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RESEARCH ARTICLE

PRODUCTION AND CHARACTERIZATION OF BACTERIAL LIPASE

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ABSTRACT

Lipases are important biocatalysts that carry out novel reactions in both aqueous and nonaqueous media. Lipases have ability to carry out a wide variety of chemo-, regio- and enantioselective transformations and are the tools of choice for organic chemists. Their general ease of handling, broad substrate tolerance, high stability towards temperatures and solvents, high enantioselectivity, and convenient commercial availability have all added to their widespread popularity among organic chemists. Lipases show immense versatility regarding their catalytic behavior and scope to search for newer lipases with desired selectivity and substrate tolerance .The unique interfacial activation of lipases has always fascinated enzymologists, and, recently, biophysicists and crystallographers have made progress in understanding the structure–function relationships of these enzymes. However, complete understanding of the lipase molecule requires greater input of research effort.

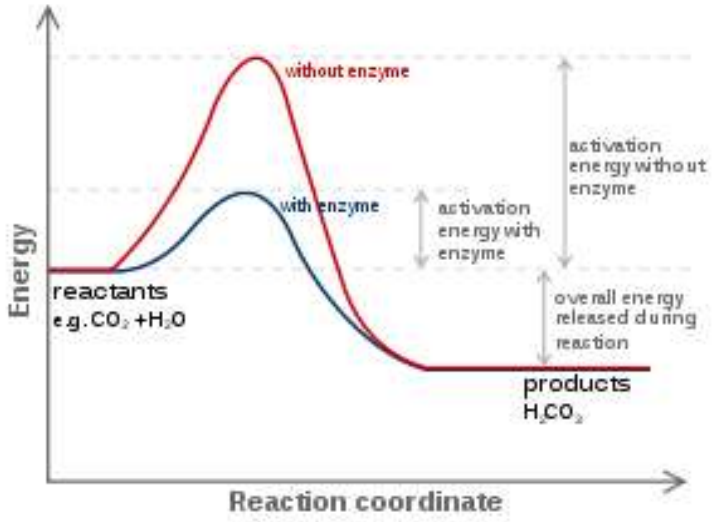
KEYWORDS: Biocatalysts, Biophysicists, Crystallographers

INTRODUCTION:

"Enzymes are the biocatalyst that changes the rate of reaction and activation energy without being used up in the reaction mixture."

The basic enzymatic reaction is

Substrate + Enzyme = Product + Enzyme



In 1835 Swedish chemist Jon Jakob Berzelius done some work ,and termed their chemical action catalytic. In 1878, German physiologist Wilhelm Kühne (1837–1900) first used the term enzyme, which comes from Greek χενζυμον, "in leaven". In 1926, first enzyme was obtained in pure form, by James B. Sumner.

Enzyme have protein and non protein part. This entire active complex is referred to as the holoenzyme; i.e., apoenzyme (protein portion) plus the cofactor (coenzyme, prosthetic group or metal-ion-activator) is calledtheholoenzyme.

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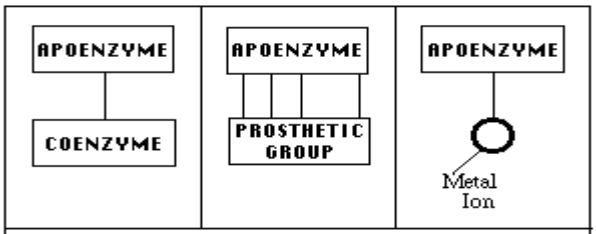


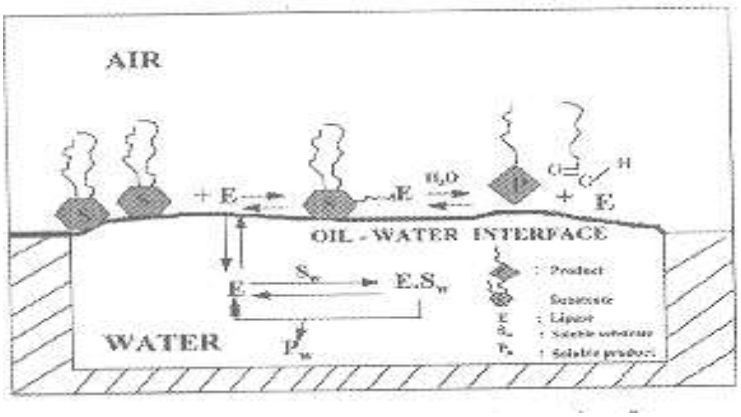
FIGURE 2
Holoenzymes - apoenzymes plus various types of cofactors

Apoenzyme + Cofactor = Holoenzyme :

Enzymes are generally globular proteins and range from just 62 amino acid residues in size, for the monomer of 4-oxalocrotonate tautomerase. Enzymes are usually very specific as to which reactions they catalyze and the substrates that are involved in these reactions. Complementary shape, charge and hydrophilic/hydrophobic characteristics of enzymes and substrates are responsible for this specificity. Enzymes can also show impressive levels of stereospecificity, regioselectivity and chemoselectivity. Some substance can inhibits (inhibitor like EDTA) and some increases (activator like Mg^{2+}, Ca^{2+}) the enzyme activity.

Enzymes serve a wide variety of functions inside living organisms. They are indispensable for signal transduction and cell regulation, often via kinases and phosphatases. They also generate movement, with myosin hydrolysing ATP to generate muscle contraction. Other ATPases in the cell membrane are ion pumps involved in active transport. Viruses can also contain enzymes for infecting cells, such as the HIV integrase and reverse transcriptase. An important function of enzymes is in the digestive systems of animals. Enzymes such as amylases and proteases break down large molecules (starch or proteins, respectively) into smaller ones. Several enzymes can work together in a specific order, creating metabolic pathways. Enzyme have different applications such as Food processing, Brewing industry , Fruit juices, Dairy industry , Starch industry , Paper industry , Biofuel industry , Biological detergent, Contact lens cleaners, Rubber xindustry, Photographic industry, Molecular biology .

