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Studies on Lipolytic Bacteria in Intestinal Tract of Marine Fishes

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ABSTRACT

The aim of my present study was Isolation and Identification of lipase producing bacteria species from various fish samples from different fish market in cuddalore, pazhaiyar. From 9 fish samples 35 isolates were observed and identified. Lipase producing Bacteria isolated by using Tributyrin Agar medium. The isolated colonies were streaked on the solidified Agar medium and incubate the plates at 37°C for 48 hours. The selection of working strain was made on the basis of their lipase activity determined by the zone formation around the culture. The isolated colonies were studied for Hicrome Bacillus Agar and different *Bacillus* Sp were identified. *Pseudomonas* sp were identified by cetrimide agar and *S. aureus* by Mannitol salt agar. Microbial lipases are high in demand due to their specificity of reaction, stereo specificity and less energy consumption than conventional method (Saxena *et.al.,* 1999). These microorganisms have been found in diverse habitat and especially oil processing industries. These enzymes are widely used in numerous biotechnological process such as cosmetic, food, leather, detergent and pharmaceutical industries (Sztajer *et.al.,* 1998). Microbial lipases production has increased for the past one decade, because of its potential application in industries.

Keywords: lipase, Hicrome Bacillus agar, *Bacillus* sps.

INTRODUCTION

The bacterial flora of the gastrointestinal tract in general represents a very important and diversified enzymatic potential, and its seems logical to think that the enzymatic mass lodged in the digestive tract might interfere in a considerable way with a major part of the metabolism of the host animal (Bairagi *et al.,* 2002). Some fish species acquire many of their intestinal enzymes from the Microflora in habiting their guts (Hamid *et al*., 1979; Cahli, 1990; Amit Kumar Sinha *et al* ., 2007; Ringo *et al.,* (2010). The bacterial species isolated from gut for the qualitative detection of different enzymatic activities. Bairagi *et al.,* (2002)

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Lipolytic enzymes, are ubiquitously found in mammalian systems. Lipases can be isolated from various living sources such as plants, animals, bacteria and fungi. The gut microbiota is enormous of digestive enzymes, especially in both marine and fresh water fishes. The endogenous digestive enzymes, which are secreted to the lumen of the alimentary canal, originate from the oesophageal, gastric, pyloric caeca and intestinal mucosa and from the pancreas (De Silva & Anderson 1995).The presence of endogenous digestive enzymes in fish has been reported in numerous studies. Bacterial lipases are widely produced by Bacillus sps and has predominant synthesizers in different extracellular enzymes whilst remarkable biochemical properties has significantly stabilized for industrial usage. Lipases enzyme catalyzes the hydrolysis of lipid. Among the various lipases, bacterial lipases are the most predominant than animal and fungal lipases.

MATERIALS AND METHODS

Collection of Samples: Fresh marine fishes were collected from fish markets of cuddalore areas such OT fish market (Old town cuddalore) and Pazhaiyar fish market and transported to the laboratory in a sterile polyethene bags and immediately processed in the laboratory.

Isolation and identification: Fresh marine fish were collected washed with water surface sterilized with alcohol and again washed with sterile saline. The intestine of the fish is taken by dissection process. The intestine is homogenized by using rotar and pestle. The homogenized sample is used for serial dilution technique. The lipid producing bacteria was isolated by using Tributyrin agar and then further it was screened and its phenotypic character was studied by using Hicrome Bacillus agar for Identifying *Bacillus* species, Cetrimide agar for *Pseudomonas* species and *Staphylococcus aureus* in MSA agar The isolates was identified based on Morphological, cultural & biochemical characters and further confirmed by molecular level taxonomy.

SCREENING OF LIPASE UTILIZERS

Sterile production medium called tributyrin agar medium was inoculated with the organism. After incubation period, the clear zone was formed . Formation of clear zone around the organism was considered as lipase utilizers.

