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ISOLATION AND CHARACTERIZATION OF LIPASE PRODUCING BACTERIA FROM RESTAURANT WASTE WATER

V. P. Sutar[1] and J. V. Kurhekar[2]*

[1]Department of Microbiology, D. A. B. Naik Arts and Science College Chikhali, Tal- Shirala, Dist- Sangli.

[2]HOD, Department of Microbiology, Dr. Patangrao Kadam Mahavidyalaya, Sangli.

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***Corresponding Author**
J. V. Kurhekar
HOD, Department of Microbiology, Dr. Patangrao Kadam Mahavidyalaya, Sangli.

ABSTRACT

The presence of Fats, Oils and Greases (FOGs) in restaurant waste water is a major problem. It is not only responsible for increased BOD and COD but also causes a number of environmental problems. In this study, five lipolytic bacteria, *Staphylococcus petrasii subsp. jettensis, Staphylococcus epidermidis, Acinetobacter junii, Pseudomonas composti and Pseudomonas aeruginosa* were isolated from a local restaurant waste water. The isolates were identified using morphological, physiological and biochemical tests. The 16 S rRNA analysis was performed for species level identification. The bacterial isolates were checked for their potential to secrete enzyme Lipase in mineral medium supplemented with various vegetable oils, used in restaurants. The maximum production of lipase (more than 6 Units/ml) was observed in presence of soybean, sunflower and cotton seed oil. The ground nut oil and palm oil showed comparatively less lipase production.

KEYWORDS: Restaurant waste water, Lipolytic bacteria, Fats, Oils and Greases (FOGs), Lipase.

INTRODUCTION

Lipids consist of Fats, Oils and Greases (FOGs). The main constituents of FOGs are animal Fats and vegetable Oils. They also comprise of a combination of glycerol and free fatty acids.[1] It is found in waste water collected from food processing industries[2], restaurant[3],[4] and from kitchen.[4] The lipids are amphiphilic molecules made up of hydrophilic heads and hydrophobic tails. The presence of lipids in the waste water creates environmental pollution.

The lipid coats the animal body surface, aquatic plants and forms a thin layer on aquatic surface. It results in reduced oxygen transfer and increased Chemical Oxygen Demand (COD).[4] The lipids cause the blocking of sewer lines which results in clogging, overflow of waste water and unpleasant odors which affects public health.[5] Chemically lipid contains glycerol and fatty acids, joined by ester linkages. Microorganisms are able to utilize the lipids by secreting Lipases. Lipases are hydrolytic enzymes (E.C. 3.1.1.3) that bring about hydrolysis of lipids by cleaving the ester bonds to release fatty acids and glycerol. Therefore, application of microorganisms for treatment and bioremediation purposes, provide a very efficient tool for purifying contaminated effluents and natural waters.[6] The indigenous microorganisms isolated from lipid contaminated environmental sites may be able to utilize the lipids as a source of Carbon and Energy. Such microorganisms may be able to clear the waste lipids more efficiently than the routine physicochemical processes. The present study is targeted at isolating indigenous Lipolytic bacteria from the restaurant waste water to remove the FOGs.

Sample Collection

A total five waste water samples were collected from a local restaurant. Four samples were collected from three different concrete chambers along the restaurant sewer line of kitchen and one from outlet of sewer pipe. The chamber is a very important part of sewer line which is used for cleaning and maintenance. The FOGs coming from kitchen gets stuck on the wall of the chamber and creates an environment suitable for growth of lipolytic bacteria. The samples were collected in sterile bottles and immediately transferred to a laboratory.

Enrichment of lipolytic bacteria

The enrichment of lipolytic bacteria was carried out in Bushnell Hans medium (BH) supplemented with soybean oil, as the sole source of Carbon and Energy. The BH medium was prepared by dissolving $(NH_4)_2SO_4$-2.38g, KH_2PO_4-1.36g, $CaCl_2 \cdot 7H_2O$-10.69 g, $MgSO_4 \cdot 7H_2O$ -0.25g, Na_2HPO4-1.42g and $FeSO_4 \cdot 7H2O$-0.28 mg per liter in distilled water. One ml of each restaurant waste water sample was used to inoculate separate 50 ml of BH medium supplemented with soybean oil and mixed thoroughly. The medium was then incubated on a rotary shaker at 30°C, at a speed of 100 rpm for 7 days. The medium was observed visually, every 24 hrs for growth.

