CHARACTERIZATION OF EDIBLE OIL INDUSTRY EFFLUENT AND BIOREMEDIATION USING LIPOLYTIC ISOLATES

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Abstract: The environmental challenge for edible oil industry is mainly associated with liquid wastewater. In India, this industry is a major component of the ever-growing food processing industry sector. The process water discharged by this industry is heavily polluted. In this study, physico-chemical and microbiological characterization of the industrial effluent was carried out. The lipolytic micro-organisms were isolated from waste sources and were identified. Bioremediation of liquid wastewater using these potent isolates was carried out in the laboratory so as to have acceptable BOD, COD, pH and Oil and Grease content in terms of regulatory standards prior to discharge. Percentage reduction of these parameters up to 96 hours was checked using Shake Flask Method as well as Lab-Scale Bioreactor Method. Around 80-100% reduction was observed in these parameters after biodegradation. These lipolytic cultures were further taken for immobilization and the activity was studied using two different immobilization materials.

Keywords: Effluent biodegradation, lipolytic microorganisms, Oil and Grease, immobilisation.

Introduction: One of the biggest problems faced by an edible oil industry is wastewater treatment, both qualitatively and quantitatively. The industry usually produces 7 million liters of wastewater per day [1]. The rapid industrial development in this sector has definitely contributed to environmental degradation, as the effluent has high pollutant profile based on pH, concentrations of BOD, COD, Oil and Grease content (O&G), total solids (TS) which is addition of total dissolved solids (TDS) and total suspended solids(TSS) and Hardness. The industry produces around 4,000-6,000 mg/l of oily waste materials/fatty contaminants in the wastewater [2]. Separation of oil from water is necessary as it can cause problems like oil slick on surface of water bodies, clogging of pipes and sewer lines and also soil contamination.

Vegetable oils are nothing but natural fats, which are complex mixtures of simple or mixed triacylglycerols [3]. Oils from food processing units are polar and biodegradable[4]. Many lipolytic microorganisms can degrade oils and fats. Hydrolysis of fats and oils is the primary reaction of lipases[5]. Microbial degradation is one of the most important methods to ameliorate the oil pollution in the environment. The ability of microbial degradation can be harnessed to remove biodegradable pollutants from the industrial wastes. This can be achieved by bioremediation.

Bioremediation involves the use of microorganisms to remove pollutants. The objective of bioremediation is to exploit naturally occurring biodegradative processes to clean up contaminated sites. There are several types of bioremediation. *In situ* bioremediation is the in-place treatment of a contaminated site.

Ex-situ bioremediation may be implemented to treat contaminated soil or water

that is removed from a contaminated site. It is currently being used to decrease the organic waste contents of soil, groundwater, chemical plants, effluents of

dairy and brewery industry and oily sludge from petroleum refineries.

In order to keep the environment clean, the oily waste should be degraded, by using eco-friendly technology. Therefore, the lipolytic microflora was isolated from various edible oil mill waste sources, and screened qualitatively and quantitatively for lipid degradation. The most obvious direct indicator of bacterial activity is reduction in the amount of oil. Gravimetric method was used to measure percentage degradation, as it is simple and economical [6].

The present paper discusses the isolation of lipolytic microorganisms from vegetable grease and oil contaminated wastes and its subsequent use for bioremediation purpose.

Materials and Methods:

The effluent from an Edible Oil Industry based in Mumbai was taken and studied for Physico-chemical and Microbiological characteristics.

Sample Collection:

Effluent generated after refining of oil, was collected from different sites of the Effluent Treatment Plant of above-mentioned industry. These samples were collected monthly, at the time when production and operation of Effluent Treatment Plant were at their maximum capacity. They were then stored in refrigerator till further processing was done. Sludge sample was collected after physico-chemical treatment. Soil sample was collected from sites where oil was spilled, in and around the edible oil industry.

Study Of Physico-Chemical Characteristics Of The Effluent:

Physico-chemical characterization of the effluent was done and the parameters selected for characterization include BOD, COD, pH, Oil & Grease content and TS, TDS, TSS. These were done according to the Standard procedures laid down by ISI [7]. Values obtained before and after ex-situ bioremediation were compared and studied.

Isolation And Identification Of Microorganisms:

Samples like effluent, sludge and soil around the industry, were used for isolation of lipolytic organisms. Organisms were isolated by serial dilution technique. Nutrient Agar and Sabaroud's Agar were used as plating media. Isolates were then screened qualitatively, for lipolytic activity by spot inoculating on Gorodkowa's Tributyrin Agar and zone of clearance was measured [8]. The lipolytic isolates were identified using standard biochemicals and with the help of Bergey's Manual of Determinative Baceriology [9]. Standard Cultures were obtained from National Chemical Laboratory, Pune.

