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Study of Fungal Species Capable of Degrading Groundnut Oil

KEYWORDS: Biodegradation, vegetable oils, fungi

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ABSTRACT *Domestic waste water, as well as industrial water contains traces of oils. Lipids (fats, oils and greases) are major organic matters in municipal and some industrial waste water and cause severe environmental pollution. Waste water produced from edible oil refinery, slaughter houses, wool scouring and dairy products industry contains a high concentration of lipids. Oily waste water causes a lot of problem for treatment plant and discharge sewers. The present study aimed to isolate various species of fungi capable of degrading ground nut oil and to determine their degradation potential. Decomposed oil seeds were used to isolate different fungal species, identified and their lypolytic activity was determined by growing them in oil containing culture medium. Dry weight of mycelium was determined. GC-MS analysis of degraded and non-degraded oil samples were carried out to confirm biodegradation. From the study 8 fungal members belonging to three genera namely Aspergillus, Fusarium and Alternaria were found to be effective in biodegradation of ground nut oil.*

Domestic waste water, as well as industrial water contains traces of oils. Lipids (fats, oils and greases) are major organic matters in municipal and some industrial waste water and cause severe environmental pollution. Fungi are very diverse organisms. They have given a very big contribution toward framing human welfare, since from many years. Their contribution extands from natural to industrial uses. As they can exist and servive in almost every habitat, they are capable of regulating the flow of nutrients and energy through their mycelial network. However, the contributions of the fungi are often neglected by professionals, engineers, scients and general public. (Harbhajan Singh, 2006). Oil is an excellant carbon source for microbial growth, having approximately twice the energy value of glucose. (M. P. Roux-Van der. Merweet.al., 2005)

Biodegradation is a process of chemical breakdown or transformation of a substance caused by micro-organisms or their enzymes. (Dr. Dmitri Kopeliovich, Emmanuel O Aluyor et.al. 2009). Biodegradation can takes place at two extents; Primary Biodegradation and Ultimate Bidegradation. In primary biodegradation, the physical and chemical properties of a substance are modified due to the activity of micro-organisms. Whereas in ultimate biodegradation, the substance gets totally utilized and converted into carbon dioxide (CO_2) or methane (CH_4), water, mineral salt and microbial cellular constituents i.e. biomass.

From many years, the research has been carried out to find out fungal sppdegradating vegetable oils. A noticible work has been carried out by Negedu A et.al. in 2012. They checked degradative ability of 9 fungal spp namely, Aspergillustamarii, A. chevallieri, A. flavus, A. rurer, A. terrus, A. niger, *Penicillium chrysogenum, Cephaliophora irregularis, Syncephalastrum recemosums* From the presented work efforts has been done to check the role of different fungal species in biodegradation of groundnut oil.

MATERIALS AND METHOD

Vegetable oil used

Ground nut (Arachis hypogeaL) oil

Ground nut seeds were collected from market and broken into small pieces with the help of mortar and pestle. Small amount of each sample was buried in soil for about 10 days, during which moisture was maintained in the soil to ensure fungal growth. Similarly some amount of the seed samples were put in separate petri-plates and were kept exposed in air for 1 day. After that covered with lid and kept in darkness for about 10 days. After 10 days seed materials were removed from soil as well as from petri plates. Each sample was cleaned properly with distilled water to remove excess of soil artifacts.

Serial dilution:

1 gm of decomposed seed sample was added to 9 ml of sterile distilled water to make 1:10 dilution. Shaken properly. Similarly dilutions of each seed sample were prepared such as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}. 0.5 ml of each dilution was aseptically poured in separate sterilized petri-plates containing czapek-dox agar medium supplemented with 50µg/ml of streptomycin to inhibit bacterial growth. All the plates were incubated at 37°C for 3-8 days for isolation of fungi having property of oil degradation. Morphological appearances of the inoculated plates were observed and distinct colonies were sub-cultured to obtain pure isolates which were maintained on czapek-dox slants and stored in refrigerator for further study.