Isolation of lipolytic microbes

Each enriched suspension of lipolytic bacteria was serially diluted (10^{-1} to 10^{-5} dilution) in sterile saline. 0.1 ml from 10^{-3}, 10^{-4} and 10^{-5} dilutions was spread on sterile Tributyrin Agar plates (TBA). TBA was prepared by dissolving peptone 5 g, yeast extract 0.3 g, tributyrin 10 ml and agar 25 g per liter. The plates were incubated at 37^0C for 48 hrs. The colonies showing clear zones were considered positive for Lipase production. The isolates were selected on the basis of diameters of clear zone around the colony.

Identification of the isolates

Identification of isolates was carried out by conventional biochemical test[7] and 16S rRNA analysis was carried out using universal primer 27F and 1492R.

Lipase production

The isolates were grown overnight in nutrient broth on a rotary shaker at 30°C, at a speed of 100 rpm. The cultures were centrifuged and washed twice with sterile phosphate buffer. The cell pellets were resuspended in sterile phosphate buffer and OD was adjusted to 0.8 at 620 nm. A 5% (V/V) inoculum of each isolate was used to inoculate sterile 50 ml Mineral Medium (MM) supplemented with vegetable oil 1% (V/V) in 250 ml Erlenmeyer flask. The different vegetable oils like Soybean oil, Sunflower oil, Cotton seed oil, Groundnut oil, Coconut oil (non refined) and Palm oil were used to study the Lipase production. The flasks were incubated on a rotary shaker at 30°C, at a speed of 150 rpm for 72 hrs. The mineral medium was prepared by dissolving NH_4Cl - 0.57 g, KH_2PO_4 - 0.43 g, K_2HPO_4 - 1.09 g, Na_2HPO_4 - 1.33 g, $MgSO_4.7H_2O$ - 0.023 g, $CaCl_2$-0.028 g and $FeCl_3.6H_2O$ – 0.025 g per lit in distilled water and added with 1% vegetable oil. The medium was sterilized by autoclaving.[8]

Lipase assay

Lipase assay was carried out using titrimetric method.[9] In this method, one ml of culture supernatant from each of the inoculated flask was removed every 12 hrs and added to 5 ml of 5% Olive oil / Gum Arabic emulsion substrate prepared in phosphate buffer of pH 7. The reaction mixture was then incubated at room temperature for 30 min at a speed of 200 rpm. After completion of reaction, the 10 ml of 95% (v/v) was added to stop the reaction. The fatty acids produced due to the action of Lipase was titrated with 0.05 N NaOH using phenolphthalein until a faint pink color appeared. A blank was prepared by addition of heat inactivated culture supernatant at 95^0C for 15 min. The volume of NaOH consumed for

titration of liberated fatty acid was calculated by subtracting the volume required for blank, from volume required for the test. One unit of lipase activity was expressed as micro equivalents of fatty acid released from a triglyceride in 30 min at pH 7.0 at 20°C.

$$1 \text{ unit of Lipase activity} = \frac{\text{Volume of NaOH consumed (ml) X Molarity of NaOH X 1000}}{5\text{ml}}$$

RESULTS AND DISCUSSION

Enrichment and Isolation of lipolytic bacteria

The waste water sample from different chambers and outlet was inoculated in BH medium supplemented with soybean oil. The soybean oil was selected because of its wide use in local restaurants, as compared to other Oils. The flask inoculated with waste water from chamber number2 turned milky because of the activity of lipolytic bacteria. This was confirmed by comparing with control. The other flask remained less milky. For isolation of lipolytic bacteria, one ml from each enriched broth was serially diluted and spread on separate TBA plates. The enriched sample from chamber number 2 yields lipolytic bacteria. None of the sample from rest of the chambers and outlet showed the presence of lipolytic bacteria. The chamber number 2 showed a presence of high amount of residual waste lipid from kitchen. The other chambers showed less quantity of waste lipids. This is one of the reason for presence of lipolytic bacteria in chamber number 2. In all nine bacterial isolates showed clear zones around the colonies, indicating Lipase production. The isolates were named as R1, R2, R 3 ……..R9. Out of these, five bacterial isolates R1, R5, R6, R8 and R9 were selected for further studies, based on the diameters of clear zones formed around the colony. The isolates were purified, streaked on nutrient agar slants and stored in refrigerator.