OPTIMIZATION OF LIPASE

Effect of pH on Lipase Production

The isolates such as OFAA14 (*Pseudomonas sps*),SFAA27 (*Staphylococcus aureus*), PFAA33 (*Bacillus sps*) were inoculated in the tributyrin broth at different pH ranges from pH 4 to 9 and incubated at 37°C for 48 hours. The lipase production was observed in spectrophotometer at OD of 560nm

Effect of Various Lipid Source on Lipase Production

The isolates such as OFAA14*(Pseudomonas sps),*SF AA27*(Staphylococcusaureus),* PFAA33*(Bacillus sps)* were inoculated in the tributyrin broth supplemented with various lipid source such as castor oil, gingely oil, olive oil, coconut oil, palm oil, groundnut oil and incubated at 37 ˚C for 48 hours. The lipase activity was observed in spectrophotometer at OD of 560nm

Effect of Various Nitrogen Source on Lipase Production

The isolates such as OFAA14*(Pseudomonas sps),*SFA A27*(Staphylococcusaureus),* PFAA33*(Bacillus sps),* were inoculated in the tributyrin broth supplemented with various nitrogen source such as ammonium chl oride,peptone,tryptone,40%urea,beef extract, yeast extract and incubated at 37°C for 48 hours. The lipase activity was observed in spectrophotometer at OD of 560nm

16srRNA typing

The following cultures OFAA 14, SFAA 27, PFAA33 were subjected for genome sequencing and the sequencing was done in PACE Microbial technology. Puducherry.

16srRNA isolation, amplification, sequencing and treeing programme protocol

1. Preparation of template DNA It is important to use a pure cultured bacterium for identification. Colonies are picked up with a sterilized toothpick, and suspended in 0.5ml of sterilizes saline in a

1.5ml centrifuge tube. Centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet is suspended in 0.5ml of InstaGene Matrix (Bio-Rad, USA). Incubated 56°C for 30 min and then heated 100°C for 10 min. After heating, supernatant can be for PCR.

2. 2. PCR Add 1µl of template DNA in 20µl of PCR reaction solution. Use 518 F/800 R primers for bacteria, and then perform 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. DNA fragment are amplified about 1,400bp in the case of bacteria. Include a positive control (*E.coli* genomic DNA) and a negative control in the PCR.

518 F	5'CCAGCAGCCGCGGTAATACG 3'
800 R	5'TACCAGGGTATCTAATCC 3'

Purification

Products remove unincorporated PCR primers and dNTPs from PCR product by using Montage PCR clean up kit (Millipore).

Sequencing

The purified PCR products of approximately 1,400bp were sequenced by using the primers (785 F 5' GGA TTA GAT ACC CTG GTA 3' and 907 R 5' CCG TCA ATT CCT TTR AGT TT 3'). Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing product were resolved on an Applied BioSystems were model 3730XL automated DNA sequencing system (Applied BioSystems , USA).

Phylogenetic Tree Construction

The culture sequence obtained were subjected to BLAST analysis, the phylogenetically similar type strains sequences and other phylogenetic related sequence were selected from the Gen Bank and they were subjected to multiple sequence alignment and then align sequences were trimmed to similar length in nucleotides and were subjected to phylogenetic tree (neighbour joining) construction using MEGA 6. In the tree the number at the nodes indicate levels of the bootstrap support [high bootstrap values (close to 100%) meaning uniform support] based on a neighbour joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated . Bar 0.005 substitutions per site.

RESULTS

A total number of 9 fresh marine fish samples were collected in OT from Cuddalore and processed in the lab.

TRIBUTYRIN AGAR BASE MEDIUM

The lipase producing bacterial strains were isolated from fish sample and different fish using Tributyrin agar base medium observed for clear zone

Table 1: Various Fish Samples & Total Number Isolates

S.No	Sample	Sampling Area	Number of Isolates	Total Number of Isolates
1	(*Asian arowana*)	OT-Cuddalore	8	35
2	*(Labio rohita)*	OT -Cuddalore	10	
3	(*Sardinella brachysoma*)	OT -Cuddalore	2	
4	(*loligo duvaucelli*)	Pazhaiyar	1	
5	(*Asian arowana)*	Cuddalore	5	
6	(*Lutjanus gibbus*)	Cuddalore	2	
7	(*Chromolaena odorata*)	Cuddalore	2	
8	(*Paenus monodon*)	Pazhaiyar	3	
9	(*Catla catla*)	Cuddalore	2	

