ISOLATION AND SCREENING OF SOIL MICROORGANISMS WITH LIPASE ACTIVITY

Project work by

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Submitted to St.Teresa's College (Autonomous), Ernakulam

affiliated to Mahatma Gandhi University, Kottayam in

Partial fulfillment of requirement for the Degree of Bachelor of Science

In Zoology.

2017-2018

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CERTIFICATE

This is to certify that the project report entitled "**ISOLATION AND SCREENING OF SOIL MICROORGANISMS WITH LIPASE ACTIVITY**" submitted by Ms. NILEENA MOHANKUMAR(AB15ZOO041) in partial fulfillment of the requirements for the Degree of Bachelor of Science in Zoology is a bonafide work done under my guidance and supervision and to the best of my knowledge, this is her original effort.

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DECLARATION

I, Ms. **NILEENA MOHANKUMAR**, hereby declare that this project report entitled "**ISOLATION AND SCREENING OF SOIL MICROORGANISMS WITH LIPASE ACTIVITY**" is a bonafide record of work done by me during the academic year **2017-18** in partial fulfillment of the requirements for the Degree of Bachelor of Science in Zoology.

This work has not been undertaken or submitted elsewhere in connection with any other academic course and the opinions furnished in this report is entirely my own.

NILEENA MOHANKUMAR

ACKNOWLEDGEMENT

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done only is die to such supervision and assistance and I would not forget to thank them.

I would like to acknowledge Kerala State Council for Science, Technology and Environment for providing financial assistance for this project under the SPYTiS-II (Letter No. 244/SPYTiS-II/2017/KSCSTE dated 30.10.2017).

I respect and thank Dr. REEMA KURIAKOSE for providing me an opportunity to do the project in St. Teresa's College, Ernakulam and giving me all support and guidance which made me complete the project duly.

I owe my deep gratitude to my project guide Dr. HELVIN VINCENT, who took keen interest on my project and guided us all along, till the completion of our project work by providing all the necessary information for developing a good system.

I would always remember Dr. MEERA JAN ABRAHAM, Dr. SOJA LOUIS, Ms. JAYA S and Ms. NIMITHA R CHANDRAN for their encouragement and more over for their timely support and guidance till the completion of my project.

I am thankful to and fortunate enough to get constant encouragement, support and guidance from all teaching staffs of Zoology department which helped me in successfully completing my project work. I would like to extend my sincere esteems to all staffs in laboratory for their timely support. Lastly, I thank almighty, my parents, brother, sister and friends for their constant encouragement without which this project would not be possible.

CONTENTS

ABSTRACT

Lipases are glycerol ester hydrolases that catalyze the hydrolysis of the carboxylic ester bond through various important reactions such as trans-esterification, alcoholysis, interesterification, esterification, aminolysis etc. to free fatty acids and glycerol. The current study includes isolation and screening of lipase producing bacteria from the soil samples. The soil samples were rich in microbial content. The soil microbes were isolated by serial dilution and pour plate method. They were screened for lipase production using 0.1% tributyrin agar plates. After incubation, among the ninety isolates 31 isolates exhibited high lipolytic activity on tributyrin plate assay. These lipase producing microorganisms may be used for different applications after proper characterization.

INTRODUCTION

Soil is defined as a mixture of organic and inorganic materials that cover the surface of the earth which supports biological activity. Soil differs from the material from which it is derived in many physical, chemical, biological, and morphological properties and characteristics. Varieties of substances were found in soils, they are categorized into four basic components: minerals, organic matter, air and water. Soil is composed of 45% minerals, 25% water, 25% air, and 5% organic matter. Soil, air and water are found in the pore spaces between the solid soil particles. The ratio of air-filled pore space to water-filled pore space changes depending on water additions through precipitation, through flow, groundwater discharge, and flooding.

Soil is essential for life, in the sense that they provide the medium for plant growth, habitat for many insects and other organisms, act as a filtration system for surface water, carbon store and maintenance of atmospheric gases.