Identification of bacterial isolates

The isolates were identified by Gram staining, colony characters and conventional biochemical tests. (Table number1).

Table number- 1 Morphological, physiological and biochemical characteristics of lipolytic isolates.

Sr. No	Characters	Lipolytic isolates				
		R1	**R5**	**R6**	**R8**	**R9**
1.	Gram Staining	Gram Positive cocci	Gram Positive cocci	Gram negative coccobacilli	Gram Negative rod	Gram Negative rod
2.	Catalase	+	+	+	+	+
3.	Oxidase	-	-	**D+**	+	+
4.	Gelatinase	-	-	-	-	-
5.	Lipase	+	+	+	+	+
6.	Lecithinase	-	-	+	+	+
7.	Caseinase	-	+	-	+	+
8.	Growth at 4^0 C	-	-	-	-	-
	41^0 C	+	+	+	+	-
	7.5% salt	+	+	-	-	-
	10% salt	+	+	-	-	-
	15 % salt	+	-	-	-	-
9.	Hemolysis	+	+	+	+	+
10.	Indole	-	-	-	-	-
11.	Methyl red	-	-	-	-	-
12.	VP	-	-	-	-	-
13.	Citrate	-	-	+	-	+
14.	L-Lysine DC	-	-	-	-	-
15.	Phenyl alanine DA	-	-	-	-	-
16.	Arginine HD	+	-	+	+	+
17.	H_2S	-	-	-	-	-
18.	Urease	-	+	-	-	+
19.	Nitrate	+	+	-	-	-
20.	Acid production from Glucose	+	+	-	-	-
21.	Galactose	+	+	-	-	-
22.	Fructose	-	-	-	-	-
23.	Lactose	+	W+	-	-	-
24.	Mannitol	+	-	+	+	-
25.	Maltose	+	+	-	+	-
26.	Xylose	-	-	-	-	-
27.	Arabinose	-	-	-	-	-
28.	Sucrose	+	W+	-	+	-
29.	O & F utilization of Glucose	O & F	O & F	O	NS	O

Key - +: Positive test, -: Negative test, W+: D: Delayed, Weak positive,

O & F: Oxidative and Fermentative, O: Oxidative, NS: Non saccharolytic,

DA: Deamination, DC: Decarboxylation, HD: Hydrolysis

From the microscopic and biochemical characteristics, it was found that isolate R1 and R5 belong to genus ***Staphylococcus***. The isolate R6 belongs to genus ***Acinetobacter*** and isolate

R8 and R9 belong to genus ***Pseudomonas***. The 16S r RNA sequencing of all isolates was performed and the resultant sequence was submitted in NCBI Blast and EzBioCloud (https://www.ezbiocloud.net, Seok-Hwan Yoon et al. 2017) for identification of closely related species. The sequence was then submitted to DDBJ, DNA Data Bank of Japan to get the accession numbers. The details are shown in Table number-2.

Odeyemi et al[10] isolated different species of *Pseudomonas, Staphylococcus, Enterococcus and Serratia* from restaurant waste water and receiving stream. **El-Bestawy et al**.[11] isolated oil and grease degrading *Pseudomonas* and *E.coli* from industrial waste water contaminated with vegetable oil and grease. **D. Mahajan et al**[12] isolated lipolytic *E.coli* from pharmaceutical industry waste. **T. Selva Mohan et al**[13] isolated Bacilli from oil mill waste.

Table no -2 Lipolytic bacterial isolates from restaurant waste water.

Sr. No	Isolate Code	Name of Organism	DDBJ Accession Number
1	R1	*Staphylococcus petrasii subsp. jettensis* VSJK-R1	LC314649
2	R5	*Staphylococcus epidermidis* VSJK-R5	LC314650
3	R6	*Acinetobacter junii* VSJK-R6	LC314651
4	R8	*Pseudomonas composti* VSJK-R8	LC314652
5	R9	*Pseudomonas aeruginosa* VSJK-R9	LC314653

Lipase assay

All five isolates are capable of producing Lipase in the presence of various vegetable Oils. The Lipase production increases with an increase in incubation period. The assay was conducted every 12 hrs, till the end of incubation. The Maximum Lipase production was observed (more than 6 Units/ml) in presence the presence of soybean oil, sunflower oil, cotton seed oil. The groundnut oil shows less Lipase production than above three Oils (about 6 Units/ml). Palm oil induces the least Lipase production, (less than 6 Units/ ml).