Table 2: Morphological & Biochemical Characteristic Results

S.No	Isolate No	Gram Staining	Spore taining	INDOLE	MR	VP	CITRATE	UREASE	TSI	LIA	CATALASE	OXIDASE	Mannitol Motility Test
1	KFAA01	Gram negative rod	Non spore forming	-	-	-	+	-	K/K	K/K	+	+	Non motile
2	KFAA02	Gram negative rod	Non spore forming	-	+	-	+	+	K/K	K/K	+	+	Non motile
3	KFAA03	Gram negative rod	Non spore forming	-	+	-	+	+	K/K	K/K	+	+	Non motile
4	KFAA04	Gram negative rod	Non spore forming	-	-	-	+	+	K/K	K/K	+	+	Non motile
5	KFAA05	Gram negative rod	Non spore forming	-	-	-	+	-	K/K	K/K	+	+	Non motile
6	KFAA06	Gram negative rod	Non spore forming	-	+	-	+	-	K/K	K/K	+	+	Non motile
7	KFAA07	Gram negative rod	Non spore forming	-	-	-	+	-	K/K	K/K	+	+	Non motile
8	KFAA08	Gram negative rod	Non spore forming	-	-	-	+	-	K/K	K/K	+	+	Non motile
9	OFAA09	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
10	OFAA10	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
11	OFAA11	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
12	OFAA12	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
13	OFAA13	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
14	OFAA14	Gram negative rod	Non spore forming	-	+	-	+	+	K/A	K/K	+	+	Motile

S.No	Isolate No	Gram Staining	Spore taining	INDOLE	MR	VP	CITRATE	UREASE	TSI	LIA	CATALASE	OXIDASE	Mannitol Motility Test
15	OFAA15	Gram negative rod	Non spore forming	-	+	-	+	+	K/A	K/K	+	+	Motile
16	OFAA16	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/A	+	+	Motile
17	OFAA17	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
18	OFAA18	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
19	KFAA19	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
20	KFAA20	Gram negative rod	Non spore forming	-	+	-	+	-	K/A	K/K	+	+	Motile
21	SFAA21	Gram negative rod	Non spore forming	-	+	-	-	-	K/A	K/K	+	+	Motile
22	KFAA22	Gram negative rod	Non spore forming	-	+	-	-	-	K/A	K/K	+	+	Non motile
23	KFAA23	Gram positive rod	Spore forming	-	+	-	-	-	K/A	K/K	+	+	Non motile
24	KFAA24	Gram negative rod	Non spore forming	-	+	-	-	-	A/A	K/A	+	+	Non motile
25	KFAA25	Gram positive cocci	Non spore forming	-	+	-	-	-	K/K	K/A	+	+	Non motile
26	KFAA26	Gram positive rod	Spore forming	-	+	-	+	-	A/A	A/A	+	+	Non motile
27	**SFAA27**	Gram positive cocci	Non spore forming	-	+	-	-	-	A/K	K/K	+	+	Non motile
28	SFAA28	Gram positive cocci	Non spore forming	-	+	-	+	-	K/K	K/K	+	+	Non motile

S.No	Isolate No	Gram Staining	Spore taining	INDOLE	MR	VP	CITRATE	UREASE	TSI	LIA	CATALASE	OXIDASE	Mannitol Motility Test
29	KFAA29	Gram negative rod	Non spore forming	-	+	-	+	-	K/K	K/K	+	+	Motile
30	KFAA30	Gram negative rod	Non spore forming	-	+	-	+	-	K/K	K/K	+	+	Motile
31	PFAA31	Gram positive rod	Spore forming	-	+	-	-	-	A/A	K/K	+	+	Motile
32	PFAA32	Gram positive rod	Spore forming	-	+	-	-	-	A/A	K/K	+	+	Motile
33	**PFAA33**	Gram positive rod	Spore forming	-	+	-	-	-	A/A	K/K	+	+	Motile
34	KFCC34	Gram positive rod	Spore forming	-	+	-	-	-	A/A	K/K	+	+	Motile
35	KFCC35	Gram positive rod	Spore forming	-	+	-		-	A/A	K/K	+	+	Motile