Lipases (triacylglycerol acylhydrolases, 3.1.1.3) belong to the class of serine hydrolases and therefore do not require any cofactor. The natural substrates of lipases are triacylglycerols, having very low solubility in water. A lipase is a water-soluble enzyme that catalyzes the hydrolysis of ester bonds in water–insoluble, lipid substrates. Lipases thus comprise a subclass of the esterases. Lipases are not involved in any anabolic processes. This enzyme acts at the oil–water interface. Lipases catalyse the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol and fatty acids, and under certain conditions the reverse reaction leads to esterification and formation of glycerides from glycerol and fatty acids



While a diverse array of genetically distinct lipase enzymes are found in nature, and represent several types of protein folds and catalytic mechanisms, most are built on an alpha/beta hydrolase fold and employ a chymotrypsin-like hydrolysis mechanism involving a serine nucleophile, an acid residue (usually aspartic acid), and a histidine . Many lipases that are produced by Gram-negative bacteria require a dedicated helper protein, a lipase-specific foldase, to obtain their native, fully folded and biologically active conformation.

Lipase widely occur in nature,but only commertially lipase is significant. They are also found in germinating oil seeds like rape, musturd, groundnut, sunflower etc. Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins (Winkler et al). Most of the bacterial lipases reported are constitutive and are

nonspecific in their substrate specificity, and a few bacterial lipases are thermostable[..] Some example are Achromobacter sp., Alcaligenes sp., Arthrobacter sp., Pseudomonas sp., Staphylococcus sp., and Chromobacterium sp. Fungus also produces lipase such as Aspergillus niger, Candida cylindracea, Humicola lanuginosa, Mucor miehei, Rhizopus arrhizus, R. delemar, R. japonicus, R. niveus and R. oryzae.

In the present day industries, lipases have made their potential realized owing to their involvement in various industrial reactions either in aqueous or organic systems, depending on their specificity. Lipases are used extensively in the dairy industry for the hydrolysis of milk fat. Current applications include flavour enhancement of cheese, acceleration of cheese ripening, manufacture of cheese-like products, and lipolysis of butter fat, and cream with the help of Mucor miehei, Aspergillus niger and A. oryzae. A. The application of lipases in the oleochemical industry is enormous as it saves energy and minimizes thermal degradation during hydrolysis, glycerolysis, and alcoholysis. Lipases also help in in synthesis of triglycerides (cocoa butter). This triglyceride and functionally similar fats are readily obtained by acidolysis of palm oil fractions which are rich in 2-palmitoyl glyceride with unsaturated fatty acid(s)[234]. Acidolysis, catalysed by 1, 3-specific lipases. Enzymic synthesis of functionally similar surfactants has been carried out at moderate temperature (60–80°C) with excellent regioselectivity. Adelhorst et al.[237] have carried solvent-free esterification of simple alkyl-glycosides using molten fatty acids and immobilized Candida antarctica lipase. Enzymic synthesis of functionally similar surfactants has been carried out at moderate temperature (60–80°C) with excellent regioselectivity. Adelhorst et al.have carried solvent-free esterification of simple alkyl-glycosides using molten fatty acid sand immobilized Candida antarctica lipase. Lipases have applications as industrial catalysts for the resolution of racemic alcohols in the preparation of some prostaglandins, steroids, and carbocyclic nucleoside analogues. Lipase used in production of biodiesel. It also used in agrochemicals and pharmaceutical. It have wide role in textile companies. It help in production of detergents The stereo selectivity of lipase is useful for synthesis of optically active polymers[.] . In the field of liquid crystals, suitable monomers can be prepared by lipase-catalyzed transesterification of alcohols.

MATERIALS AND METHODS:

Isolation and Screening and lipase producing microorganisms:

1) Collection of soil samples from oil mills.
The samples from different areas of oil mills in and around the Bangalore in sterile plastic bags were collected.

2) Isolation of organisms by serial dilution and agar plate method.
The soil sample was serially diluted using sterile saline(0.8%) ,10^{-1},10^{-2}…etc form each dilution 0.1ml of sample was inoculated in nutrient agar plates which was sterilized at 121oC for 20min. the plates were incubated for 24hrs at 37oC. (K.R. Aneja, 2005)
Number of cells per g of soil was calculated as

No. of cells /g= No. of colonies X reciprocal of dilution factor
Dry Wt. of soil

3) Sub culture preparation.
After incubation, the discrete or isolated colonies has been examined and some of the cells from one of the colony are sub cultured to separate agar slants with a sterile needle or loop for further examination(H.J. Conn et al, March 1919).

4) Identifying the organisms by physical appearance and microscopic characteristics.
The isolated organism was identified by differential staining i.e. Gram Staining. In this process the fixed bacterial smear is subjected to four different reagents like Crystal violet (primary stain), iodine solution (mordant), alcohol (decolorizing agent) and saffrarin (counter stain).The isolated bacteria retain the primary stain (appears dark blue)(Bergey et al, 1994)

5) Screening the microorganisms on specific media for the production of lipase.

