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Production of Microbial Lipases Isolated From Curd Using Waste Oil as a Substrate

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ABSTRACT

Lipase (triacyl glycerol acylhydrolases, EC 3.1.1.3) catalyses hydrolysis of long chain acyl glycerol at an oil water interface. Microbial lipase has various applications in industries so microorganism producing lipase was isolated and screened for the lipolitic activity from curd and was biochemically characterised. Organism from curd sample was found to be of Lactobacillus sps. It was subjected to submerge fermentation process using waste oil as an substrate. The extracellular enzyme obtained was partially purified using ammonium sulfate precipitation & the purified enzyme was used to carry out titrimetric assays, the molecular weight was found to be 29 kDa for Lactobacillus sps & the specific activity was found to be 0.082 U/mg.

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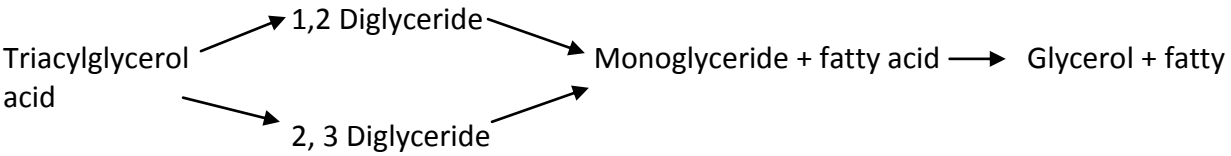
INTRODUCTION

Lipases belong to the class of serine hydrolases and therefore, do not require any cofactor. The natural substrates for lipases are triacylglycerols, which have very low solubility in water. Under natural conditions, lipases catalyse the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase where the enzyme remains dissolved. Lipases occur widely in nature, but only microbial lipases are commercially significant. Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields, ease of genetic manipulation and rapid growth of micro organisms and inexpensive media.[1]

Lipases hydrolyze triacylglycerols as shown in the reaction pathway:



Lipases show variations in their specificity. Thus from the fatty acid side, some lipases have an affinity for short-chain fatty acids (C_2, C_4, C_6, C_8 and C_{10}), some have a preference for unsaturated fatty acids (oliec, linoleic, linolenic,etc.) while many others are non-specific and randomly split the fatty acids from the triglycerides.

Lipases can be classified in several groups according to their specificity as non-specific lipases, 1, 3-specific lipases, 2-specific lipases and fatty acyl lipases.

Lipases constitute the most important group of biocatalysts because of its various industrial applications such as microbial lipases in the detergent, food, flavour industry, biocatalytic resolution of pharmaceuticals, esters and amino acid derivatives, making of fine chemicals, agrochemicals, use as biosensor, bioremediation, cosmetics and perfumery.

MATERIALS AND METHODS

Isolation of Lipolytic Bacteria [2,3]:

0.1ml of Two day old curd sample from dairy was spread plated on the modified Isolating agar medium containing: - Peptone, yeast extract, olive oil and agar, pH 7.0 and plates were incubated at 37°C and checked after 24 hrs and 48 hrs.

Monitorizing Lipolitic Activity on Agar Plates [4, 5]:

The fluorescence dye Rhodamine B agar plate assay is described as a true lipase assay which indicates the zone of lipolysis as an orange fluorescence under UV light at 350nm. Regardless of substrate, the indicator dye used must undergo a definite color change with a

change in pH. No fluorescent halos are formed when esterase is tested in that assay. Rhodamine B in the presence of olive oil forms a fluorescence complex with free fatty acids.

Culturing and Characterization of the Isolates:

The production of lipase activity was verified from the sample on a modified medium containing fluorescent dye Rhodamine-B (%0.005) (dissolve in distilled water and sterol filtrated). Isolate showing maximum zone of clearance were preserved as a pure culture on the nutrient agar slant containing 1% olive oil as a substrate and was subjected for identification by Morphological and biochemical characterization.

Fermentation:

1. Modified broth/liquid medium containing waste oil as a substrate was used for fermentation (Basal medium)

2. The pre-culture formed was inoculated into production medium (basal medium).

3. Incubated in an orbital shaker at 160 rpm at 37°C for 6 days.

4. Extracellular enzyme production was checked every 24h for 6 days. & assays were carried out.

Extraction of Enzyme:

1. After 6 days of incubation the flasks were retrieved from shaker. Culture broth was transferred to sterile centrifuge tubes, centrifuged at 8000rpm for 10min. and supernatant was filtered in a sterile filter paper. Extraction was done under sterile conditions to prevent any microbial contamination. The clear supernatant obtained was used as the extracellular enzyme source. The crude filtrate containing enzyme is assayed for the specific activity and stored at 4°C.