TRIBUTYRIN AGAR BASE

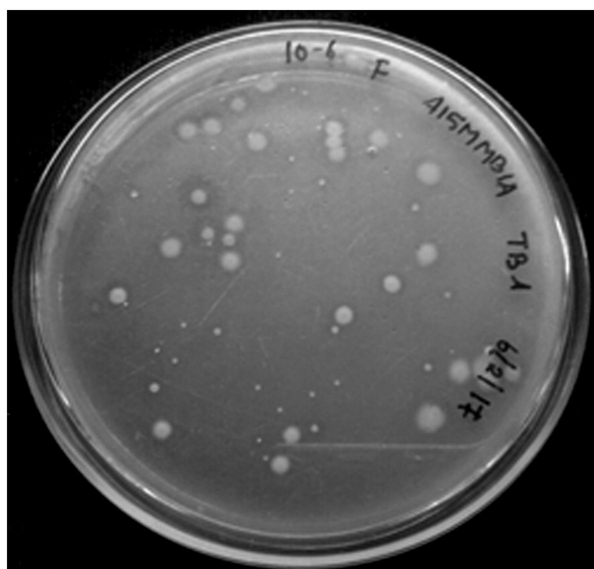


Plate 1: Shows the Lipase Producing Bacteria in Clear Zone Formation

HICROME BACILLUS AGAR

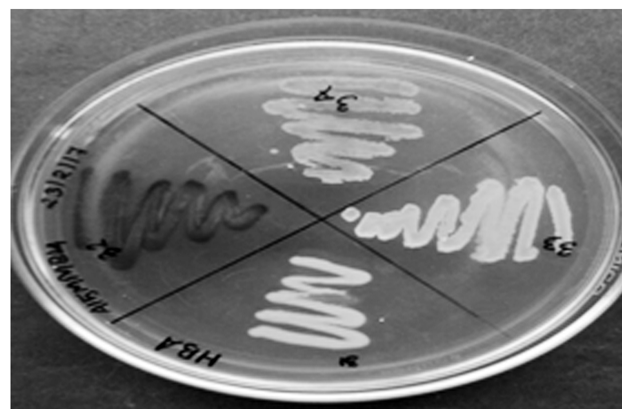


Plate 2: Shows the Growth of Different *Bacillus* Species Forming Colourful Colonies

CETRIMIDE AGAR BASE

Plate 3: *Pseuodomonas* Species Shows Green Colour Formation in Cetrimide Agar

MANNITOL SALT AGAR

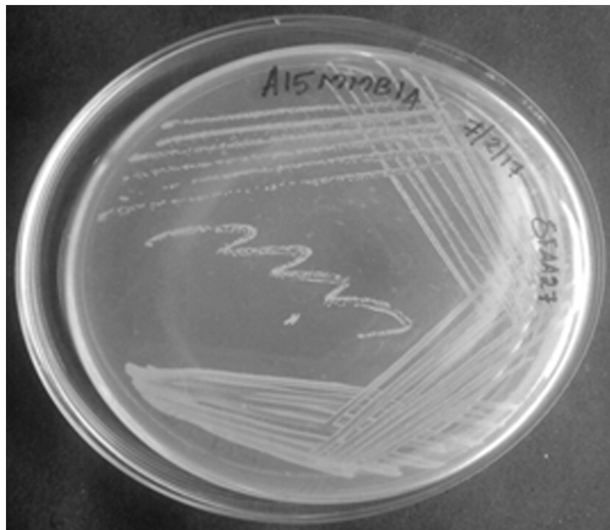


Plate 4: Shows the Yellow Colour Colonies of *Staphylococcus Aureus* In Mannitol Salt agar