At the end of 72 hrs, isolate R6 showed maximum Lipase production at 8 Units/ml in presence of the presence of soybean oil (Figure 1). Isolates R1 and R6 showed maximum production of Lipase at 7.5 Units/ml in the presence of sunflower oil (Figure 2). In the presence of cottonseed oil, the isolates R1, R5 and R9 produced 7 Units/ml of Lipase (Figure 3). The isolates R5, R6, R8 and R9 showed Lipase activity at 6 Units/ ml in the presence of ground nut oil (Figure 4). The isolate R8 showed maximum Lipase production at 6.5 Units/ml in the presence of palm oil (Figure 5). The lowest Lipase production at 3 Units/ml was recorded by R5 isolate in the presence of palm oil (Figure 5).

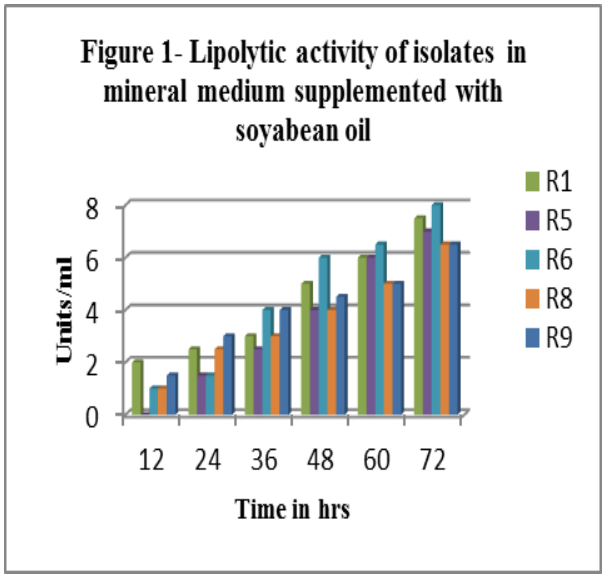
Figure 1- Lipolytic activity of isolates in mineral medium supplemented with soyabean oil
Units/ml
0
2
4
6
8
12 24 36 48 60 72
Time in hrs
R1
R5
R6
R8
R9

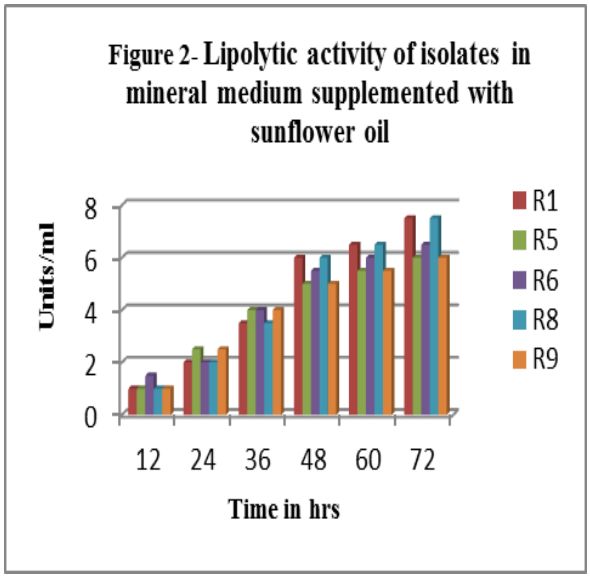
Figure 2- Lipolytic activity of isolates in mineral medium supplemented with sunflower oil
Units/ml
0
2
4
6
8
12 24 36 48 60 72
Time in hrs
R1
R5
R6
R8
R9