2. After incubation, cells were harvested by centrifugation at 10,000 rpm and the supernatant was collected. To this phosphate buffer having pH 7 was added.

Partial Purification of Lipase:

Lipase was partially purified using Ammonium sulphate.

1. Solid ammonium sulphate was added to the extract with stirring to bring the saturation to 35% under ice bath and after standing it for 4 hrs at 4^{o}C, precipitates was removed by centrifugation.
2. More Ammonium sulphate was added to the supernatant to bring the saturation to 70% and the mixture was kept overnight.

3. Precipitates were collected and were dissolve in distilled water and dialyze against water for 36 hrs using a bladder membrane.

4. The enzyme obtained was used for the assays and for treating the applications.

Lipase Assay:

Titrimetric Determination of Lipase Activity: [6]

In this procedure, native substrates (triacylglycerols) are hydrolyzed to yield fatty acids. Subsamples are withdrawn from reactive mixtures at predetermined intervals, and reactivity is quenched by the addition of ethanol. The amount of fatty acids released during the reaction is determined by direct titration with NaOH to a Phenolphthalein end point.

Procedure:

1. Into each of six 25-ml Erlenmeyer flasks, add 10 ml of 95% (v/v) ethanol and 2 to 3 drops of 1% (w/v) Phenolphthalein indicator. *(This titration cocktail is used to quench the reactivity of subsamples of the reaction mixture. Six flasks are used for five time points plus a reagent blank.)*

2. Into a 50-ml Erlenmeyer flask with stopper, place 50 ml of 5% (w/v) olive oil/gum Arabic emulsion substrate and pre-incubate 15 min in a 37°C water bath with magnetic stirring.

3. Add an appropriate amount of enzyme to initiate lipolysis on the emulsion substrate, start timer, and continue stirring.

4. At five suitable reaction intervals (e.g., 5, 10, 15, 20, and 25 min), remove 5 ml reaction mixture and transfer each subsample to a separate flask containing titration cocktail prepared in step 1. Swirl contents immediately to stop the reaction.(The quenched subsamples may be turbid. Samples may be put aside (up to 2 to 3 hr at 20°C to 22°C) for later titrimetric analysis).

5. Into the last 25-ml Erlenmeyer flask containing titration cocktail, add 5 ml phosphate buffered olive oil/gum Arabic emulsion substrate and mix well this serve as a reagent blank.

6. Titrate the contents of each flask with 0.05 N NaOH until a light pink color appears.

Enzyme Activity Calculation: [6]

1. Calculate the quantity of fatty acids liberated in each subsample based on the equivalents of NaOH used to reach the titration end point, accounting for any contribution from the reagent, using the following equation:

ISSN: 0975-8585

$$\mu\text{mol fatty acid/ml subsample} = \frac{[(\text{ml NaOH for sample} - \text{ml NaOH for blank}) \times N \times 1000]}{5\ \text{ml}}$$

Where *N* is the normality of the NaOH titrant used (0.05 in this case) & 5ml is volume of reaction mixture used.

2. Create a reaction progress curve by plotting the quantity of fatty acid liberated over the time of reaction. Determine the activity (initial velocity, V_0) of the lipase from the slope of the linear portion using the following equation:

$$V_0 = \text{slope} = (Y_2 - Y_1)/(X_2 - X_1)$$

Where units are µmol/ (ml x min), equivalent to mM/min.

3. Determine the specific activity (sp. act.) of the lipase preparation, if the protein content of the added enzyme preparation is known, using the following equation:

$$\text{sp. act.} = V_0 \div [\text{a mg protein}/ (50 + b)\ \text{ml reaction volume}]$$

Where ***a*** *is* mg protein added in ***b*** volume to the reaction mixture, and units are µmol/ (min x mg protein preparation).

(If a liquid form of the enzyme is added, such as lipases in broth samples from microbial cultures, calculate specific activity on a normalized basis by substituting a ml enzyme sample added (instead of mg protein) using the equation above.)

4. Determine the number of units (U) of lipase activity, which is defined as the amount that produces 1 µmol of fatty acid per minute under the specified assay conditions.

(Depending on the nature of the original enzyme sample, specific activity can be expressed as U/mg protein or as U/ml for liquid forms of an enzyme of unknown concentration.)

