lipase producing.

Characterization of microorganism

Morphological Identification of the organisms look like black mold. It has a welvety appearance and spores were powdery in nature. The conidia were proceeded at the tip of certain vertically growing hyphae. Vegetative growth was seen in many foods especially, if held at 30°C-50°C.The organism have characters such as it is tolerant to 70-95% humidity. It does not require moisture content. Most of the fungi can grow at temperature between 10°C-40°C. The optimum temperature is between 25°C-35°C.

Fermentation

Production Media

The basal medium for lipase production contained (gl^{-1}); $NaNo_{3\text{-}5}$; $MgSo_4$ $7H_2O$ – 0.5, KCl -0.5, $KH_2PO_{4\text{-}1}$, $Na_2HPO_{4\text{-}3}$, $ZNSO_4$ $7H_2O$-0.01, $MnSo_4$ H_2O-0.01, Sucrose 5 and olive oil 1%. The optimization of enzyme production was carried out by altering, the nitrogen and carbon sources, as well as the phosphate and salts in the culture medium.

Culture Conditions

An aqueous spore suspension was used as the inoculum. 0.3ml of the spore suspension was added to a series of 250ml conical flask containing 50ml of the medium. The flasks were incubated for 72h on a rotary shaker. The flask was removed at 25h intervals and the mycelium was separated by filtration and the filtrate was used for the enzyme assay.

Enzyme Assay

Lipase activity was assayed by titrimetric method[5] with Slight modification. 75ml of 2% polyvinyl alcohol was homogenized with 25ml of olive oil and used as the substrate. The substrate emulsion (5ml) and 4ml of 0.1M sodium phosphate buffer, pH 7.0 were pre-incubated at 37°C for 10min and 1ml of the enzyme solution was then added and incubated at 37°C for 20min. The reaction was terminated by the addition of 20ml of acetone and titrated against 0.01M Sodium hydroxide. A control was carried out similarly, except, one ml of the heat – inactivated enzyme was added to the reaction mixture. One unit of lipase activity was defined as the amount of enzyme that released free fatty acids in 1min under standard assay conditions.

Partial identification of fungal isolates

Partial identification of fungal isolates was done by the visual observation in pertidish culture. For the observation, the isolates grown in c were used. The mode of mycelial growth, color, odor and changes in the medium color of isolate were examined.

Characterization of Lipase

Lipase activity was measured at different pH 2 to 10 using 0.1M glycine HCL for pH 2 to 4 pH range. pH 5 to 8 with 0.1M Sodium phosphate buffer and pH 9 to 10 with 0.1 glycine NaOH buffer at 37°C and at different temperatures (25°C-60°C) at pH 7.0with olive oil as the substrate.

To investigate the pH and temperature stability of the lipase the enzyme was pre-incubated with buffers of different pH for 30min at 30°C and incubated at different temperature for 30min at pH 7.0. The activity was assayed titrimetrically as mentioned above.

Invitro inhibition of lipase activity

The effect of temperature, pH, different carbon source, nitrogen source on the activity of lipase were studied.

Temperature

The assay mixture was incubated at various temperature for a period of 10min and the enzyme activity was assayed as described previously. The various temperature studied were 27°C, 30°C, 37°C, 40°C, 45°C, 50°C and 60°C.

pH

To determine the optimum pH for the activity of lipase, the assay mixture was incubated at 70°C for a period of 10min at various pH conditions with pH range of 2-10 were used.

Carbon source

Effect of carbon source on the lipase activity was determined by growing the culture in the basal medium pH 7.0 for 24h at 37°C in rotary shaker. The carbon source is substituted like. Fructose, Galactose, Dextrose, Maltose, Starch, and sucrose.

Nitrogen source

Effect of different sources on the lipase activity was determined by growing on the culture in the basal medium for 24hrs at 37°C in rotary shaker. The basal medium was substituted by different nitrogen sources like urea (0.5), urea+beef extract (0.5), urea+beef extract (1.0), urea+beef extract (1.5), urea+beef extract (0.5) urea+beef extract (1.0), urea+beef extract (1.5). The lipase assay was done using the supernatant following the procedure described previpously.