They were as follows:

i. *Pseudomonas aeruginosa* (NCIM 2053), and

ii. *Aspergillus flavus* (NCIM 650)

The bacterial and fungal cultures (Isolates and standards) were maintained on Nutrient Agar and Potato Dextrose Agar slants, respectively.

Quantitative Lipolytic Activity :

Using Tributyrin as the substrate:

Flasks containing 50ml of sterile Bushnell & Hass Mineral medium, 1%(v/v) Tributyrin and 10%(v/v) saline suspension of the respective isolates (18-24 hrs. old) with optical density 0.1, were incubated on rotary shaker at RT for 3 days. A total of 10 isolates were taken in this study. After incubation, the contents of the flasks were mixed vigorously with a total of 50 ml petroleum ether (B.P. 60-80° C) in separating funnel and

allowed to stand, to separate the aqueous layer and the solvent layer. Aqueous layer was discarded and solvent layer was collected in pre-weighed beaker/crucible. Solvent was evaporated in a hot air oven at 80° (+/-1°) C. The residual oil was weighed. Percentage degradation was measured as loss in weight of solvent extractable components in comparison with that of uninoculated control flask [6].

Quantitative lipolytic activity for Various oils:

Different vegetable oils like soya bean oil, sunflower oil, groundnut oil, coconut oil, which are the major products of the above Edible Oil Industry, were taken for analysis, employing them as substrates. Waste oil (a mixture of above oils) extracted from effluent was also used as a substrate for biodegradation purpose along with the vegetable oils. Method used was the same as above except various oils selected were substituted for Tributyrin.

Methods Used For Bioremediation:

Shake Flask Method: The capacity of the isolates and consortia to degrade oily wastes was tested on a non-continuous model, using Shake flask method. A total of five potent isolates and three different consortia were used for study and the results were compared. Effluent was taken in a flask along with 10% saline suspension of the respective isolates with Optical Density of 0.1, and kept on shaker for 3 days [10]. Effluent samples before and after bioremediation were characterized for parameters like pH, BOD, COD, Oil and Grease Content.

Lab-scale Bioreactor Method: Bioremediation was carried out on lab- scale using a pilot scale bioreactor. The bioreactor with five liter capacity was employed for lab scale bioremediation. The reactor vessel was made up of glass with inlet as well as outlet device and also air sparger for aeration purpose. This study was carried out using two potent isolates and four different types of consortia. Effluent was taken in the reactor along with 10% saline suspension of the respective isolates with Optical Density of 0.1. Effluent was characterized after 24, 48, 72 and 96 hours for reduction in parameters like pH, BOD, COD, Oil and Grease content, TS, TDS, TSS and Hardness.

Bioremediation with Immobilized Lipolytic Isolates: The two potent isolates and two different consortia were immobilized using materials like Calcium alginate and Polyacryl amide gel. These immobilized/entrapped cells are capable of producing extracellular lipase, and can degrade oil. The residual oil was checked by gravimetric method.

In this method, 3% Sodium alginate and 4% Calcium chloride were prepared and autoclaved. Culture suspension (adjusted to 0.2 Optical Density) of overnight grown lipolytic isolates was taken. Culture was added in proportion of 1 ml of suspension to 100 ml of cooled Sodium Alginate solution. Beads of sodium alginate-culture were prepared in refrigerated Calcium Chloride using pipette with continuous swirling. These beads were kept overnight in refrigerator for hardening. They were washed 4-5 times with distilled water and were kept in Gorodkowa's Tributyrin Broth, an activation medium. They were washed again with distilled water and were used for bioremediation purpose.

In second method for entrapment, 1 g of packed intact cells of the selected isolate were suspended in 4 ml of physiological saline. To the suspension, acrylamide monomer (0.75 g), BIS (40 mg), TEMED (0.5 ml), and 2.5% potassium persulfate (0.5 ml) were added, and the mixture was incubated at 37° C for 30 minutes to yield the polyacrylamide gel containing the cells. Polymerization was carried out under anaerobic conditions, because oxygen prevents the polymerization. The gel was washed with physiological saline after being made to the proper shape.

The activity of entrapped cultures using both methods was studied using shake flask method. In 500 ml flask 250 ml of effluent was taken and around 250 beads were added to it. The flask was incubated for 3 days at RT Percentage reduction in various parameters like pH, BOD, COD, Oil and Grease content after 72 hours was studied.