Lipase is a hydrolytic enzyme which degrades lipid molecule into glycerides and free fatty acid. The lipase production by the microorganisms can be tested by inoculating the organisms on Tributyrin agar media which contain tributyrin as lipid substrate. If a clear zone is appeared around the bacterial colony, it illustrates the presence of lipase.
(Cappuccino, 2003)

Production and Purification of the enzyme:

1) Production of the lipase enzyme on the production media.
Prepare the production media at three different concentration of peptone. Now take the organisms and

inoculate in respective media. Then incubate for 72 hours at 37^0c (Aisaka K, 1980,)

2) Assay

2.1) Activity of enzyme can be determined by Titrimetric method.

Pipette (in milliliter) the following reagents into suitable container.

Reagents	Test	Blank
Distilled water	2.5	2.5
Reagent A (tris HCl)	1.0	1.0
Reagent B (olive oil sample)	0.3	0.3

Mix by swirling and incubate at 37^0c for half and hrs. Add 0.1 ml reagent F (enzyme solution). Incubate it for half an hr at 37^0c. Add 1.5 ml of reagent c (95% ethanol) in each test tube. Firstly take blank, add phenolphthalein, titrate it with std NaOH and take the reading. Take the test, add phenolphthalein and titrate it with std NaOH and take the reading.

2.2) Specific activity can be determined by Follins Lowery Method.

TEST TUBE NO.	VOLUME OF BSA (ml)	VOLUME OF DISTILLED WATER (ml)	VOLUME OF REAGENT C (ml)	INCUBATION FOR 10 MINUTE	VOL. OF FOLLINS REAGENTS (ml)	IN CUBATION FOR 30 MINUTES	OPTICAL DENSITY (nm)
BLANK	0	1	5		0.5		
1	0.2	0.8	5		0.5		
2	0.4	0.6	5		0.5		
3	0.6	0.4	5		0.5		
4	0.8	0.2	5		0.5		
5	1	0	5		0.5		
UNK 1	0.1	0.9	5		0.5		
UNK 2	0.1	0.9	5		0.5		
UNK 3	0.1	0.9	5		0.5		

3) Purification of the lipase enzyme by standard method.

The purification of lipase enzyme was done by applying different methods such as Ammonium sulphate precipitation, Dialysis, Ion exchange chromatography.

3.1) Ammonium sulfate precipitation

Measure the volume of enzyme solution, pour it into a beaker with a magnet bar and place it in an ice bath at 4 ^{0} c. Calculate the required amount of ammonium sulphate, taking 0.6 gm of salt/ml of the protein solution. Start stirring the solution and add salt to it in small portion, allow salt to dissolve before adding next portion. When all the salt has been added, take beaker of stirring and leave it at 4^0c for some time. The precipitated protein of interest is recovered by centrifugation and dissolved in fresh buffer for the next stage of purification. (Neerupama Nawani et al, 2000 march)

3.2) Dialysis of Proteins

Firstly activate the dialysis bag by heating it in a beaker of distilled water. Add sodium bi carbonate in beaker, heat it upto boil. Change the distilled water, heat it upto boiling. The concentrated protein solution is placed in dialysis bag with small holes which allow water and salt to pass out of the bag while protein is retained**.** The dialysis bag is placed in a large volume of buffer and stirred for many hours (16 to 24 hours). (Max Lechner et al, 1988 June)

3.3) Ion-exchange chromatography

A sample is introduced, either manually or with an autosampler, into a sample loop of known volume. A buffered aqueous solution known as the mobile phase carries the sample from the loop onto a column that contains some form of stationary phase material (cellulose glass beads).

The target analytes (anions or cations) are retained on the stationary phase but can be eluted by increasing the concentration of a similarly charged species that will displace the analyte ions from the stationary phase (Hamish Small ISBN, 0-306-43290-0, Ion exchange chromatography)

Characterization of the enzyme:

1) To study the enzyme characteristics in different parameters by using temperature, pH, substrate concentration, inhibitor(EDTA), activator ($CaCl_2$), thermo stability, and effect of nitrogen

1.1) Effect of pH

The enzyme was incubated in the buffer of different pH via like 3, 5, 7, 8 and 9. Assay for enzyme activity was carried out by titrimetric method.

1.2) Effect of temperature

The enzyme solution was incubated for half an hrs at different temperature viz like 4^0c, 27^0c, 37^0c, 80^0c and 100^0c. Assay for enzyme activity was carried out by titrimetric method.

1.3) Effect of substrate concentration

The enzyme was subjected to different concentration of substrate viz like 50 µl, 150 µl, 250 µl, 350 µl and 450 µl. Assay for enzyme activity was carried out by titrimetric method.

1.4) Effect of activator ($CaCl_2$)

The enzyme solution was incubated for half an hrs at different concentration of activator ($CaCl_2$) viz like 0.2mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml and 1 mg/ml. Assay for enzyme activity was carried out by titrimetric method.

1.5) Effect of inhibitor (EDTA)

The enzyme solution was incubated for half an hrs at different concentration of inhibitor (EDTA) viz like 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml and 1 mg/ml. Assay for enzyme activity was carried out by titrimetric method.

1.6) Thermostability of enzyme

The enzyme solution was incubated at 37^0c and 55^0c for different time periods. Assay for enzyme activity was carried out by titrimetric method.

2) The molecular weight of the enzyme is assayed by SDS-PAGE method.