A lipase is any enzyme that catalyzes the hydrolysis of fats (lipids). Lipases are a subclass of the esterase. Lipases perform essential roles in the digestion, transport and processing of dietary lipids in most living organisms. Most lipases act at a specific position on the glycerol backbone of a lipid substrate. Several other types of lipase activities exist in nature, such as phospholipases and sphingomyelinases. Lipases are generally animal sourced, but can also be sourced microbial.

Lipases are involved in diverse biological processes which range from routine metabolism of dietary triglycerides to cell signaling and inflammation. Thus, some lipase activities are confined to specific compartments within cells while others work in extracellular spaces. Pancreatic lipases are secreted into

extracellular spaces where they serve to process dietary lipids into more simple forms that can be more easily absorbed and transported throughout the body. Fungi and bacteria may secrete lipases to facilitate nutrient absorption from the external medium. Certain wasp and bee venoms contain phospholipases that enhance the effects of injury and inflammation delivered by a sting. *Malasseziaglobosa*, a fungus that is thought to be the cause of human dandruff, uses lipase to break down sebum into oleic acid and increase skin cell production, causing dandruff. As biological membranes are integral to living cells and are largely composed of phospholipids, lipases play important roles in cell biology.

Lipases 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. Biotechnological advances has brought recombinant lipase enzymes to market for use in applications such as baking, laundry detergents and even as biocatalysts in alternative energy strategies to convert vegetable oil into fuel. High enzyme activity lipase can replace traditional catalyst in processing biodiesel, as this enzyme replaces chemicals in a process which is otherwise highly energy intensive, and can be more environmentally friendly and safe. Industrial application of lipases requires process intensification for continuous processing using tools like continuous flow microreactors at small scale.

There must be three factors to detect a lipase-positive bacterium by culturing it. These factors include (i) growth of the organism, (ii) production of lipase by that organism under suitable growth conditions and (iii) the presence of a sensitive method to detect lipase activity. Growth conditions affect the synthesis of lipase by microorganisms. Carbon and nitrogen sources, the presence of activators and inhibitors, incubation temperature, pH, inoculum amount and oxygen tension can

influence lipase production. The carbon source has been reported as the major factor that affects lipase expression, since lipases are inducible enzymes. The production of these enzymes depends on the presence of a lipid, such as olive oil or any other inducer, such as triacylglycerols, fatty acids and tweens (Muthumari*et al.,* 2017).

Considering the ever increasing demand for the better lipases in the industry and the need to understand the mechanisms of lipase actions and the scope to research for novel sources of lipase the present study has been carried out to isolate and characterize the novel lipase producing bacteria from soil samples. Soil is a rich source of many types of microbial strains which can provide a particular group of microbial strains necessary for the degradation of different contaminants thrown in to the soil. Hence the soil samples can be used to isolate the novel strains that may be used as a part of the microbial pool for the production of lipase at research labs and industries (Bhavani*et al.,* 2012).

REVIEW OF LITERATURE

Lipases (triacylglycerol lipases EC 3.1.1.3) are enzymes that catalyze the degradation of fats and oils and convert them into fatty acids and glycerol. Triacylglycerols are the main substrates for lipases. Lipases are produced by substrates such as natural oils, synthetic triglycerides and esters of fatty acids. Bacterial lipases are used extensively in food and dairy industry for the hydrolysis of milk fat, cheese ripening, flavour enhancement and lipolysis of butter fat and cream. Lipases are used in detergent industry as additives in washing powder, in textile industry to increase the absorbency of the fabrics also for synthesis of biodegradable polymers and for different trans-esterification reactions.

Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. Most of the bacterial lipases reported so far are constitutive and are non-specific in their substrate specificity and a few bacterial lipases are thermostable. Among bacteria, *Achromobacter sp., Alcaligenes sp., Arthrobacter sp., Pseudomonas sp., Staphylococcus, Serratia sp.* and*Chromobacterium* spp. have been exploited for the production of lipases (Jaiswal*et al*., 2017)

The major factor for the expression of lipase activity has always been carbon, since lipases are inducible enzymes and are thus generally produced in the presence of a lipid source such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, tweens, bile salts and glycerol. However, their production is significantly influenced by other carbon sources such as sugars, polysaccharides, whey and other complex sources. Among the different carbon sources used, olive oil was found to be the most suitable source. (Mobarak*et al*., 2011).

In a study, soil sample was collected from oil mills, garage and petrol bunks for screening of lipase producing microorganism using tributyrin agar plate method. The isolated microorganisms were screened using qualitative assay. The isolated bacteria were grown on three different substrates. Among which *Pongamiapinnata* showed maximum enzyme activity (0.4675 U/ml) as compared to *Jatrophacurcus* (Asmita and Naik, 2016).

A measure of the significance of the lipase gene family is the importance of its members. The lipase gene family members are involved in a wide array of metabolic pathways, ranging from lipid digestion, absorption, fatty acid uptake, lipoprotein transformation, and inflammation. While much has been learned about the lipases, it is also clear that the complexity of this enzyme class extends far beyond our current understanding. There remains considerable uncertainty about basic processes like the mechanism of lipolysis and the numerous nuances of enzyme function that have yet to be solved. Due to the central importance of lipase function in lipid metabolism and transport, and its implication in serious diseases of the Western world such as obesity, diabetes, and atherosclerosis, it is imperative to know how lipases normally work. The lipase gene family has given us the opportunity to glimpse into those inner workings, and our view will continue to expand with the advent of new structural information (Howard and Michael, 2002).

Immobilization of the lipase improved its thermal stability in comparison to the free form and could allow its utilization in oil hydrolysis. (Bárbara *et al*., 2014).

METHODOLOGY

SAMPLE COLLECTION

Soil samples were collected from three different locations of Ernakulam district. The soil samples were collected using a spatula from 2-3 cm depth, where most microbial activities will take place and were transferred into a sterile plastic bag. Care was taken to make sure that it should contain the traces of organic matter and should contain moisture content to yield good growth of bacteria. These samples were taken to the lab and stored in refrigerator.

SERIAL DILUTION

Collected soil samples were mixed in 100ml of sterile distilled water in a conical flask. Now samples in conical flask has 10^{-2} dilution factor. Autoclaved 6 test tubes were taken containing 9ml each of sterile distilled water and they are numbered as 1, 2, 3, 4, 5& 6. Using micropipette 1ml of soil sample solution is taken from conical flask containing 10^{-2} as dilution factor. Now the test tube number 1 dilution ratio is marked as 10^{-3}. Again 1ml of soil sample solution from the test tube number 1 is transferred to test tube number 2 using micropipette and the dilution ratio is termed as 10^{-4}. Similarly remaining 4 test tubes containing 9ml distilled water were diluted and ratio marked as 10^{-5}, 10^{-6}, 10^{-7} & 10^{-8}.

POUR PLATE TECHNIQUE

Nutrient agar is used as a general purpose medium for the growth of a wide variety of microorganisms. It consists of peptone, nutrient broth and agar. This relatively simple formulation provides the nutrients necessary for the replication of a large number of microorganisms.1.33 g of nutrient broth and 2 g of agar is taken in a conical flask and is dissolved in 100 ml distilled water. The contents are mixed properly and sterilized by autoclaving at121°C for 15 min. Sixpetriplates were autoclaved and labelled as $10^{-3,}$ 10^{-4}, $10^{-5,}$ 10^{-6}, 10^{-7}, & 10^{-8}. Add 1ml each of diluted soil sample from appropriate tubes to the respective petriplates. Collect the bottle of autoclaved agar medium inside Laminar Airflow Chamber. Make sure that the nutrient broth has pinna bearable temperature. Hold the bottle in the right hand; remove the cap with the little finger of the left hand. Flame the neck of the bottle. Lift the lid of the Petri dish slightly with the left hand and pour the sterile molten agar into the Petri dish and replace the lid. Flame the neck of the bottle and replace the cap. Gently rotate the dish to mix the diluted soil sample and the medium thoroughly and to ensure that the medium covers the plate evenly. Do not slip the agar over the edge of the petri dish. Allow the agar to completely gel without disturbing it, it will take approximately 10 minutes. Seal and incubate the plate in an inverted position at 37°C for 24-48 hours. Observe the petriplates for the presence of microbial colonies after the incubation time.