Results and Discussion: Industrial regulations enforce primary treatment of the wastewater. However, these treatments are often characterized as physico-chemical like addition of alum, aeration, etc. Samples were collected post primary treatment by industry, and the effluent was characterized for various parameters like BOD, COD, Oil and Grease Content, TS, TDS, TSS, Hardness. The values were exceeding ISI standards for disposal, even after primary treatment by the above mentioned Industry. Various effluent samples were collected on monthly basis and, ranges of values for various parameters are presented in **table1**

Table 1: Physicochemical characters for Effluent after Primary Treatment by the Industry (values in mg/L)

Sample No.	pH	BOD	COD	O&G	TS	TDS	TSS
Limiting Values	**7 ± 0.3**	**50**	**250**	**10**			**50**
1	6.8	1,500	2,160	1,000	2,300	2,000	300
2	6.7	1,500	2,100	250	2,100	1,950	150
3	6.5	1,540	80,000	1,200	2,100	950	1,150
4	7.0	740	12,000	1,350	5,000	3,900	1,100
5	7.3	660	3,200	200	2,250	1,900	350
6	7.5	1,520	2,180	250	2,900	2,600	300
7	7.5	1,520	2,360	250	5,200	2,900	2,300
8	7.6	600	2,000	100	2,100	900	1,200
9	6.8	1,380	10,000	1,000	5,100	4,500	600
10	7.5	1,500	32,800	110	2,500	1,950	550

Qualitative And Quantitative Analysis Of Lipolytic Activity Of Isolates:

Lipolytic microorganisms were isolated from various sources like effluent, sludge and soil sample around the industry. Oil stressed areas show a number of isolates having good oil degradation capacity. Two Standard Strains - *Pseudomonas aeruginosa* (NCIM2053) and *Aspergillus flavus* (NCIM650) were also included for comparative studies.

Primary screening for lipase activity was performed using Gorodkowa's Tributyrin Agar (GTA) and zones of clearance were measured. The isolates showing good zone of clearance above 10 mm on the media plate were taken for further studies. These isolates were identified using various standard biochemical tests and with the help of Bergey's Manual Of

Determinative Bacteriology. They were identified as *Pseudomonas, Bacillus, Staphylococcus* and *Micrococcus* species. The fungal isolate was identified as *Aspergillus* species with the help of 'Fungi Imperfecti, A Manual of Soil Fungi'[11]. **Fig 1**. shows the distribution of lipolytic isolates. They were further tested quantitatively for their activity.

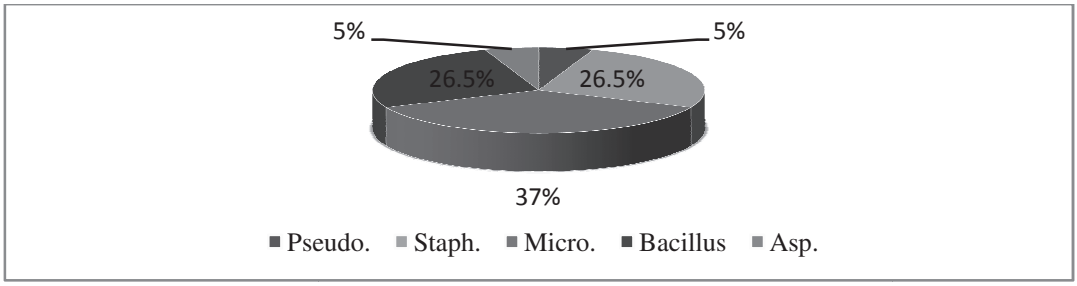


Figure 1: Distribution of lipolytic isolates

Gruiz, K[10] and Kavitha *et al* [12] had isolated similar fat-degrading bacteria from food industry waste and from oilseed and commercially available vegetable oils, respectively. Results agree with isolates obtained by Ebtesam El-Bestawy *et al* [13].

Oil degradation capacity of above five potent lipid degraders and two standard strains was then checked quantitatively using Gravimetric Method. **Fig. 2** represents percentage degradation with Tributyrin substrate as a sole source of Carbon.