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein_folding, posttranslational modifications and other factors).The SDS gel electrophoresis of samples having identical charge per unit mass due to binding of SDS results in fractionation by size and is probably the world's most widely used biochemical method. The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary_structure. This is known as Native_PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

SDS binds in a ratio of approximately 1.4 g SDS per 1.0 g protein (although binding ratios can vary from 1.1-2.2 g SDS/g protein), giving an approximately uniform mass: charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the size of the protein. A tracking dye may be added to the protein solution (of a size smaller than protein) to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run. (David R Caprette)

RESULTS:

Isolation and screening of lipase producing microorganism:

1) The colonies of following characteristics were isolated and show a clear zone when screened in Tribytrin media

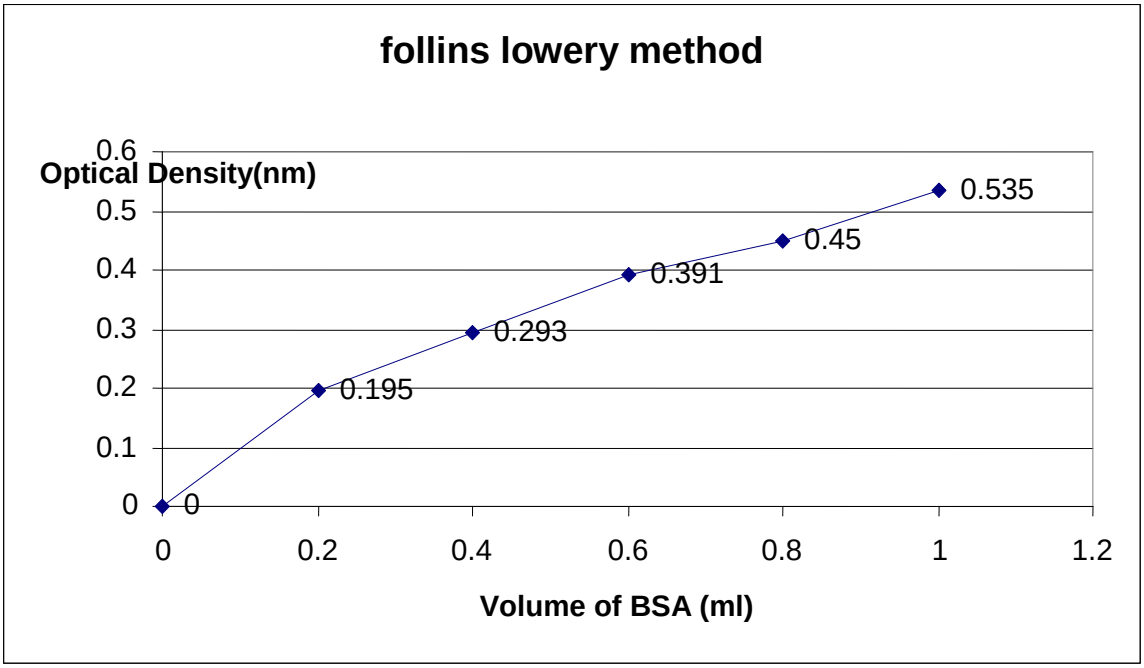
SAMPLE	NUMBER OF PLATES	DILUTION	NUMBER OF COLONIES	CHARACTERISTICS/GRAM STAINING	NO. OF CELLS=NO OF COLONY*DILUTION FACTOR/WEIGHT OF SOIL
SAMPLE 1	1	10^{-2}	18	PALE YELLOW, OPAQUE, SMOOTH, ROUND/GRAM POSITIVE	1800
	2	10^{-4}	20	PALE YELLOW, OPAQUE, SMOOTH, ROUND/GRAM POSITIVE	20000
	3	10^{-6}	76	PALE YELLOW, OPAQUE, SMOOTH, ROUND/GRAM POSITIVE	76000000
SAMPLE 2	1	10^{-3}	14	PALE YELLOW, OPAQUE, SMOOTH, ROUND/GRAM POSITIVE	14000
			2	PINK, OPAQUE ,SMOOTH, ROUND/GRAM POSITIVE.	2000

			1	YELLOW, OPAQUE, SMOOTH, ROUND/GRAM POSITIVE.	1000
	2	10^{-5}	46	PALE YELLOW, OPAQUE, SMOOTH, ROUND/GRAM POSITIVE.	4600000
			4	PINK, OPAQUE, SMOOTH, ROUND/GRAM POSITIVE.	400000

Production and purification of enzyme:

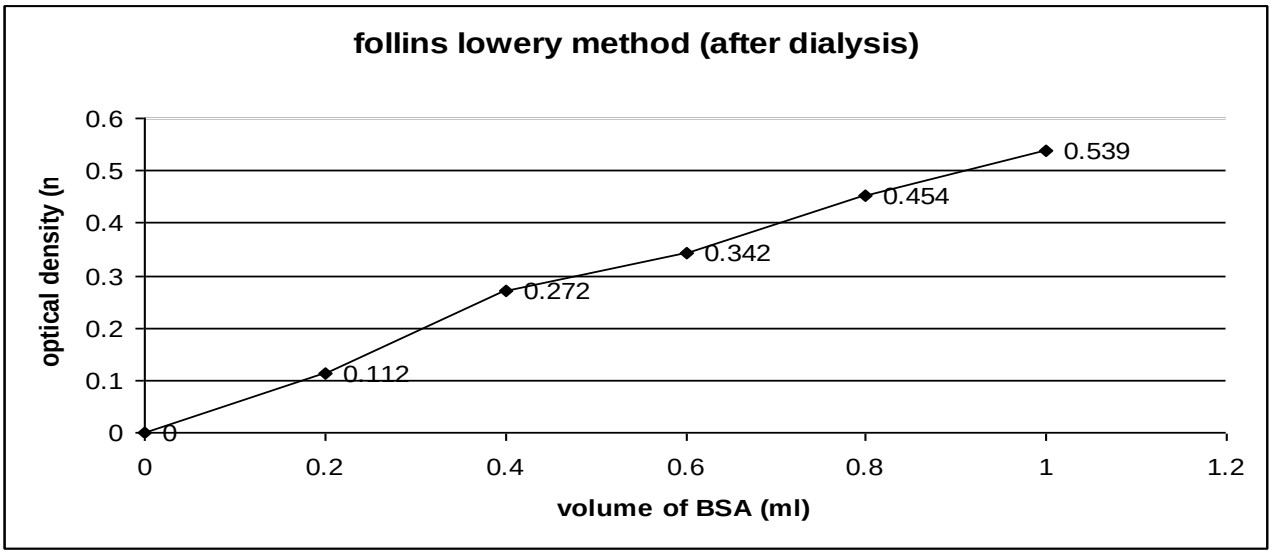
1) The enzyme was produced in production media by the microorganisms in incubation period of 72 hrs at 37^0c of temperature.