QUADRANT STREAKING

The transfer loop was sterilized before taking the bacterial culture. To streak a culture from a pour plate culture, metal loops were first sterilized by flaming the wire loop held in the light blue area of the Bunsen burner until it is red hot. Once sterile, the loop is allowed to cool it or blow on it. The culture plate was opened and the sample was collected using sterile loop. The lid of the culture plate was opened just sufficiently enough to take the culture. The mouth of the plate was exposed to heat. The loop was inserted into the plate and a loop full of culture was taken. The lid of the plate was replaced. The agar plate was taken and the lid opened just sufficiently enough to streak the plate with inoculation loop. A loop full of the culture was placed on the agar surface. The loop was flamed and cooled by touching an unused part of the agar surface close to the periphery of the plate, and then drag it rapidly several times. The loop was removed and the petri dish was closed. The loop was reflamed and cooled, and the petri dish was turned 90 degree. The loop was then touched to a corner of the culture and dragged it several times hitting the original streak a few times. This was repeated once more. The plate was incubated in an inverted position in an incubator for 18-24 hrs.

PREPARATION OF NUTRIENT AGAR SLANT

100ml distilled water was taken in a conical flask. The required amount of nutrient broth and agar was added into it. The conical flask was then boiled taking care not to overflow while boiling. The heat was turned off after the agar was boiled. The test tubes were placed without the caps on a test tube rack. The test tube was filled with 5ml of the molten agar from

the conical flask. The cotton plugs were then replaced. All the test tubes were sterilized. After sterilization, when the agar was still hot, the test tubes were tilted on a solid surface making sure not to touch the cotton plugs. The medium was allowed to cool and was solidified at this angle, which increases the surface area of the agar. The caps of the test tubes were tightened after the agar was cooled. The slants were ready to use once the agar had solidified. They can be stored at room temperature or in the refrigerator for future use. The slants were inoculated by transferring cells with an inoculating loop from a single-colony microorganism on a plate to the slant. The loops were then moved across the surface of the slant and the tubes were capped. The slants were incubated until there were evidence of growth. The tubes were kept in refrigerator.

TRIBUTYRIN AGAR (TBA) PLATE ASSAY (Asmita and Naik., 2016)

0.8 g of nutrient broth and 1.5 g of agar is taken in a conical flask and is dissolved in 100 ml distilled water. To it add 1 ml of Tributyrin. Mix and heat for boiling to dissolve the medium completely. Sterilize by autoclaving at 121°C (15lbs pressure) for 15 minutes. The whole process of pouring the molten tributyrin agar medium is done inside a LAF. Collect the bottle of autoclaved medium. Hold the bottle in the right hand; remove the cap with the little finger of the left hand. Flame the neck of the bottle. Lift the lid of the Petri dish slightly with the left hand and pour the sterile molten medium into the Petri dish and replace the lid. Flame the neck of the bottle and replace the cap. Gently rotate the dish so as to maintain uniform turbidity and to ensure that the medium covers the plate evenly. Allow the agar to completely gel without disturbing it, it will take approximately 10

minutes. Inoculate the soil microbes isolated by patching on the surface of the agar medium with sterile loop. Seal and incubate the plate in an inverted position at 37°C for 24-48 hours. Following incubation, the plates were observed for the zone of clearance around colonies, indicative of lipase activity.