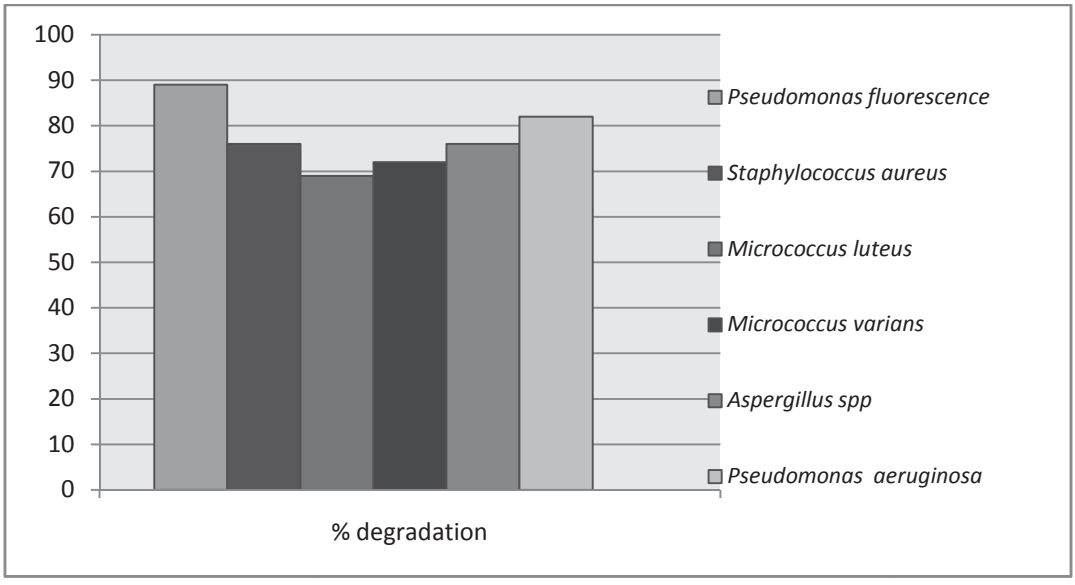


Figure 2: % Degradation of Tributyrin

Pseudomonas spp. showed percentage degradation of Tributyrin in the range of 80-90, while *Staphylococcus, Micrococcus* and *Aspergillus* spp. showed 60-80 % degradation of Tributyrin.

Biodegradation of various individual oils such as sunflower, soya bean, coconut, groundnut, sesame, mustard using these isolates was

studied. Also, the effect of the isolates on mixed oil extracted from wastewater was analyzed. **Figure 3** gives the details about degradation of these various oils. *Pseudomonas spp*.

was found to have comparatively better lipolytic activity, degrading different oils in the range of 60-90%, whereas *Staphylococcus spp.* and *Micrococcus spp.* were found to have degradation capacity of around 30-70%. *Aspergillus spp*. also had a degradation capacity of about 30-50%.