ENZYME IMMOBILIZATION

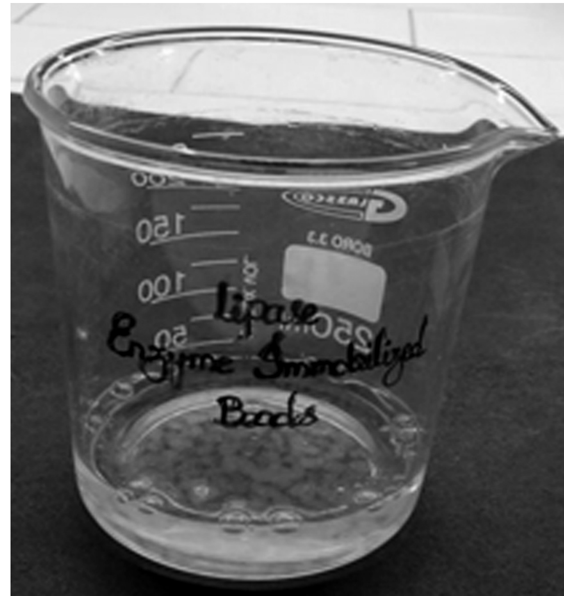


Plate 5: Shows the immobilized Lipase Beads of OFAA14 – Pseudomonas sps

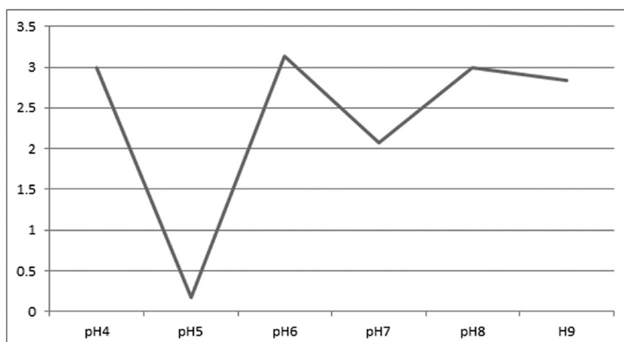
OPTIMIZATION OF LIPASE ENZYME AT VARIOUS PH

The isolates such a OFAA14*(Pseudomonas sps),* SFAA27*(Staphylococcusaureus),* PFAA33 *(Bacillus sps),* were inoculated in tributyrin broth at different pH ranges from pH 4 to 9 and incubated at 37°C for 48 hours. The lipase production was observed in spectrophotometer at OD of 560nm.

Table 3: Effect of various pH on Lipase Production by Isolate OFAA14 – Pseudomonas sps

S.No	TRIBUTYRIN BROTH at Different pH	Od At 560nm
1	4	3.0000
2	5	0.1774
3	6	3.1400
4	7	2.0653
5	8	3.0000
6	9	2.8413

Graph 1: Effect of Various pH on Lipase Production by Isolate OFAA14 – Pseudomonas sps



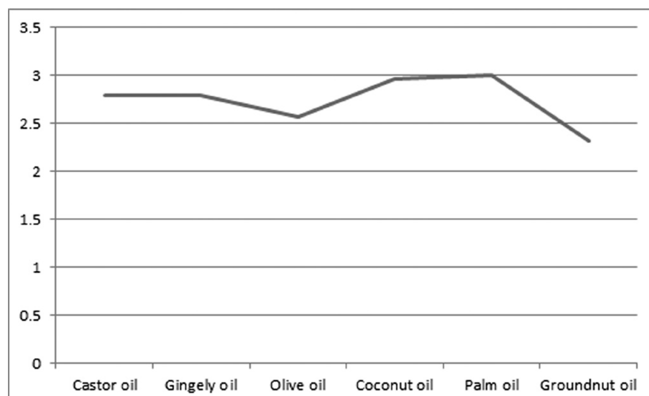
EFFECT OF VARIOUS LIPID SOURCE ON LIPASE PRODUCTION

The isolates such as OFAA14 *(Pseudomonas sps),* SFAA27 *(Staphylococcus aureus),* PFAA33 *(Bacillus sps),* were inoculated in tributyrin broth at various lipid source such as castor oil, gingelly oil, olive oil, coconut oil, palm oil, groundnut oil and incubated in 37°C and 48 hours. The lipase activity was observed in spectrophotometer OD at 560nm.

Table 4: Effect of Various Lipid Source on Lipase Production by Isolate OFAA14 – Pseudomonas sps

S.No	TRIBUTYRIN BROTH With Different Lipid Source	OD at 560nm
1	Castor oil	2.7886
2	Gingely oil	2.7948
3	Olive oil	2.5688
4	Coconut oil	2.9697
5	Palm oil	3.0000
6	Groundnut oil	2.3196

Graph 2: Effect of Various Lipid Source on Lipase Production by Isolate OFAA14 – Pseudomonas sps