All the isolated fungal forms from ground nut oil seeds were tested for their lipolytic activity by inoculating them on tri-butyrin agar medium containing 5.0 gm peptone, 3.0 gm yeast extract, 10.0 ml tri-butyrin, 20.0 gm agar per lit at pH 7.0. Plates were incubated at 37°C for 3-5 days. The culture medium contains tri-butyrin as a reactant; degradation of this compound gives rise to clear zones surrounding the lipolytic colonies in the otherwise turbid culture medium (Bharati P et al. 2012). Isolates showing zone of clearance were further confirmed by growing them on czapek-dox ager medium supplemented with respective vegetable oil (50 gm/ lit) as a source of carbon instead of glucose.

All the fungal forms extensively grown on culture plates were observed under light microscope. On the basis of microscopic examination and morphological characteris-

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tics, all the fungal forms were identified with the help of "Manual of Soil Fungi" by Gilman (2012) and found to be species of genus *Aspergillus, Fusarium and Alternaria*

Preparation of the Inoculum
A pure culture of each of the isolates was grown for 5 days on Czapek-Dox Agar supplemented with 50µg/ml of streptomycin (to inhibit bacterial growth) in petri plates. One disk of agar (about 5mm diameter) and mycelium of each isolate were aseptically introduced into the flasks containing nutrient medium using a sterile cork borer.

Microbial Biodegradation Test
The mineral salt medium (MSM) was modified from (Bharati P et al. 2012). The composition of the medium was:

i. K2HPO4 : 1.0 gm

ii. NH4NO3 : 1.0 gm

iii. MgSO4.7H2O : 0.2 gm

iv. CaCl2.2H2O : 0.1 gm

v. NaCl : 0.1 gm

vi. FeCl3.6H2O : 0.01 gm

vii. pH : 7.0

viii.D/W : 1000 ml

The medium was autoclaved at 121°C temperature and 15 Lbs pressure for 15-20 min. 100 ml of MSM medium plus 30 gm of ground nut oil was prepared in 15 conical flasks. Each flask seeded with a disk of respective inoculum. They were kept on rotating shaker and readings were taken from day 0 to day 30 with an interval of 5days. One microorganism free and one oil free flasks were kept as a control. During each extraction the MSM medium was centrifuged and the mycelium was removed. The supernatant was added to separating funnel. To this added 98 ml of Dichloromethane. It was shaken vigorously, to form 2 layers; an aqueous and an organic layer. The organic layer was then collected in a beaker. Then this extract which contains the residual oil was then heated on hot water bath for the evaporation of dichloromethane.

Dry mycelium weight determination
Growth was determined by taking dry weight of the mycelia produced on the medium as referred by Oso B A (1979). The mycelium from each flask at the time of each reading was filtered through dried and pre-weighed whatman's No. 1 filter paper. The mycelia were washed with chloroform to remove any adhering oil. The washed mycelia were dried in an oven (Bio-Techniques India) at 80°C for 1 day and then mass was determined using an electronic balance. The difference in the weight gives the mycelia dry weight.

Gravimetric analysis:
Biodegradation is a natural process carried out by microorganisms such as bacteria and fungi in combination with oxygen. Enzymatic processes lead to the degradation of the original compound and to the formation of smaller organic molecules. Some of these are used for production of biomass and others are converted to carbon dioxide, water and minerals. CO_2 as byproduct of biodegradation can be measured by number of methods. In this study we used a unique method for CO_2 estimation.

GC-MS Analysis:
A gas-chromatography coupled with mass spectrometer (GC-MS) is a combined analyzer that has a superior ability in analyzing organic compounds qualitatively and quantitatively. It inherits the feathers of high resolution and accurate mass measurement with simple operation and high sensitivity.

Gas Chromatography - Mass Spectrometry (GCMS) are two essential techniques applied for analysis of edible oils. GCMS reveal the compounds eluted at different retention times with mass spectra corresponding to compounds present, indicative of the fatty acid compositions. Conventionally the resultant signals from both instruments are analyzed with the software equipped at the workstations for peak integration. The GC-MS analysis was done at SAIF Department, IIT- Pawai, Mumbai, Maharashtra, India.