Figure 1: % degradation of various oils

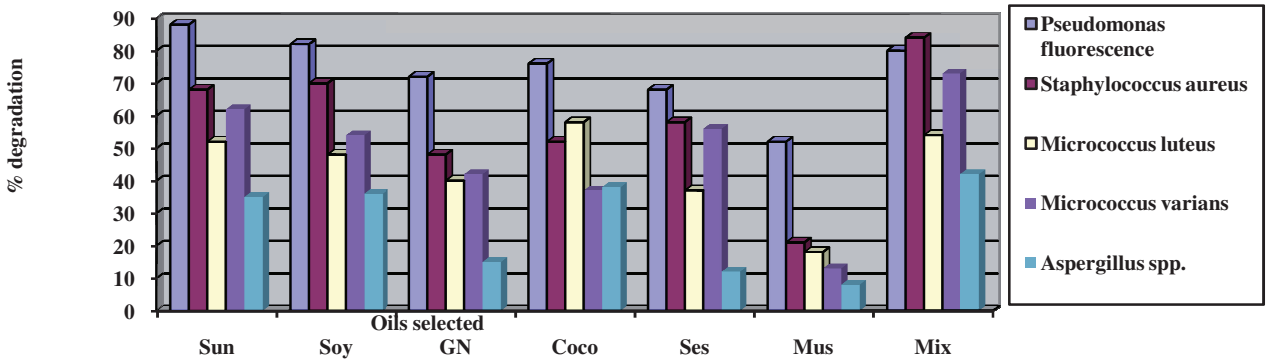


Figure 3: % degradation of various oils

Table 2: Bioremediation using shake flask method, after 72 hours

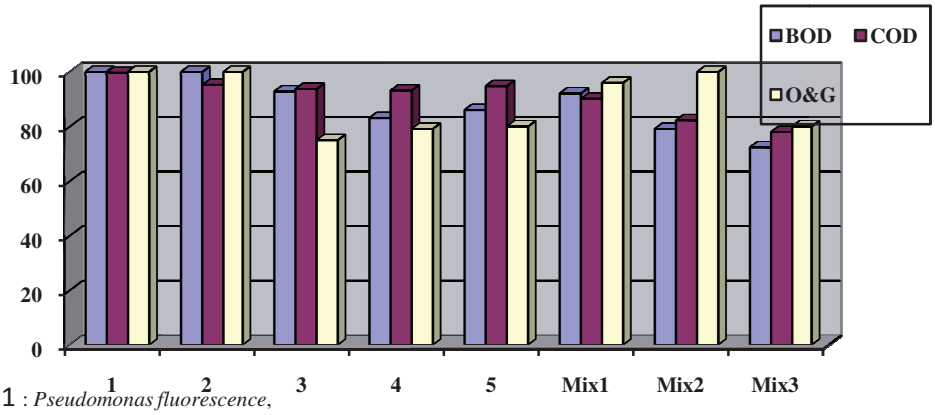
Culture Suspension	pH B.T.	pH A.T.	BOD B.T.	BOD A.T.	COD B.T.	COD A.T.	O&G B.T.	O&G A.T.
Pseudomonas fluorescence	6.5	7.0	1,540	0	80,000	210	1,200	0
Staphylococcus aureus	7.5	7.2	1,520	0	2,360	112	250	0
Micrococcus luteus	6.5	7.2	1,540	110	80,000	5,000	1,200	300
Micrococcus varians	6.5	7.6	1,540	260	80,000	5,445	1,200	250
Aspergillus spp.	7.5	7.0	1,520	212	2,180	113	250	50
Mix. 1	7.5	7.2	1,520	120	2,180	215	250	10
Mix. 2	7.6	7.5	600	125	2,000	355	100	0
Mix. 3	7.5	7.6	1,520	420	2,180	480	250	50

(Key: B.T. = Before Treatment, A.T. = After Treatment)

Above results are in line with earlier analysis by Kavitha et al [12].Bioremediation studies were performed using Shake Flask Method to study the use of these isolates for effluent bioremediation.

Percentage reduction in various parameters like BOD, COD and O & G was studied using five potent isolates and three consortia. Results are shown in Table 2 and same is depicted in **Fig.4.**

Figure 4: % reduction in various parameters after bioremediation



1 : *Pseudomonas fluorescence*,
2 : *Staphylococcus aureus*,
3:*Micrococcus luteus*,
4: *Micrococcus varians*,
5: *Aspergillus spp.*
Mix1: Isolate 6, 7, and 13
Mix2: Isolate 5, 11 and 17

From the Table 2 it can be seen that almost all the five isolates and consortia were showing reduction in Oil & Grease Content of around 70-90%, reduction in BOD of around 80-100% and reduction in COD of around 90%. *Pseudomonas spp.* showed the best degradation capacity among all.

In Lab Scale bioremediation Method, bioremediation of 5 liters of effluent was carried out using bacteria and fungi & four consortia. The effluent was characterized for BOD, COD, Oil & Grease Content and Hardness after duration of 24, 48, 72, and 96 hours. The results are represented graphically in Fig. 5, 6, 7 and 8. [Key: Mix1- *Micrococcus varians, Micrococcus luteus, Bacillus spp.* Mix2- *S. aureus, Micrococcus cryophilus, Bacillus carotarum* Mix3- *Micrococcus spp, P. fluorescence* and Mix5- *S.aureus, Micrococcus cryophilus, Bacillus spp.*]

During bioremediation, the biomass generally settles down in the reactor or adheres to the sides of the reactor. Hence, values for TS, TDS and TSS are not taken into consideration.

From the figure 5,6,7,8 it can be seen that though there was no appreciable reduction in BOD, COD and Oil & Grease Content for up to 48 hours, there was good reduction in the above parameters after 48 hours. Reduction reaches its maximum value after 96 hrs. Treatment with *Micrococcus variants* gave 70-90 % reduction in all the parameters. There was 100 % reduction in BOD and O&G contents, while reduction in COD was more than 90 %. *Aspergillus spp*. was chosen for Lab Scale Bioreactor Method as it gave best results at Shake Flask Level.