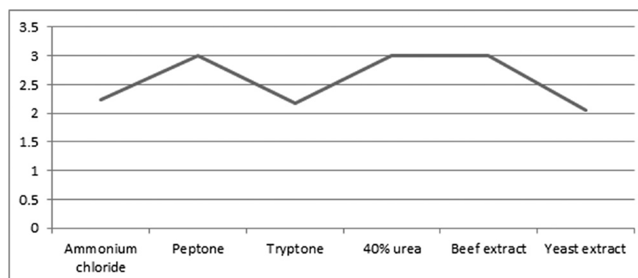
EFFECT OF VARIOUS NITROGEN SOURCE ON LIPASE PRODUCTION

The isolates such as OFAA14 *(Pseudomonas sps)* SFAA27 *(Staphylococcusaureus),* PFAA33 *(Bacillus sps),* were inoculated in tributyrin broth at various nitrogen source such as ammonium chloride, peptone, Tryptone, 40% urea, beef extract, yeast extract and incubated at 37°C and 48 hours. The lipase activity was observed in spectrophotometer OD at 560nm.

Table 5: Effect of Various Nitrogen Source on Lipase Production by Isolate OFAA14--Pseudomonas sps

S.No	TRIBUTYRIN BROTH With Different Nitrogen Source	OD at 560 Nm
1	Ammonium chloride	2.2380
2	Peptone	3.0000
3	Tryptone	2.1764
4	40% urea	3.0000
5	Beef extract	3.0000
6	Yeast extract	2.0564

Graph 3: Effect of Various Nitrogen Source on Lipase Production by Isolate OFAA14 – Pseudomonas sps



RESULTS OF ENZYME OPTIMIZATION

pH: The isolates such as OFAA14 *(Pseudomonas sps),* SFAA27 *(Staphylococcusaureus),* PFAA33 *(Bacillus sps),* on different pH were studied the isolate OFAA14 shows higher production of lipase in pH6(3.1400), the isolate SFAA27 shows higher production of lipase in pH5 (2.5504) and the isolate PFAA33 shows higher production of lipase in pH7 (3.0000), these values determine the highest production of proteases based on their OD value.

Lipid source: The isolates such as OFAA14 *(Pseudomonas sps),* SFAA27 *(Staphylococcus aureus),* PFAA33*(Bacillus sps),* on different lipid source were studied the isolate OFAA14 shows higher production of lipase in palm oil (3.0000), the isolate SFAA27 shows

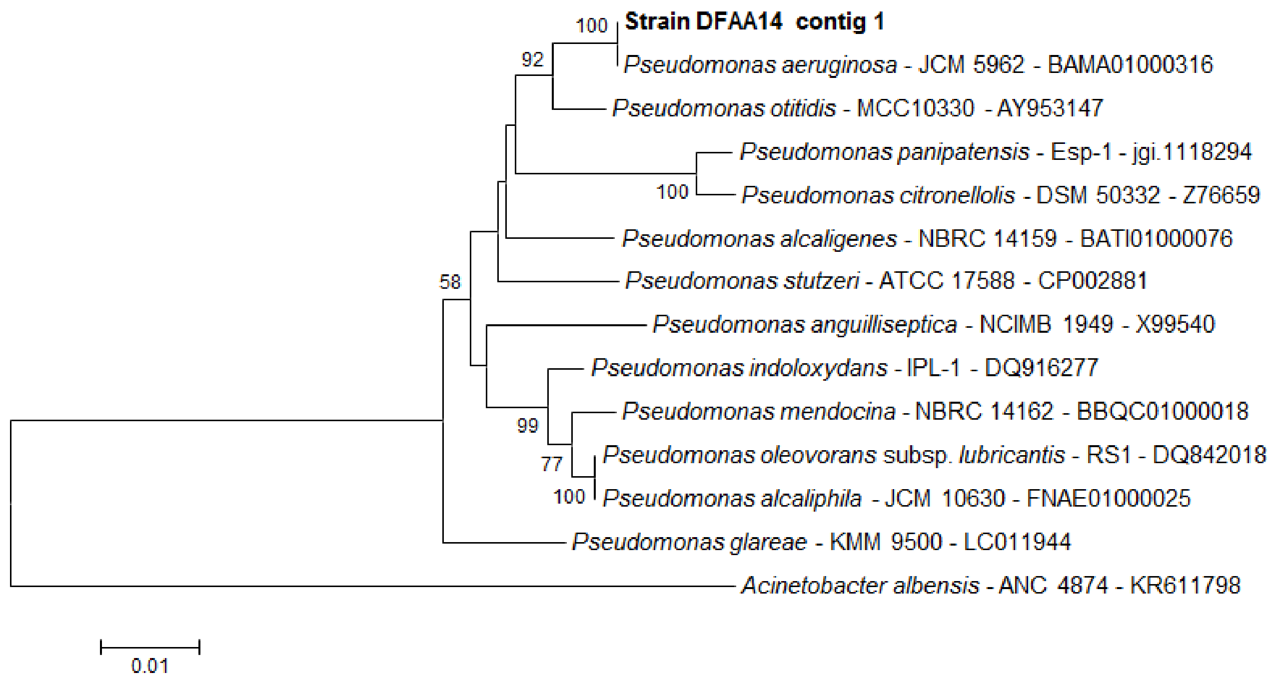
higher production of lipase in coconut oil (2.0627) and the isolate PFA33 shows higher production of lipase in castor oil (2.0903). These values determine the highest production of lipases based on their ODvalue