S.no.	Vol of BSA (ml)	O.D (nm).
1	0	0
2	0.2	0.195
3	0.4	0.293
4	0.6	0.391
5	0.8	0.45
6	1	0.535
7	Unknown1 (V)	0.418
8	Unknown2 (P)	0.55
9	Unknown 3 (A)	0.558



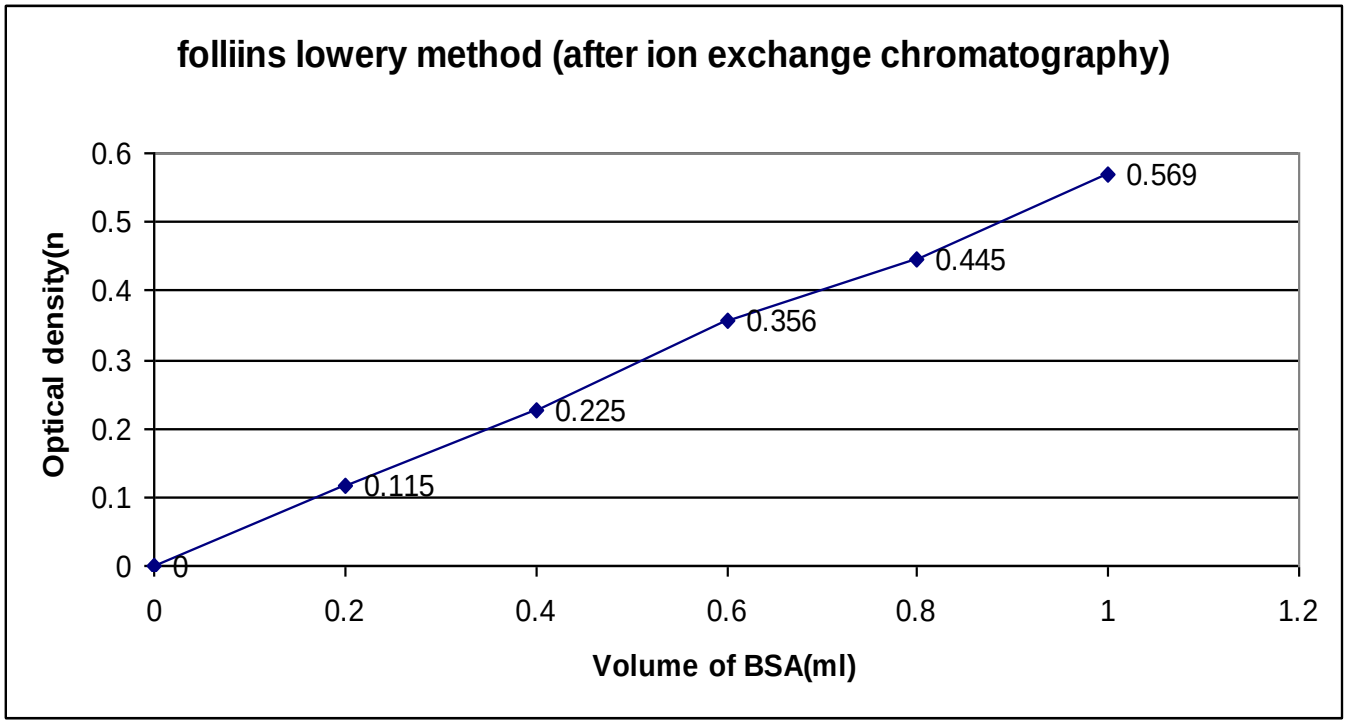
2) The purification of the enzyme was done by standard method (dialysis)

S.no.	Volume of BSA(ml)	O.D(nm)
1	0	0
2	0.2	0.112
3	0.4	0.272
4	0.6	0.342
5	0.8	0.454
6	1	0.539
7	unknown 1 (V)	0.315
8	unknown 2 (P)	0.327
9	unknown 3(A)	0.094



3.) Ion exchange chromatography

S.no.	Vol of BSA (ml)	O.D.(nm)
1	0	0
2	0.2	0.115
3	0.4	0.225
4	0.6	0.356
5	0.8	0.445
6	1	0.569
7	unknown 1(A)	0.199
8	unknown 2(A)	0.308



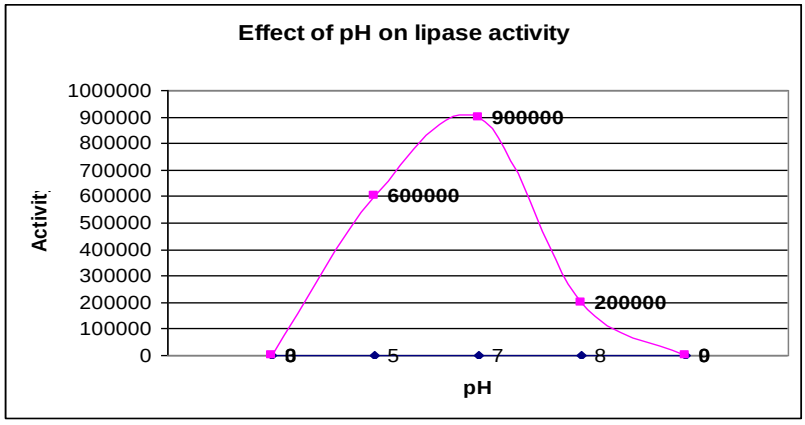
4) The enzyme activity and specific activity of enzyme at different phases.