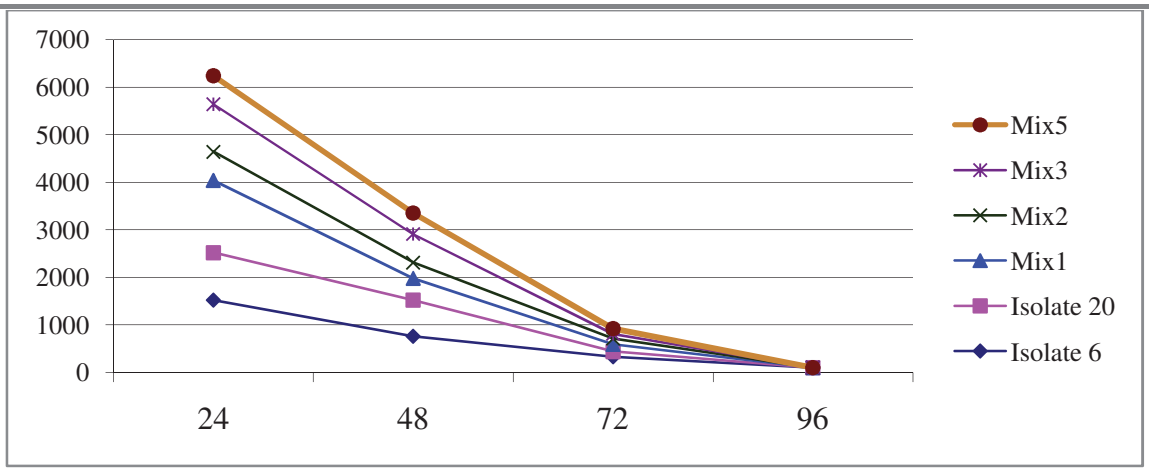


Figure 5: Sequential Decrease in BOD after Lab Scale Bioremediation

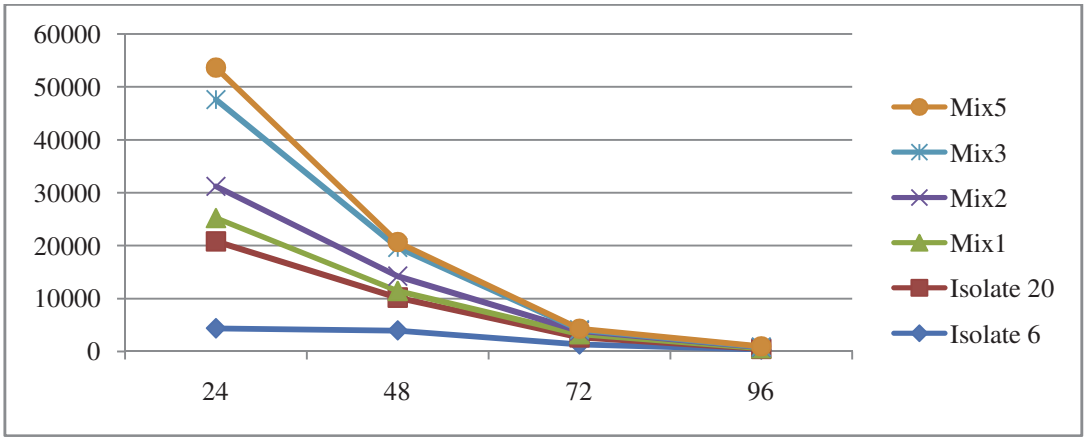


Figure 6: Sequential Decrease in COD after Lab-Scale Bioremediation

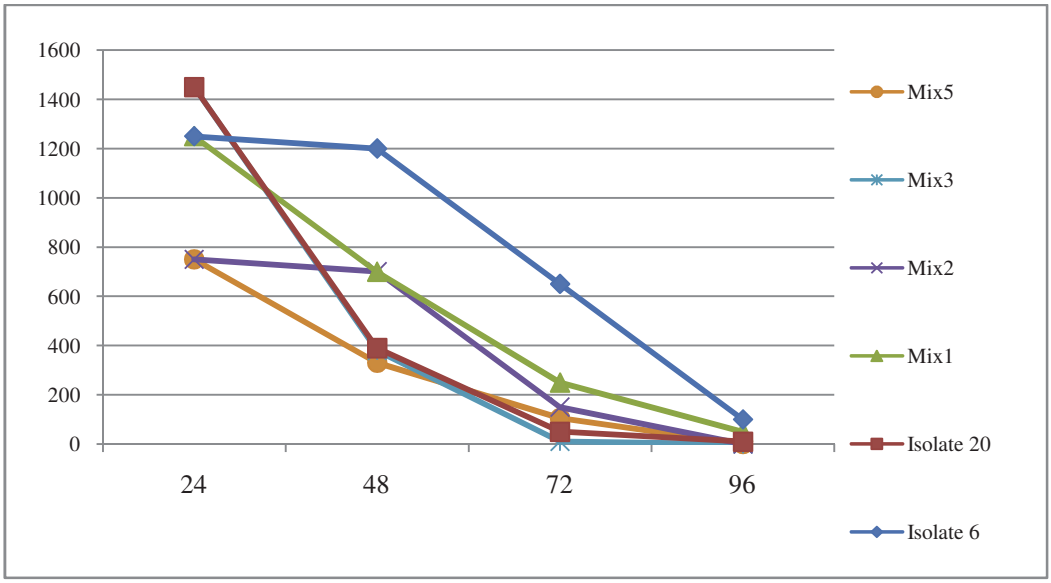


Figure 7: Sequential Decrease in O&G Content after Lab-Scale Bioremediation

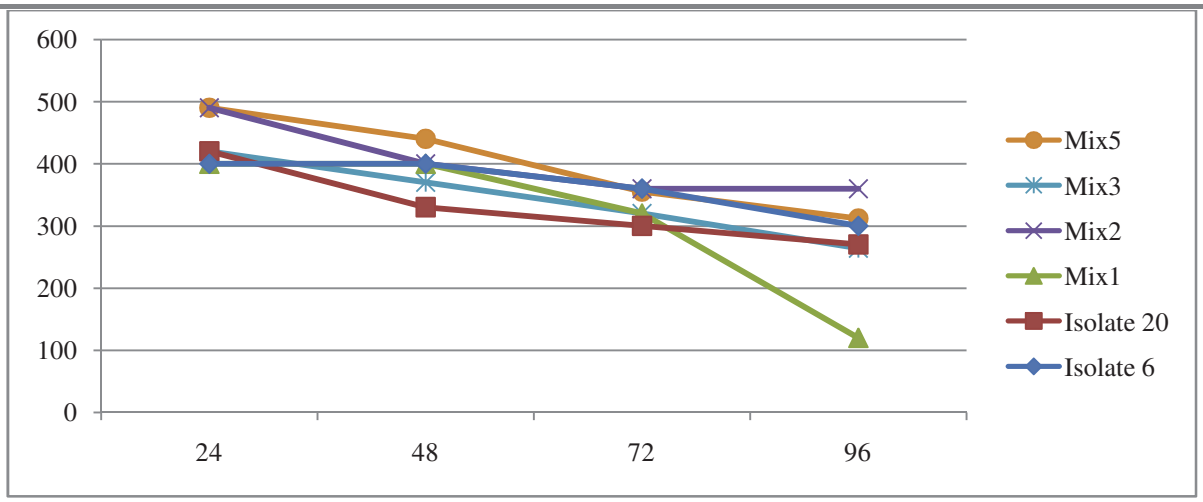


Figure 8: Sequential Decrease in Hardness after Lab-Scale Bioremediation

Table 3: Bioremediation using Calcium Alginate Beads (shake flask method)

Sample	Culture Beads (Alginate Method)	O&G B.T.	O&G A.T.	COD B.T.	COD A.T.	BOD B.T.	BOD A.T.	pH B.T.	pH A.T.
Effluent	*S. saprophyticus*	1,200	0	80,000	6,200	1,540	0	6.5	7.4
Effluent	*S. saprophyticus* (2nd use)	1,200	20	80,000	7,500	1,540	50	6.5	7.5
Effluent	*Micrococcus varians*	1,200	210	80,000	5,640	1,540	210	6.5	7.2