Nitrogen source: The isolates such as OFAA14 *(Pseudomonassps)*,SFAA27 *(Staphylococcus aureus)*, PFAA33*(Bacillus sps)*, on different nitrogen source were studied the isolate OFAA14 shows higher production of lipase in peptone, beef extract, and 40% urea (3.0000), the isolate SFAA27 shows higher production of lipase in beef extract and yeast extract (3.0000) and the isolate PFAA33 shows higher production of lipase in beef extract(1.9381)these values determine the highest production of lipases based on their ODvalue.

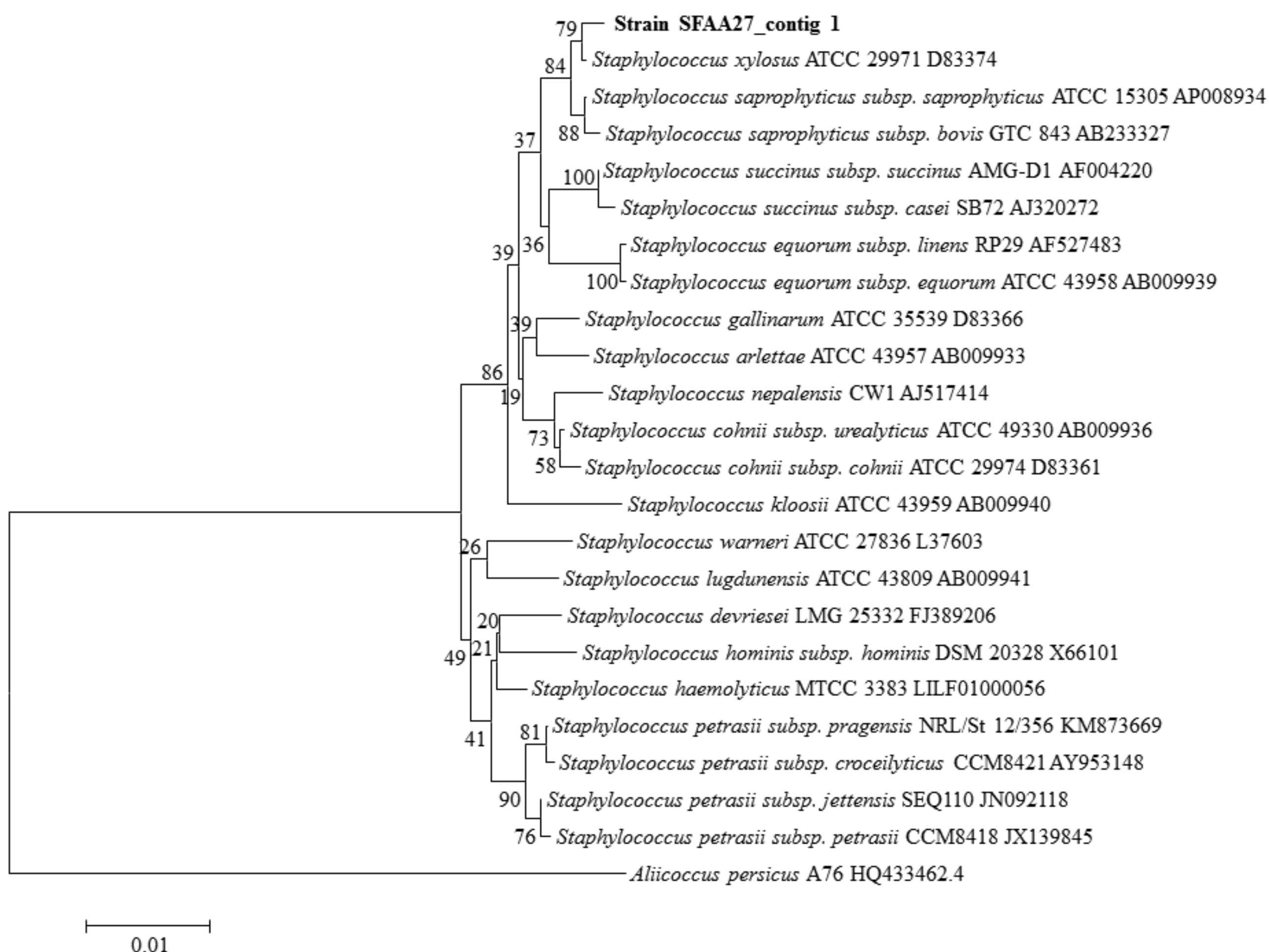
Output of Enzyme optimization: Amongst 39 isolates. The isolate such as OFAA1-*Pseudomonas* sps were found to be increase in lipase activity apart from SFAA27 – *Staphylococcus aureus* and PFAA33-*Bacillus* sps strains .