S.NO	CRUDE EXTRACT	CON.OFPROTEIN (µG/M)	ACTIVITY (UNITS/MI)	SPECIFIC ACTIVITY (UNITS/Mg)	YIELDS (%)	PURIFICATION FACTOR
1	MEDIA 1 (V)	200µg/ml	100000	13333.33	-	-
	MEDIA 2 (P)	-	600000	63157.89	-	-
	MEDIA 3 (A)	-	300000	30000	-	-
2	AFTER DIALYSIS	-				
	1(V)	-	200000	125000	0.002	0.00070
	2(P)	-	500000	90909	0.000139	0.0000228
	3(A)	-	400000	70175	0.00044	0.000078
3	After ION- EXCHANGE CHROMATOGRAPHY					
	1(A)	-	100000	114285	0.00011	0.000127
	2(A)	-	400000	57142	0.00044	0.0000635

Characterization of enzyme.

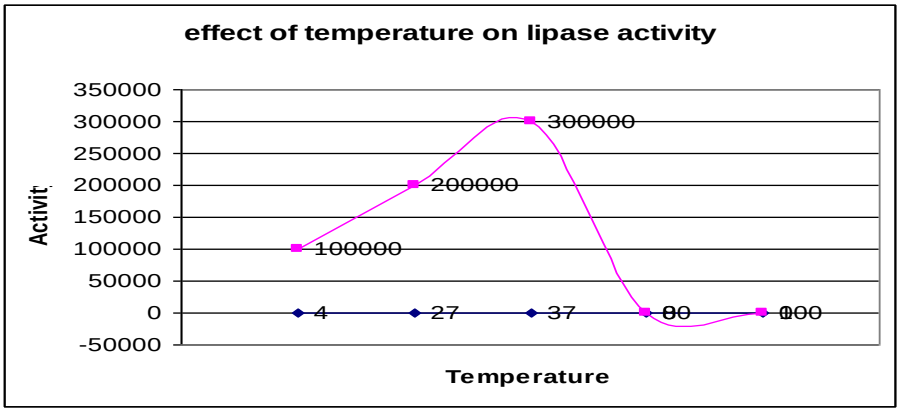
1) Effect of pH on enzyme activity.

S.no	Ph	Activity(units/ml)
1	3	0
2	5	600000
3	7	900000
4	8	200000
5	9	0



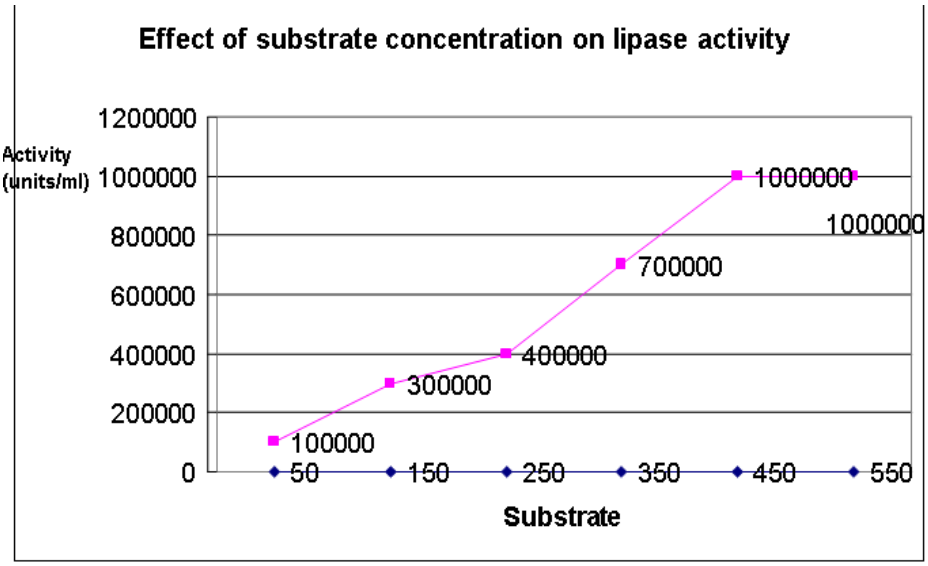
2) Effect of temperature on enzyme activity.

S.no	Temperature (^{0}c)	Activities(units/ml)
1	4	100000
2	27	200000
3	37	300000
4	80	0
5	100	0



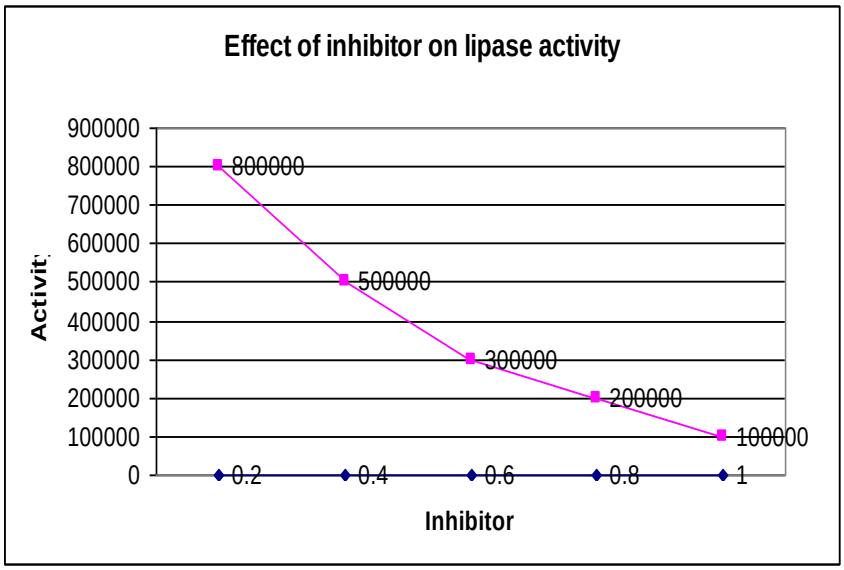
3) Effect of substrate concentration on enzyme activity.