Effluent	*Micrococcus varians* (2nd use)	1,200	250	80,000	8,100	1,540	260	6.5	7.4
Effluent	*Micrococcus luteus*	1,200	300	80,000	4,540	1,540	100	6.5	7.2
Effluent	*Micrococcus luteus* (2nd use)	1,200	320	80,000	6,000	1,540	110	6.5	7.2
Effluent	*P. fluorescence*	1,200	0	80,000	210	1,540	0	6.5	7.2
Effluent	*P. fluorescence* (2nd use)	1,200	10	80,000	280	1,540	0	6.5	7.0
Effluent	*S. aureus*	250	0	2,360	100	1,520	0	7.5	7.2
Effluent	*S. aureus* (2nd use)	250	0	2,360	212	1,520	50	7.5	7.2
Effluent	Mix.5	250	0	2,180	50	1,520	0	7.5	7.0
Effluent	Mix.5 (2nd use)	250	10	2,180	220	1,520	110	7.5	7.2
Effluent	Mix.6	100	0	2,000	345	600	120	7.6	7.6
Effluent	Mix.6 (2nd round)	100	10	2,000	765	600	300	7.6	7.5

(Key: B.T. = Before Treatment, A.T. = After Treatment). Results after 72 hours of incubation (Values in mg/L.)