Results of Genome Sequencing

The following isolates OFAA14, SFAA27, PFAA33 were subjected for genome sequencing and the results were identified by molecular method as, OFAA14-*Pseudomonas* sp, SFAA- *Staphlococcus aureus*, PFAA 33- *Bacillus* sp and the results are as follows. The phylogenetic tree also been constructed for this isolates.



Phylogenetic tree derived from 16sr RNA sequence data of strain OFAA14 and other related species. The OFAA14 was determined by molecular chronometers analysis has Pseudomonas sps



Phylogenetic tree derived from 16sr RNA sequence data of strain SFAA27 and other related species. The SFAA27 was determined by molecular chronometers analysis has Staphylococcus aureus

DISCUSSION

Lipases are wide spread among organisms being produced by bacteria, algae, fungi etc., among these bacterial strains has the high activity lipase production. They are involved in the production of lipase, a lipase source in various cell metabolisms. Lipase producing Bacteria isolated by using Tributyrin Agar medium. Lipase activity were also studied by using different carbon ,nitrogen and lipid sources showed remarkable stability in various sources. The selection of working strain was made on the basis of their lipase activity determined by the zone formation around the culture. Enzyme immobilization technique was carried out using sodium alginate and calcium chloride, with standard protocol, using this standard protocol lipase immobilized beads were produced, these immobilized lipase beads is the important preservation technique for various enzymes and it is considered to be a great industrial importance. Five different species of *Bacillus* were isolated from different fishes such has *Bacillus megaterium, Bacillus pumilus, Bacillus subtilis, Bacillus cereus*, 8 *Pseudomonas aeruginosa* was isolated and screened and two *S. aureus* other 20 isolates are detected as Gram negative rods and, all the isolates are identified as lipase producers. All these lipase producers appeared promising and good source of lipase but it requires a detail characterization of growth and nutrient conditions. The 16srRNA typing results provides a detailed information on lipolytic bacteria which is signified in Phylogenetic tree under different genera. The present study was aimed to isolate and characterize lipase producing bacteria from various fish samples. Five different species of *Bacillus* were isolated such *Bacillus megaterium, Bacillus pumilus, Bacillus subtilis, Bacillus cereus*, 8 *Pseudomonas aeruginosa*

was isolated and screened and two *S. aureus* other 20 isolates are detected as Gram negative rods and, all the isolates are identified as lipase producers. All these lipase producers appeared promising and good source of lipase but it requires a detail characterization of growth and nutrient conditions. The optimization process of lipase enzyme at various pH with different isolates was standard, and with different lipid and nitrogen sources was also reasonable in producing lipase. The genome sequencing results provides a detailed information on lipolytic bacteria which is signified in Phylogenetic tree under different genera. In our studies *Pseudomonas* sp-OFAA14 shows higher amount of lipase activity.

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