S.no.	Substrate concentration(ug/ml)	Activity(units/ml)
1	50	100000
2	150	300000
3	250	400000
4	350	700000
5	450	1000000
6	550	1000000



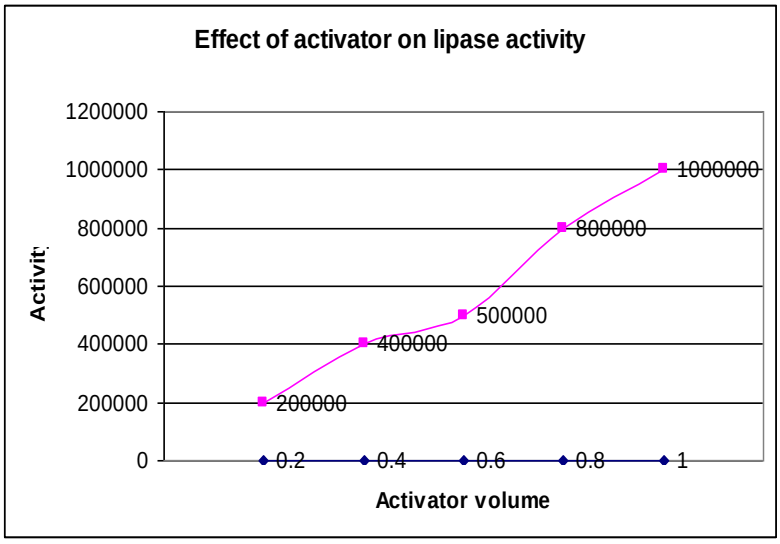
4) Effect of inhibitor on enzyme activity.

S.no.	Inhibitor(mg/ml)	Activity(units/ml)
1	0.2	800000
2	0.4	500000
3	0.6	300000
4	0.8	200000
5	1	100000



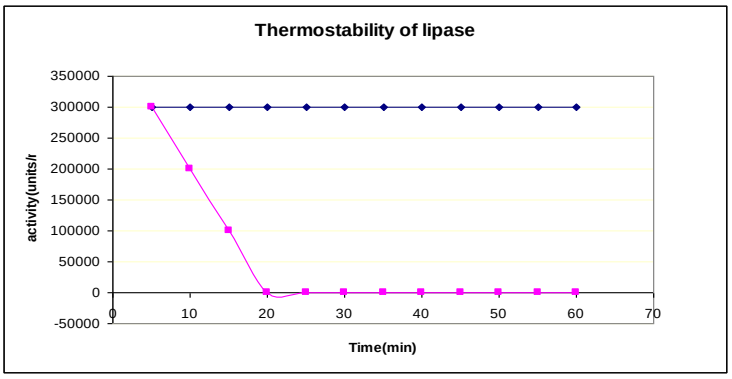
5) Effect of activator on enzyme activity.

S.no.	Activator concentration (mg/ml)	Activity(units/ml)
1	0.2	200000
2	0.4	400000
3	0.6	500000
4	0.8	800000
5	1	1000000



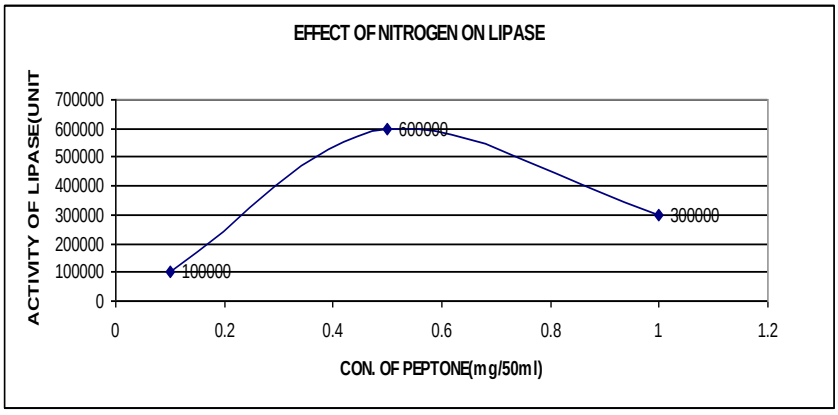
6) Thermostability of enzyme at 37^0c and 55^0c

S.no.	Time(min)	Activity of lipase at 37^{0c}	55^{0c}
1	5	300000	300000
2	10	300000	200000
3	15	300000	100000
4	20	300000	0
5	25	300000	0
6	30	300000	0
7	35	300000	No
8	40	300000	No
9	45	300000	No
10	50	300000	No
11	55	300000	No



7) Effect of nitrogen on enzyme activity

S.NO	CONCENTRATION OF PEPTONE IN MEDIA	ACTIVITY OF LIPASE
MEDIA 1	0.1 mg/50 ml	100000
MEDIA 2	0.5 mg/50ml	600000
MEDIA 3	1 gm/50 ml	300000



DISCUSSION:

Lipolytic activity is commonly considered as a characteristic of taxonomic importance; point up the wide variability that may occur in lipase production by the same organisms in different media.The organisms shows variability in the enzyme activity, when grow in three

different media having different peptone concerntration.This generally shows the effect of nitrogen on the enzyme activity produce by the same organisms. (Corzo and Revah, 1999).