As mentioned in Materials and Methods, two methods were used for immobilization of lipolytic microorganisms. It was done using Alginate method and by entrapment in PolyAcryl Amide Gel method.

Four oil degrading isolates (*Staphylococcus spp*., *Micrococcus spp*., *Bacillus spp*. and *Pseudomonas spp*.) and two consortia were immobilized and taken for ex-situ bioremediation using Shake Flask Method. The immobilized beads were used for two rounds of Bioremediation. The bioremediation activity of immobilized cultures is shown in **Table 3** and **4**

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Table 4: Bioremediation using Polyacryl amide gel entrapment (shake flask method)

Sample	Culture Beads (Polyacryl Amide Gel Method)	O&G B.T.	O&G A.T.	COD B.T.	COD A.T.	BOD B.T.	BOD A.T.	pH B.T.	pH A.T.
Effluent	*Micrococcus luteus*	750	110	6,000	1,260	600	210	9.5	7.2
Effluent	*Micrococcus luteus* (2^{nd} use)	750	112	6,000	1,350	600	200	9.5	7.6
Effluent	*S. aureus*	1,250	40	4,400	240	1,520	-	6.0	7.4
Effluent	*S. aureus* (2^{nd} use)	1,250	48	4,400	310	1,520	-	6.0	7.6

(Key: B.T. = Before Treatment, A.T. = After Treatment). Results after 72 hours of incubation (Values in mg/L.)

Conclusions: Five lipolytic isolates were identified, analyzed and optimized to form consortia. Consortia gave better results for bioremediation of the effluent at Secondary level / biological stage. After the biological treatment using these cultures, the high values for various parameters like BOD/COD, Oil & Grease and Hardness can be brought down to permissible limits of disposal.

In bioremediation, addition of nutrients can encourage the growth of indigenous microbes that can consume the contaminating substances. In case of Edible Oil Industry, the effluent generated itself is rich in various nutrients. So, the process of bioremediation is much easier and cost effective.

Onsite secondary treatment of oily effluent using lipolytic microorganisms can be made feasible. This bioremedial procedure will not only be helpful to industry in maintaining compliances and regulations but also to maintain a healthy environment around Edible Oil Mills.

References:

1. Yadav, Khushal P. S., The small big polluter (Cover Story), Down To Earth, Vol.11, No.10, pp. 25-28, 2002.
2. Patterson, J. W., Industrial Wastewater Treatment Technology, Second Edition, pp 273-297, Butterworth Publishers, 1985.
3. Lehninger, A., Principles of Biochemistry, PP 303-400CBS Publishers & Distributors, 1984.
4. Jogdand, S. N., Environmental Biotechnology, (Industrial Pollution Management), pp 60-81,

Himalaya Publishing House, 2000.

5. Khare S. K., Nabetani H. & Nakajima M., Indian Food Industry, Vol. 19. No. 1, pp. 29, 2000.
6. Nikarge S. A., Some Aspects Of Biodegradation Of Bombay High Crude Oil, M.Sc. Thesis, University of Mumbai, pp 29, 1988.
7. Trivedy, R. K. & Goel, P. K., Chemical and Biological Methods for Water Pollution Studies, pp 43-80, Environmental Publications, Karad, India, 1986.
8. Collins C. H., Lyne P. M. & Grange J. M., Collins and Lyne's Microbilogical Methods, 7th edition, pp. 114, Butterworth and Heinemann Publications, 1995.
9. Bergey's Manual of Determinative Bacteriology, 8th edition, 1974.
10. Gruiz, K. Microbiological Intensification of Food Industrial Effluent Treatment, Biotechnology and Food Industry, Proceedings of The International Symposium held on 5-9Oct.1987, pp.667-678, 1988.
11. Gilman J. C., Fungi Imperfecti, A Manual Of Soil Fungi, PP 194-400, Oxford & IBH Publishing Company, 1967.
12. Kavitha N. S., Hilda A., Gopinath S. & Latha K. (1997). Hydrolysis of Oils and Marine Environmental Ethics (Abstract), Bioethics in India : Proceedings of the International Bioethics Workshop in Madras : Biomanagement of Biogeoresources, (J Azariach , H Azariach and Darryl R J Macer ed).
13. Ebtesam El-Bestawy, Mohamed H. El-Masry & Nawal I. El-Adl. Bioremediation of Vegetable Oil and Grease from Polluted Wastewater using a sand Biofilm System, World Journal of Microbiology and Biotechnology, 20(6), pp. 551-557(7), 2004.

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