RESULT AND DISCUSSION

During the study 3 soils samples were collected from different places of Ernakulam for collecting lipase producing bacteria. The first sample was collected from college campus and named as ERJ. The second sample was collected from Thripunithura and named as ERK and third was collected from Mulanthuruthy and named as ERN (Table 1).

Table 1 showing different samples isolated

SL NO.	**LOCATION**	**SAMPLE NAME**
1	St.Teresa's College	ERJ
2	Thripunithura	ERK
3	Mulanthuruthy	ERN

Serial dilution of the 3 samples was made for the isolation of bacteria. 30 morphologically distinct bacteria have been isolated from the studied sample. The 30 bacteria from first soil sample were named from ERJ-1 to ERJ-30. The bacteria from second samples were named from ERK-1 to ERK-30 and the third from ERN-1 to ERN-30. The 90 isolated bacteria were taken and streaked to get pure cultures. It was then transferred to nutrient agar slants (Figure 1) and stored in refrigerator.

Figure1showing nutrient agar slant containing bacterial isolate

The 90 isolated bacteria were transferred toTributyrin agar plate and incubated for 24 hours. Clear patches were produced around bacteria that produced lipase enzyme.Out of 90 bacteria 31 showed lipolytic activity (Figure 2).

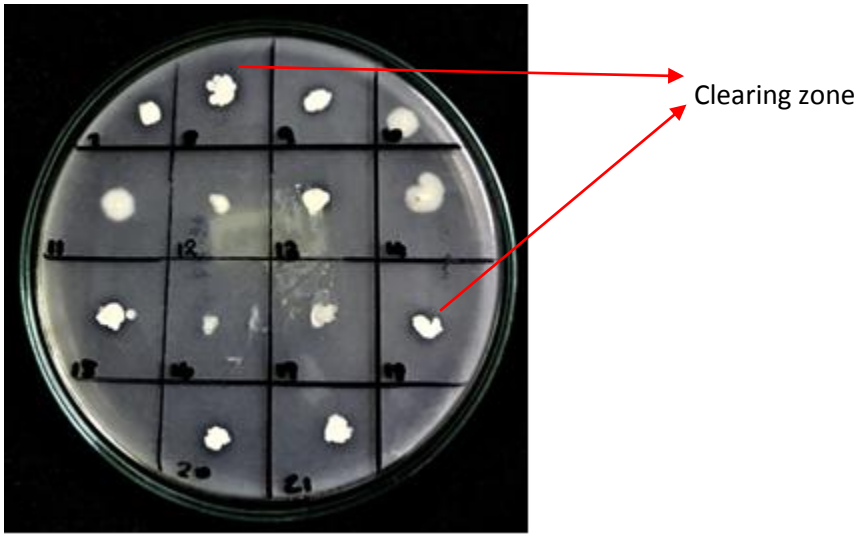
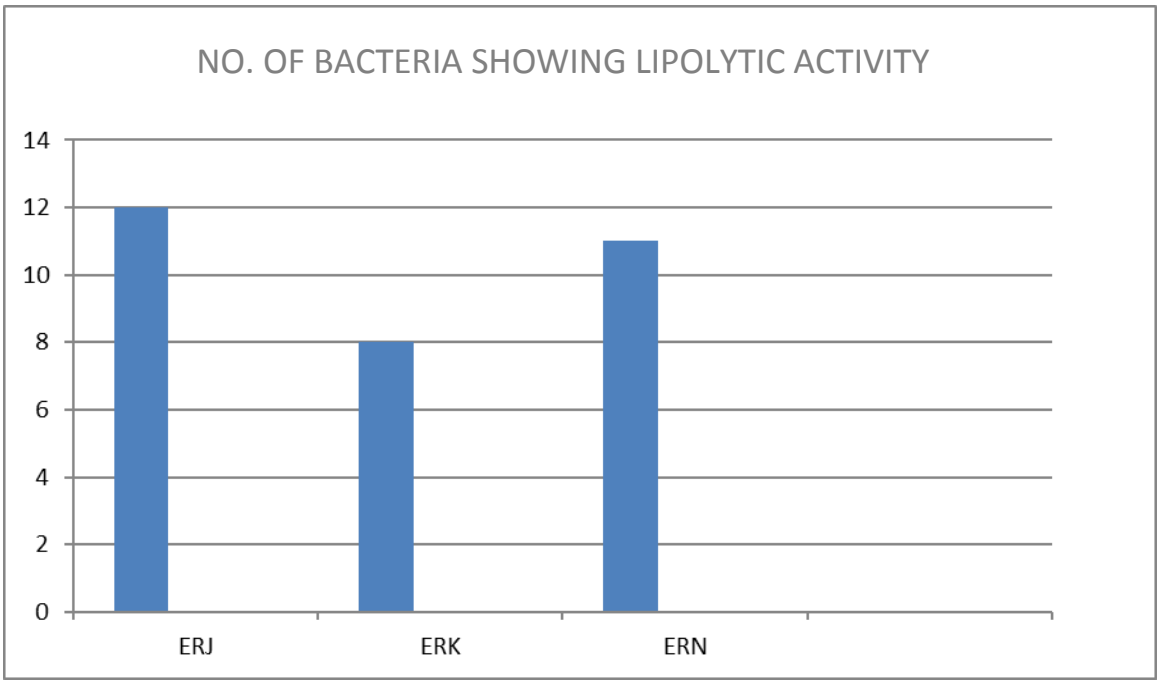