The results also indicate that the substrate concentration also affects the enzyme activity. Surface area occupied by the substrate was an important parameter in the enzyme production by the organisms. The amount of 10gm of substrate yields maximum production of lipase. The fewer yields at higher levels were due to the low mass transfer rate and difficulty in the penetration of the organisms. (Rao et al 2003) (Rohit et al 2001.)

Evaluation of the lipase producing efficiency based on the clear zone around colony indicated that all of them could produce lipase enzyme. The result of the lipase production by the organisms was outstanding when they were supplied on the emulsion tributyrin agar and incubated at 37^{oc} for 24 hours.

The extracellular lipase produced from bacillus species was optimum at pH-7 and temperature 37^{oc}.The activity of lipase incubated at PH-7 and 37^{oc} for half an hour showed the highest activity. Generally the lipase from bacteria were stable up to 70oc (Kulkarni and Gadre, 1999); but the results show that activities of all lipases incubated in buffer of pH 7-8 were decreased by higher temperature.

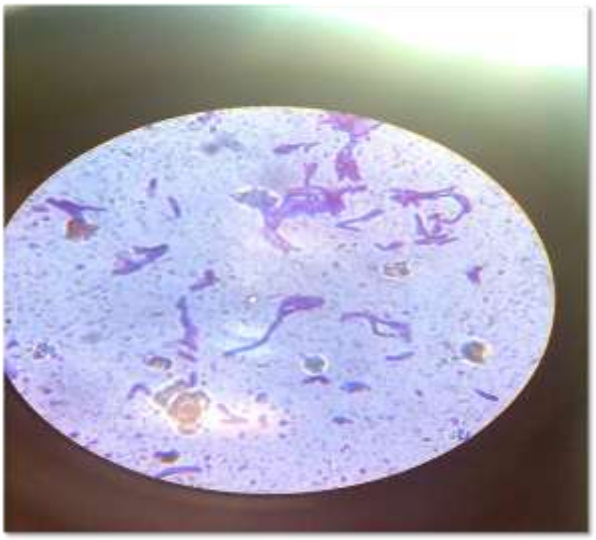
Klump et al 1992 and Jaeger et al 1994 described that thermal stability of enzyme is related to the content of hydrophobic amino acids in protein. However the results showed that the enzyme is stable at pH-7 and temperature 37^{0c}, the stability of enzyme however decreases when incubated at 55^{oc} for different time periods.

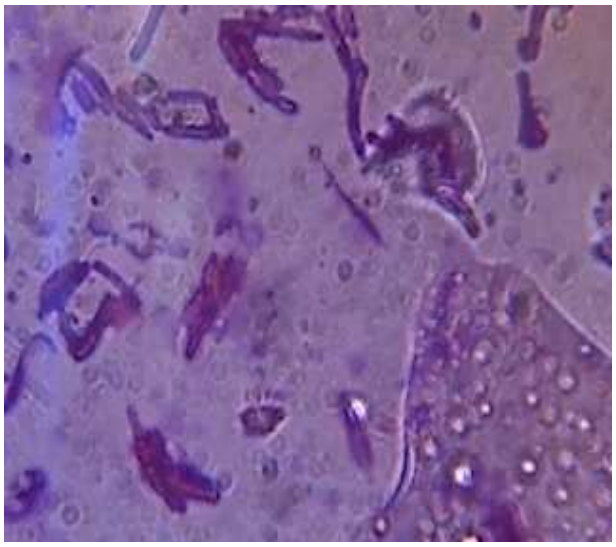
CONCLUSION:

Lipases are amongst the most important biocatalysts that carry out novel reactions in both aqueous and nonaqueous media. Lipases have the remarkable ability to carry out a wide variety of chemo-, regio- and enantioselective transformations. Thus lipases are the tools of choice for organic chemists. Their general ease of handling, broad substrate tolerance, high stability towards temperatures and solvents, high enantioselectivity, and convenient commercial availability have all added to their widespread popularity among organic chemists. Today, lipases find immense applications in various areas of industrial microbiology and biotechnology. This statement is well documented by the enormous number of research investigations undertaken in the last one and a half decades. Lipases show immense versatility regarding their catalytic behaviour. Therefore, there is a lot of scope to search for newer lipases with desired selectivity and substrate tolerance.

To widen the usage level of lipases, there is an urgent need to understand the mechanisms behind the lipase-catalysed reactions. The unique interfacial activation of lipases has always fascinated enzymologists, and, recently, biophysicists and crystallographers have made progress in understanding the structure–function relationships of these enzymes. However, complete understanding of the lipase molecule requires greater input of research effort.

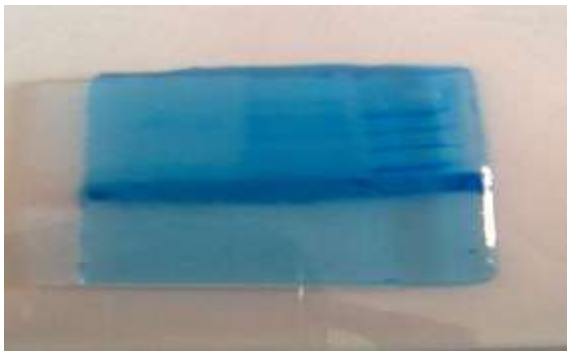
GRAM STAINING OF MICROORGANISM:





SCREENING OF MICROORGANISM:

(d) SDS PAGE:



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