OBSERVATIONS
5 Aspergillus species namely *Aspergillus fumigates* Fresenius (Syn. *Aspergillusoryzae* (Ahlburg.) Cohn.), *Aspergillus flavus* Link., *Aspergillus glaucus* Link., *Aspergillus wentii* Wehmer and *Aspergillus parasiticus* Speare and *Fusarium solani* (Martius) *Saccardo, Alternaria tenuis* Nees and *Fusarium oxysporum* (Martius) Appel Wollenweber obtained from decomposed groundnut oil seeds. All the isolates when subjected for screening to check their lipolytic activity by using tributyrin agar assay method, showed different results as shown in Table 1. Pure cultures of these isolates were prepared and maintained on czapekdox agar medium. Morphological identification of all the isolates were carried out.

Source	Isolate	Lipolytic activity
Ground nut seeds	*Aspergillus fumigates* Fresenius (Syn. *Aspergillusoryzae* (Ahlburg.) Cohn.)	+
	Aspergillus flavus Link.	++
	Fusarium solani (Martius) Saccardo	+
	Fusarium oxysporum (Martius) Appel Wollenweber	+
	Aspergillus glaucus Link.	+++
	Aspergillus wentii Wehmer	+
	Alternaria tenuis Nees	+
	Aspergillus parasiticus Speare	++

Table no.1: Isolates obtained from ground nut seeds with their lypolytic activity on tributyrin agar plate (showing zone of clearance)

Sr. No.	Name of the fungus	CO2 produced gm/liter		
		2 weeks	4 weeks	8 weeks
1	*Aspergillus fumigates* Fresenius (Syn. *Aspergillusoryzae* (Ahlburg.) Cohn.)	0.0132	0.0308	0.0396
2	*Aspergillus flavus* Link.	0.0308	0.0836	0.1628
3	*Fusarium solani* (Martius)Saccardo	0.0000	0.0044	0.0176
4	*Fusarium oxysporum* (Martius) AppelWollenweber	0.022	0.0396	0.0748
5	*Aspergillus glaucus*-Link.	0.0176	0.044	0.0924
6	*Aspergillus wentii* Wehmer	0.000	0.0176	0.0308
7	*Alternaria tenuis* Nees	0.0044	0.0132	0.0308
8	Aspergillusparasiticus-Speare	0.0352	0.0616	0.088

Table no. 2: CO_2 Produced during fungal degradation of ground nut oil

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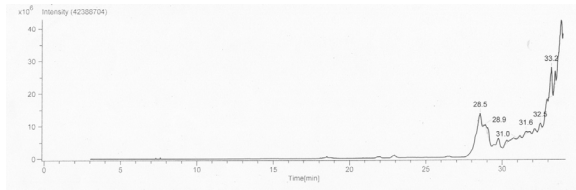
RESULTS AND DISCUSSION

8 different fungal species belonging to three genera; Aspergillus, Fusarium and Alternariawere obtained decomposed oil seeds. All the isolates were subjected for screening to check their lipolytic activity by using tributyrin agar assay method and their zone of clearance was measured (Table no. 1). Pure cultures of these isolates were prepared and maintained on czapekdox agar medium (HiMedia). Morphological identification of all the isolates were carried out, and identified as *Aspergillus fumigates* Fresenius (Syn. *Aspergillusoryzae* (Ahlburg.) Cohn.), *Aspergillus flavus* Link., *Aspergillus glaucus* Link., *Aspergillus wentii* Wehmer and *Aspergillus parasiticus* Speare and *Fusarium solani* (Martius) *Saccardo, Alternaria tenuis* Nees and *Fusarium oxysporum* (Martius) Appel Wollenweber.

The biodegradation test which was carried out in MSM broth medium using vegetative oil as a sole carbon source, revealed that almost all the three specie started the biodegradation from 3rd day of inoculation at different rate of oil degradation. Out of 8 species Aspergillusflavus Link. can be considered as best for biodegradation of ground nut oil as its dry weight was maximum i.e. 1.67 gm while *Fusarium oxysporum* (Martius) Appel Wollenweber showed least activity with a dry weight of 0.98 gm.