Protein Estimation by Bradford's Method: [7]

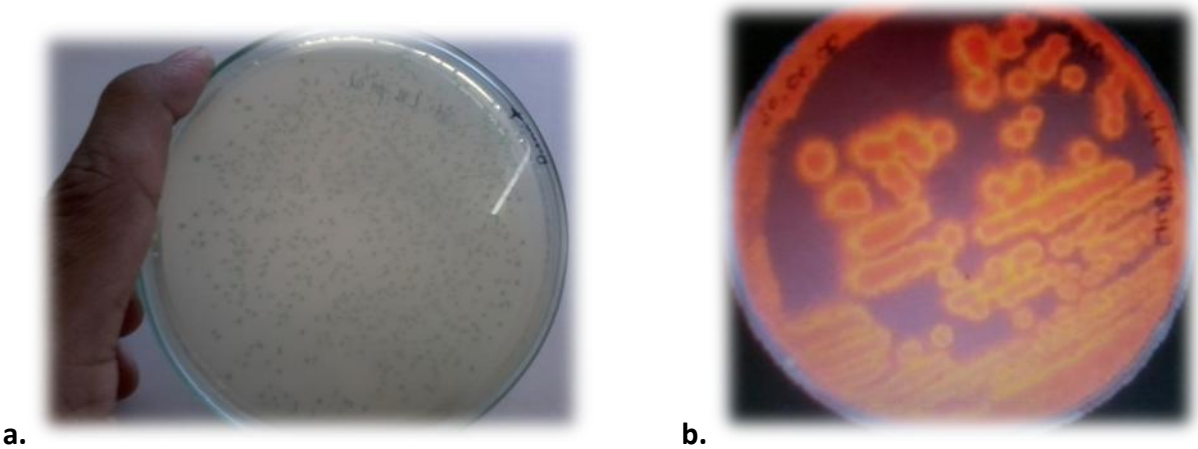
Bradford's assay relies on the affinity of the dye Coomassie brilliant Blue G250 to bind with protein and the quantity of the protein can than be estimated colorimetrically by determining the amount of dye present in the bound form. The dye binds most readily to arginyl and lysyl residue of proteins and is measured colorimetrically at 620nm.

RESULTS

Few colonies from the isolation plate were selected and were placed on the plate containing fluorescent dye Rhodamine-B (%0.005)(dissolve in distilled water and sterol filtrated) and olive oil to check out the one producing maximum orange-coloured fluorescent

ISSN: 0975-8585

halos around lipase producing colonies which were seen when these agar plates were expose to UV light at 350nm.



a. **b.**

Figure 1: a. Isolation of curd sample by spread plate method. b. Lipase positive colonies on agar Containing Rhodamine B dye and olive oil.

Table 1: Morphological and biochemical characterization of isolate from curd.

Substrate, Test	Result for colony from curd isolate
Gram Staining	Gram positive
Morphology	Chains
Motility	Non motile
Citrate	-
Catalase	-
Nitrate reduction	-
Oxidase	-
Urease	-
Glucose	+
Sucrose	-
Lactose	+
Indole	-
Methyl red	+
Voges-Proskauer	+

Key: - + positive and **-** negative

ISSN: 0975-8585

Table 2: Protein Estimation by Bradford's Method

S .No	Concentration	Stock (100µg/ml)	Diluent Phosphate buffer	Total volume (ml)	Bradford's reagent (ml)	O.D at 620 nm
1	20	0.2	0.8	1ml	5ml	0.07
2	40	0.4	0.6			0.13
3	60	0.6	0.4			0.17
4	80	0.8	0.2			0.21
5	100	1	0.0			0.27
6	Blank	0.0	1			0.00(adjusted)
7	Test	1	0			0.14

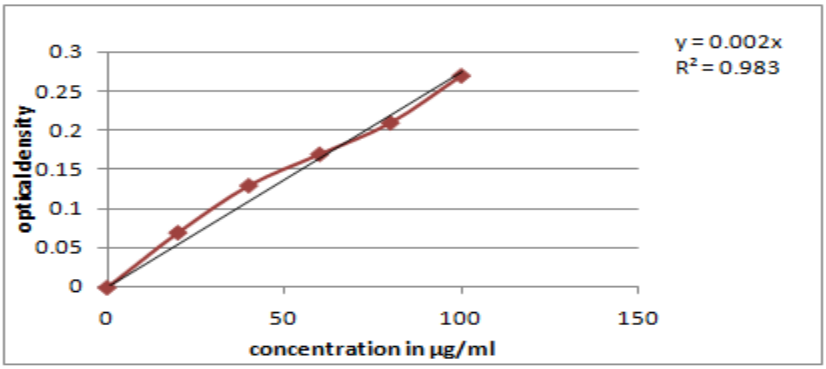


Figure 2: Protein Estimation by Bradford's Method

Table 3: Titrimetric Method for Lipase Assay

Time intervals (mins)	Readings (ml of NaOH for)		Test-Blank (ml)
	Sample	Blank	
5	10.1	9.6	0.5
10	10.7	9.8	0.9
15	10.6	9.4	1.2
20	10.9	9.2	1.7
25	11.4	9.4	2.0
30	12	9.6	2.4