Figure 2showing clearing zone around cultures indicating lipase activity

Table 2 showing isolates with lipase activity

Sl No.	Bacterial Isolate	Lipolytic Activity	Sl No.	Bacterial Isolate	Lipolytic Activity	Sl No.	Bacterial Isolate	Lipolytic Activity
1	ERJ-1	-	31	ERK-1	-	61	ERN-1	-
2	ERJ-2	-	32	ERK-2	-	62	ERN-2	+
3	ERJ-3	-	33	ERK-3	-	63	ERN-3	-
4	ERJ-4	+	34	ERK-4	-	64	ERN-4	-
5	ERJ-5	-	35	ERK-5	-	65	ERN-5	+
6	ERJ-6	-	36	ERK-6	-	66	ERN-6	-
7	ERJ-7	+	37	ERK-7	-	67	ERN-7	+
8	ERJ-8	+	38	ERK-8	-	68	ERN-8	-
9	ERJ-9	+	39	ERK-9	-	69	ERN-9	-
10	ERJ-10	-	40	ERK-10	+	70	ERN-10	+
11	ERJ-11	-	41	ERK-11	-	71	ERN-11	-
12	ERJ-12	-	42	ERK-12	-	72	ERN-12	+
13	ERJ-13	+	43	ERK-13	+	73	ERN-13	-
14	ERJ-14	-	44	ERK-14	-	74	ERN-14	-
15	ERJ-15	+	45	ERK-15	+	75	ERN-15	+
16	ERJ-16	-	46	ERK-16	-	76	ERN-16	+
17	ERJ-17	-	47	ERK-17	-	77	ERN-17	-
18	ERJ-18	+	48	ERK-18	+	78	ERN-18	-
19	ERJ-19	-	49	ERK-19	-	79	ERN-19	-
20	ERJ-20	+	50	ERK-20	-	80	ERN-20	+
21	ERJ-21	+	51	ERK-21	+	81	ERN-21	-
22	ERJ-22	-	52	ERK-22	-	82	ERN-22	-
23	ERJ-23	-	53	ERK-23	+	83	ERN-23	+
24	ERJ-24	+	54	ERK-24	+	84	ERN-24	-
25	ERJ-25	-	55	ERK-25	-	85	ERN-25	-
26	ERJ-26	-	56	ERK-26	-	86	ERN-26	+
27	ERJ-27	+	57	ERK-27	-	87	ERN-27	-
28	ERJ-28	-	58	ERK-28	+	88	ERN-28	-
29	ERJ-29	-	59	ERK-29	-	89	ERN-29	+
30	ERJ-30	+	60	ERK-30	-	90	ERN-30	-