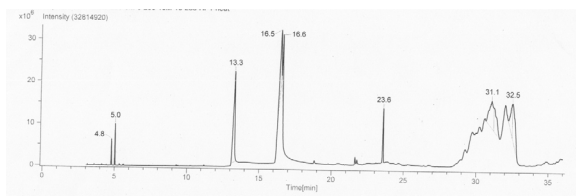
Evolution of CO_2 indicates the biodegrative ability of respective fungal members. Aspergillusflavus Link used maximum vegetable oil from culture medium evolving 0.1628 gm/lit CO_2 indicating maximum potential of oil degradation than other fungal members.

GC-MS analysis of degraded and non-degraded groundnut oil sample also confirms the role of these fungal species in biodegradation. (GR-1 and GR- 2)



Peak Number	Time [min]	Type	Peak Width(FWH [min]	Area [Intens. * sec]	Height	Description	Start Point Time[min]	Start Point Height	End Point Time[min]	End Point Height
1	7.6	BB	0.0246	675216.54	426744.61		7.6	39885	7.7	42646
2	28.5	BV	0.0322	22315.71	731349.65		28.5	13473110	28.7	10429532
3	28.7	VB	-0.0417	-561628.42	-419457.54		28.7	10429532	28.7	9794968
4	28.9	BV	0.0652	964757.11	693683.88		28.9	10109791	29.0	7554332
5	29.0	VB	0.0898	1844347.10	2071618.70		29.0	7554332	29.2	4040492
6	30.3	BV	0.1098	10365435.59	986530.73		30.1	3182290	30.3	4788890
7	30.4	VB	0.0732	1276646.46	407247.91		30.3	4798890	30.4	5202925
8	30.9	BV	0.0090	23346.10	39546.65		30.9	5850444	30.9	5908298
9	31.0	VV	0.0148	44157.68	33835.38		30.9	5908298	31.0	5975805
10	31.2	VB	0.1090	6138788.53	747775.64		31.0	5975805	31.3	6371254
11	31.6	BV	0.1030	7987390.94	936259.30		31.4	6461267	31.6	7471878
12	31.6	VB	0.0819	2486363.29	638198.53		31.6	7471878	31.7	7876050
13	31.8	BB	0.0633	1145595.37	262839.01		31.8	8112348	31.9	7539951
14	31.9	BV	0.0061	-73639.73	31496.36		31.9	7539951	31.9	7718858
15	32.1	VB	0.1010	3163531.88	542750.92		31.9	7718858	32.2	8711750
16	32.5	BB	0.1464	17634106.65	1891216.46		32.3	7947112	32.6	9387535
17	33.2	BB	0.1319	71533623.98	8968742.70		33.1	18482924	33.3	19180434
18	33.5	BB	0.0803	24461347.38	4231424.60		33.3	19180434	33.5	23776771

GR 1 : GC-MS Analysis of non-degraded ground nut oil



Peak Number	Time [min]	Type	Peak Width(FWH [min]	Area [Intens. * sec]	Height	Description	Start Point Time[min]	Start Point Height	End Point Time[min]	End Point Height
1	4.8	BB	0.0254	10631131.63	6236575.57		4.7	93599	4.9	88399
2	5.0	BB	0.0241	15132289.27	9869370.77		5.0	80079	5.1	83862
3	13.3	BB	0.1106	151907582.88	21417519.05		13.1	964118	13.4	1579543
4	16.5	BB	0.0878	101857072.45	12594761.31		16.2	10733566	16.5	21893750
5	16.6	BB	0.0881	99899493.89	19173178.12		16.5	21893750	16.6	6298402
6	23.6	BB	0.0582	47089071.55	12876958.10		23.5	1636976	23.7	1613298
7	31.1	BV	0.4360	127343615.72	6553299.73		30.7	11853974	31.2	9499190
8	31.2	VB	0.2276	80452819.90	5637571.03		31.2	9499190	31.6	7556296
9	32.5	BV	0.2385	65054537.75	7652166.26		32.2	11990897	32.5	7832901
10	32.5	VB	0.1862	118657723.52	8390085.68		32.5	7832901	32.9	2321969

GR 2 : GC-MS Analysis of degraded ground nut oil by *Aspergillus flavus Link.*

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