Purification of lipase

The enzyme extract obtained was precipitated with 80% Ammonium Sulphate. It is calculated by using formula

$$g = \frac{533\ (S2-S1)}{100-0.3 \text{ x } S2}$$

S1 = initial concentration of protein solution
S2 = final concentration of protein solution
S3 = amount of ammonium sulphate to be used for precipitation.

The precipitation was obtained as pellet after centrifuging. The pellet is dissolved in 50mM phosphate buffer (pH 7.0). The sample was loaded in the bags and dialysed in 10mM Tris HCl buffer for 6h. The buffer was changed in between after testing for presence of ammonia using Nesseler's reagent (obtained commercially).In the dialysis again, protein estimation and lipase activity assay was done. Then 30mg of protein was loaded in NATIVE PAGE and SDS PAGE as the same was subjected to purification.

RESULTS:

Isolation of Lipase Producing Colonies

The *Aspergilus sp.,* was produced the lipase enzyme by checked with lipid hydrolysis with Czapek Dox Agar medium. The lipase producing fungal strain was isolated by enrichment technique. The organism isolated was identified by lactophenol cotton blue staining. Morophological and microscopical views were the organism look like black mould and welvetly mat like appearance. The spores were powdery in nature. Microscopically the organism has the filamentous nature with hyphae collectively mycelium. The condium seen on the tip of the hyphae and the organism may be aspergillus sp., the single colonies were isolated by growing on czapek dox agar medium which shows the positive result for the production of lipase were obtained using Victoria blue B, Tween 80 was the substrate with Victoria blue B as the indicator. The initial clearance is probably due to agar diffusion. Lipolytic activity was determined by the formation of intensification of the indicator colour after 24 hours.

Production of Purification of Lipase Enzyme

After 4 days incubation of production medium olive oil broth with fermentation the crude extract was prepared by centrifuge. An aqueous spore suspension was used as the inoculum. 0.3ml of the spore suspension was added to a series of 250ml conical flask containing 50ml of the medium. The flasks were incubated for 72h on a rotary shaker. The flask was removed at 25h intervals and the mycelium was separated by filtration and the filtrate was used for the enzyme assay. The fermentation of the crude extract was prepared by centrifugation after that supernatant was used as enzyme source. The crude extract was successively precipitated at saturation of 30% and 80% in magnetic stirrer. The precipitate was collected and dissolved in 10 ml of 50mM phosphate buffer. The diluted precipitate was subjected to dialysis and the dialysed enzyme was purified.

Effect of Substrates For Production of Lipase *Aspergilus* sp,.

The *Aspergilus* sp,. was cultured in basal medium for the production of lipase enzyme in liquid fermentation by various substrates of the groundnut oil, sunflower oil, Gingelly oil, tween 80 and tween 20. The present study indicated that the higher lipase production was observed in Tween 80 used as a source of lipid substrate. The lipase enzyme activity was also high (5.85) u/ml/min) in tween 80 substrate, followed by tween 20 (5.45 u/ml/min), sunflower oil (4.34 u/ml/min), groundnut oil (23.33 u/ml/min) and coconut oil (2.9) by assay by the titrimetric method of Yamda et al , with slight modification.. The maximum enzyme production was observed by tween 80 > tween 20 > sunflower oil > groundnut oil and coconut oil respectively (figure), when compare to other tween 80 substrates was containing high content of lipid substrates were subjected to the was *Aspergilus sp,.* easily degradation of tween 80. In the present study concluded the high level of lipase enzyme production, enzyme activity and growth of *Aspergilus sp,.* Was observed in basal medium broth followed by 72 hours of incubation is optimal for enzyme production in the fermentation. Lipase activity was assayed by the titrimetric method[5]. Table 1 to 5

Table-1 Effect of different temperature on lipase activity by *Aspergillus* sp,.