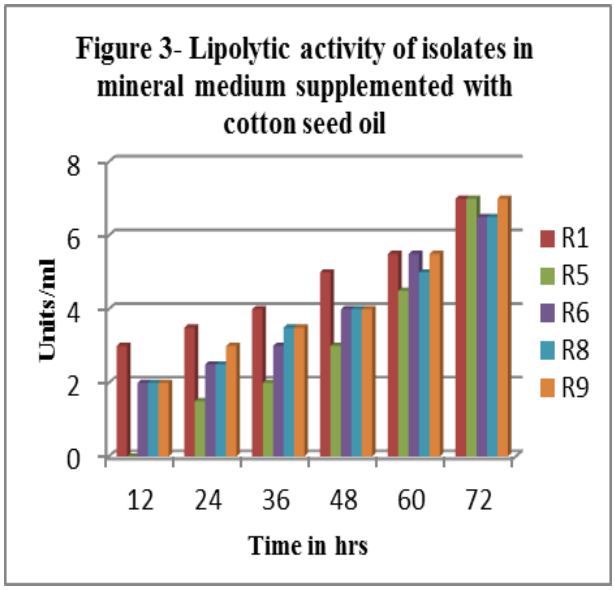
Figure 3- Lipolytic activity of isolates in mineral medium supplemented with cotton seed oil
Units/ml
0
2
4
6
8
12 24 36 48 60 72
Time in hrs
R1
R5
R6
R8
R9

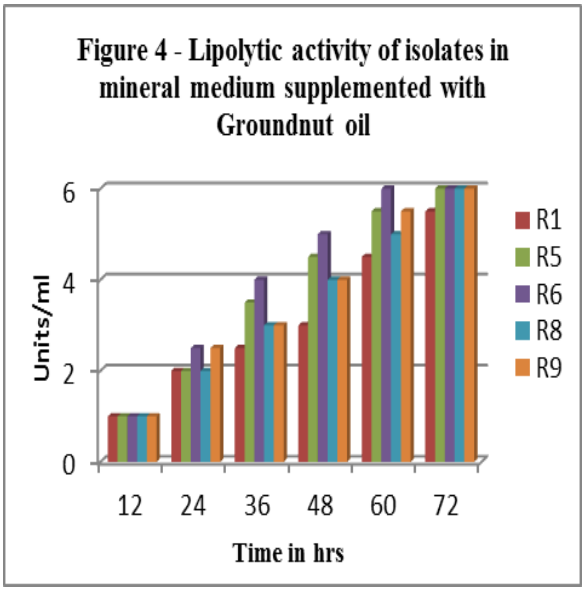
Figure 4 - Lipolytic activity of isolates in mineral medium supplemented with Groundnut oil
Units/ml
0
2
4
6
12 24 36 48 60 72
Time in hrs
R1
R5
R6
R8
R9

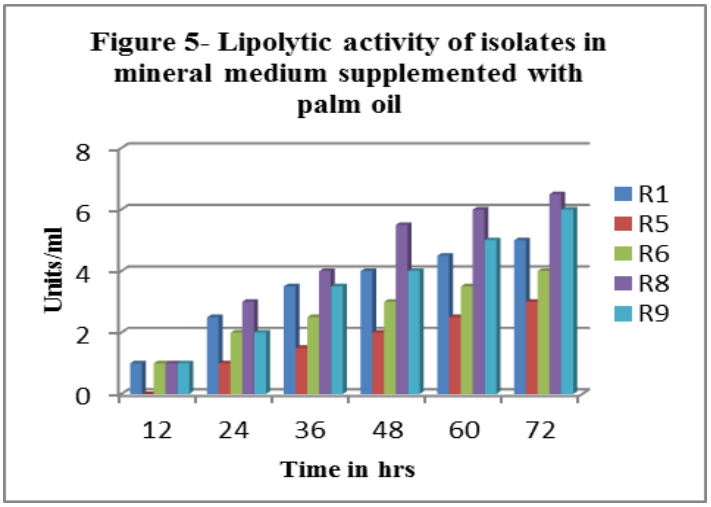
Figure 5- Lipolytic activity of isolates in mineral medium supplemented with palm oil
Units/ml
0
2
4
6
8
12 24 36 48 60 72
Time in hrs
R1
R5
R6
R8
R9

CONCLUSION

Five bacterial strains were isolated from the restaurant waste water. The presence of heavy load of waste lipids has stimulated the growth of bacteria in chamber number 2, along the sewer line of restaurant. The sample taken from this chamber showed the presence of lipolytic bacteria. The isolates had shown maximum lipolytic activity greater than 6 Units/ml for soybean, sunflower and cotton seed oil. The highest lipolytic activity shown by the isolates can be explained on the basis of common use of these oils in the restaurants. Lipolytic bacteria from restaurant waste water are well adapted to these oils as compared to groundnut and palm oil which are less commonly used. These isolates can be further studied for their bioremediation potential for restaurant waste waters containing the soybean, sunflower and cotton seed oil.

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