Graph 1 showing lipase producers of different samples



Sample ERJ had highest number of lipase producing microorganisms

This result was promising and gives the profitable method for future on this medium to scale up for protease enzyme production. The industrial importance of lipases is much considerable. Different new areas like single cell protein, cosmetics, paper pulping, lubricants, and pharmaceuticals are there where this enzyme have been employed functionally. As microorganisms are the cheapest source of enzyme, bacterial production of lipase has gained much importance than any other sources. Among various environmental ecotypes, soil or water contaminated with various kinds of oil are prime sources of lipase producing microorganisms (Manali*et al*., 2016).

In a study done by RachanaChoudhary (2017) mainly based on isolation and screening of lipase producing bacteria from oil mill effluent, thirteen bacterial colonies were isolated from the sample. They were screened for their potential lipase producing ability using Tween 20 as a substrate. Thirteen bacteria were then identified. The identified bacteria were *Vibrio parahemolyticus, Aeromonashydrophila, Micrococcus lilac, Branhmella sp., Vibrio vulvificus, Pseudomonas aeruginosa, Acinetobactercalcoaceticus, Neisseria pharynges, Lactobacillus brevis, Providensiapseudomallei, Lactobacillus viridescens, Micrococcus luteus 4* and*Obesumbacteriumproteusbiogrop*.

In a study conducted by MobarakQamsari and others (2011) lipase activity was determined spectrophotometrically at 30°C using p-nitrophenolpalmitate (pNPP) as substrate. In the work done by RachanaChoudhary (2017) lipolytic activity of isolates was tested qualitatively by plate test method of sorbitanmonolaurate, Tween 20 was used as lipid substrate. In study done by Jaiswal and others, (2017) qualitative analysis of lipase producing micro-organism was carried out by single line streaking of pure culture inoculates obtained through zigzag streaking on mineral lipid emulsion agar media and egg yolk agar media.

Bacterial strains showed high yield of lipase production at pH 6 ± 0.5. It has been reported that maximum lipase production was achieved at pH at 6–7 (Larbidaouadi*et al*., 2015).

Optimum temperature plays a vital role for the production of enzyme in shake flask method. Higher biomass concentration of lipase was observed at temperature 37°C. Researchers reported that the slight increase in temperature up to 38°C enhance the lipase production (Zhang and Hebin, 2005; Yuan *et al*., 2016; Gaur *et al*., 2008).

CONCLUSION

Soil samples were collected from different places of Ernakulam district and used for isolation and screening of lipase producing bacteria. From 90 isolated colonies 31 isolates showed zone of clearance on tributyrin agar plate which indicated their ability to produce lipase enzyme. Hence the strains were identified as lipase producers.

Lipases have many potential applications in various industries and are selected for each application based on its substrate specificity, position and stereo specificity as well as temperature and pH stability. They are mostly used in the detergent, food, pharmaceutical industries.

Lipases serve important roles in human practices. However, lipases are also being exploited as cheap and versatile catalysts to degrade lipids in more modern applications. For instance, a biotechnology company has brought recombinant lipase enzymes to market for use in applications such as baking, laundry detergents and even as biocatalysts in alternative energy strategies to convert vegetable oil into fuel. High enzyme activity lipase can replace traditional catalyst in processing biodiesel, as this enzyme replaces chemicals in a process which is otherwise highly energy intensive, and can be more environmentally friendly and safe. Industrial application of lipases requires process intensification for continuous processing using tools like continuous flow micro reactors at small scale.

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