S.No	Temperature	Lipase Activity
1.	27°C	3.6
2.	30°C	5.3
3.	37°C	6.1
4.	40°C	4.5
5.	50°C	4.1
6.	60°C	3.7

Table-2 Effect of different pH on lipase activity by Aspergillus sp

S.No	pH	Lipase Activity (U/ml)
1.	2	3.8
2.	3	4.1
3.	4	4.5
4.	5	5.6
5.	6	6.1
6.	7	6.3
7.	8	6.0
8.	9	3.2
9.	10	2.0

Table-3 Effect of different carbon source on lipase activity

S.No	Carbon source	Lipase Activity (U/ml $^{-1}$)		
		24h	48h	72h
1.	CMC	6.1	6.6	6.5
2.	Fructose	2.85	6.4	5.9
3.	Glucose	6.05	5.75	5.4
4.	Lactose	5.45	7.7	6.75
5.	Maltose	4.15	6.55	5.9
6.	Starch	3.9	6.7	6.5
7.	Sucrose	8.85	9.6	9.35

Table -4 Effect of different nitrogen source on lipase activity

S.No	Nitrogen source	Lipase activity		
		24h	48h	72h
1.	Urea (0.5)	2.6	10.95	12.7
2.	Urea+Beef extract (0.5)	2.45	10.5	9.85
3.	Urea+Beef extract (1.0)	3.05	10.9	10.8
4.	Urea+Beef extract (1.5)	3.65	11.25	11.0
5.	Urea+Beef extract (0.5)	3.35	10.55	10.55
6.	Urea+Beef extract (1.0)	3.5	10.85	10.75
7.	Urea+Beef extract (1.5)	4.05	11.1	10.9

Table-5 Effect of different substrates on lipase activity

S.No	Substrate	Lipase Activity (U/ml)
1.	Groundnut oil	3.84
2.	Sunflower oil	4.34
3.	Coconut oil	2.9
4.	Tween 80	5.85
5.	Tween 20	5.45

SDS-PAGE AND NATIVE PAGE

For determining the molecular mass of the lipase SDS-PAGE (7.5-1.5% Polyacrylamide Gradient) was performed on electrophoretically purified lipase extracted (by Ammonium Sulphate Precipitation) from slices of non-denaturing gel corresponding to the active lipase bands. Enzyme preparation from different sources were run in parallel to a standard known protein mixture (High Molecular Weight, Kit from Sigma) silver staining was used to detect protein bands Silver stains are useful for the detection of Nanogram amount of proteins are nucleic acids in Acrylamide Gels (or) on various membranes. They are more sensitive than organic stains[1].

Silver images of protein or nucleic acid patterns are produced by a difference in the oxidation –reduction potential in regions occupied by nucleic acid or proteins compare to the surrounding gel or membrane[2& 3]. This redox potential catalysis the reduction of ionic to metallic sliver. A positive (dark) image will be produced if the region occupied by nucleic acid has higher redox potential than the surrounding region. Experiments with nucleic acids and their components have implicated the purines as the active subunits in the silver staining reaction[3]. Estimation of molecular weight of lipase by gel filtration revealed 19000 and 31000 Daltons respectively. These values correspond to data previously published for microbial lipases[2].

DISCUSSION:

In the present study *Aspergillus sp* was used to produce the lipase enzyme by fermentation. The *Aspergillus sp,.* was cultured in basal medium for the production of lipase enzyme in liquid fermentation by various substrates of the groundnut oil, sunflower oil, Gingelly oil, tween 80 and tween 20. The present study indicated that the higher lipase production was observed in Tween 80 used as a source of lipid substrate. The lipase enzyme activity was also high (5.85) u/ml/min) in tween 80 substrate, followed by tween 20 (5.45 u/ml/min), sunflower oil (4.34 u/ml/min), groundnut oil (23.33 u/ml/min) and coconut oil (2.9) by assay by the titrimetric method of Y amda et al, with slight modification. The maximum enzyme production was observed by tween 80 > tween 20 > sunflower oil > groundnut oil and coconut oil respectively (figure), when compare to other tween 80 substrates was containing high content of lipid substrates were subjected to the was *Aspergilus sp,.*easily degradation of tween 80.The lipase enzyme precipitated by ammonium sulphate by 80% salt cut then the pellet was subjected to dialysis. The dialyzed enzyme solution was precipitated by respectively. The precipitated was dialyzed. The dialyzed enzyme further purified with 12% separating gel by SDS- PAGE and NATIVE PAGE along with molecular weight of the enzyme proteins examined. The optimum pH for aspergillus sp., is found to be 7.0.

and the temperature 37 °C is found to be enhanced the lipase activity.

CONCLUSION:

In the present study concluded that the maximum enzyme production was observed liquid fermentation using tween 80 as substrate was found to be more effective in lipase production medium by *Aspergillus* species.

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