Quantification of Fatty Acids Liberated

Observations for the quantity of fatty acids liberated in each subsample based on the equivalents of NaOH used to reach the titration end point, accounting for any contribution from the reagent, using the following equation:

$$\mu\text{mol fatty acid/ml subsample} = \frac{[(\text{ml NaOH for sample} - \text{ml NaOH for blank}) \times N \times 1000]}{5\ \text{ml}}$$

Where *N* is the normality of the NaOH titrant used (0.05 in this case) & 5ml is volume of reaction mixture used

ISSN: 0975-8585

Time intervals (mins)	[(ml NaOH for sample –ml NaOH for blank) x *N* x1000] 5 ml	µmol fatty acid/ml subsample
5	[(10.1ml –9.6ml) x*0.05* x1000] ÷ 5	5
10	[(10.7ml –9.8ml) x*0.05* x1000] ÷ 5	9
15	[(10.6ml –9.4ml) x*0.05* x1000] ÷ 5	12
20	[(10.9ml –9.2ml) x*0.05* x1000] ÷ 5	17
25	[(11.4ml –9.4ml) x*0.05* x1000] ÷ 5	20
30	[(12ml –9.6ml) x*0.05* x1000] ÷ 5	24

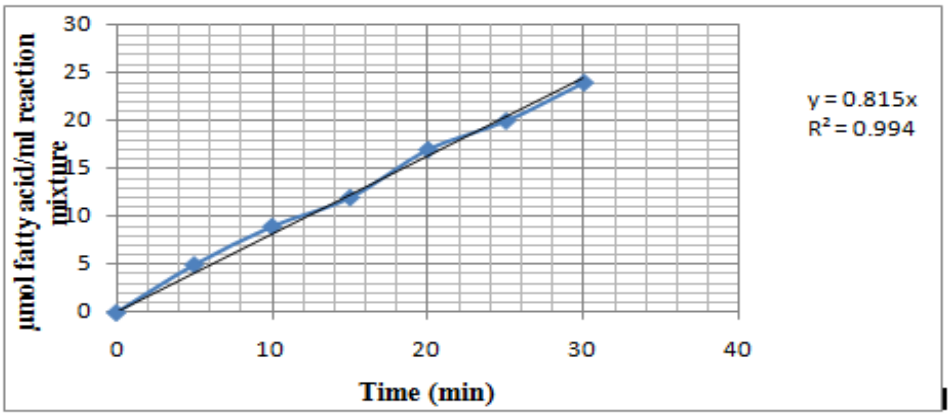


Figure 3: Reaction Progress Curve

CALCULATIONS

For the Activity (Initial Velocity, V_0)

Calculations for the activity (initial velocity, V_0) of the lipase from the slope of the linear portion using the following equation:

$$V_0 = \text{slope} = (Y_2 - Y_1)/(X_2 - X_1)$$

Where units are µmol/(ml x min), equivalent to mM/min.

For the Specific Activity

Calculation for the specific activity (sp. act.) of the lipase preparation, using the following equation:

$$\text{Specific activity} = V_0 \div [a \text{ mg protein}/ (50 + b) \text{ ml reaction volume}]$$

Where ***a*** is mg or ml protein added in ***b*** volume to the reaction mixture, and units are µmol/ (min x mg protein preparation).

ISSN: 0975-8585

Particulars	Values
Activity(V_0)	0.8154 mM/min
Specific activity	0.082 U/mg

CONCLUSION & DISCUSSION

Micro organism producing lipases were isolated using curd as a sample on a modified isolating agar medium and were verified using same medium containing Rhodamine B dye. Organism showing high lipolitic activity was found to be of *Lactobacillus sps* on morphological and biochemical characterization. This organism was used for lipase production in submerge fermentation using waste (cooked) oil as a substrate and The extracellular enzyme obtained was partially purified using ammonium sulfate precipitation method & the purified enzyme was used to carry out titrimetric assays.

The specific activity of the lipase of *Lactobacillus sps* was = 0.082 U/mg which is in comparison to the lipase obtained from fermentation using refined olive oil is fair enough so it can be concluded that even waste oil can be used as a good source of substrate. As lipase has number of applications in various industries on implementation of this method of production may help in reducing production cost.

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