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Studies On GC-MS Analysis of Fungal Biodegraded Vegetable Oils

KEYWORDS: Fungi, Vegetable oils, GC-MS, Biodegradation

Prachi A. Joshi
Research Laboratory, Department of Botany, J. S. M. College, Alibag, Dist Raigad

Dr. R. L. Mishra
Head of Department and Associate Professor,Department of Botany, J. S. M. College

ABSTRACT *Oils derived from vegetable sources are of immense use. Being important food ingredient, they have a huge industrial demand.Vegetable oil spills can cause serious effects on marine life and coastal environment. Physical, mechanical and chemical methods are not sufficient for removal of oil traces. For this problem the biodegradation is the ultimate solution.The present study aimed to isolate various species of fungi capable of degrading some commonly used vegetable oils like coconut oil, sesame oil, sunflower oil etc. 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. Quantitative analysis was of biodegradation was carried by using several methods like dry weight of mycelium, weight of residual oil after degradation, peroxide values, iodine number and free fatty acid content. GC-MS analysis of degraded and non-degraded oil samples were carried out to confirm biodegradation. From the above 6 different fungal members were isolated namely; Aspergillus flavusLink.,Aspergillus fumigates Fresenius (Syn. Aspergillus oryzae (Ahlburg.) Cohn.)Aspergillus niger gr., Aspergillus glaucus Link., Penicillium chrysogenum Thom and Rhizopus nigricans Ehrenberg*

Introduction :
Fats and oils are naturally occurring substances which consist of predominantly, the mixture of fatty acid esters of trihydroxy alcohol or glycerol [11]Vegetable oils have many uses. Most commonly they are used as an important ingredient for food in house, restaurants. Besides, they have a huge industrial demand. They are not only used for cooking purpose but also for killing the bacteria, fitting against dental cavities. Although vegetable oils are economically important, they are one of the members responsible for environmental pollution. Oil contamination is one of the most dangerous polluting agents known today. It can cause severe threats to the environment [4].

Owing to the problems associated with physical, mechanical and chemical methods, there is a need for a safer and less expensive approach to remediation of polluted environments. Bioremediation is a means of cleaning up contaminated environments by exploiting the diverse metabolic abilities of microorganisms to convert contaminants to harmless products by mineralization, generation of carbon di-oxide and water, or by conversion into microbial biomass[3], [10].

Biodegradation is the processes by which organic substances are broken down by living organisms It is a process of chemical breakdown or transformation of a substance caused by micro-organisms or their enzymes [6]. Biodegradation can takes place at two extents; primary biodegradation and ultimate biodegradation.Fungi form diverse group of organisms. They have given a very big contribution toward framing human welfare since ancient times.

Fungi are known to degrade or cause to deteriorate, a wide variety of materials and compounds. When fungi are added in a growth medium containing fats and oils, triglycerols are hydrolyzed by fungal lipases to yield diacylglycerol, monoacylglycerol, free fatty acids and glycerol. These hydrolyzed products are then taken up by the cell and reassembled within the cell [7].Many fungal species producing lipases are capable of degrading oils like *Rhizopus, Mucor, Geotrichumcandidum, Aspergillus* and Yeast have been well studied [1][5][8].The production of lipases is influenced by many factors such as pH, temperature, carbon and nitrogen[9].Many genera as *Penicillium, Rhizopus, Aspergillus* and *Fusarium have* been noted as producers of lipases with desirable properties[13].

Before thinking of bioremediation of domestic waste water containing vegetable oil traces, it is very important to know regarding microbial diversity capable of degrading that particular oil. Thus by investigating fungal diversity for biodegradation of particular oil and by observing their maximum activity under different parameters, optimum conditions for their activity, can be determined and thus can be effectively employed in waste water treatments.

Materials and Methods :
Collection of seed samples:
For the presented work four different oils seeds were used.

a) Coconut: *Cocos nucifera L.*

b) Sunflower: *Helianthus annus* L.

c) Sesame: *Sesamum indicum L.*

d) Ground nut: *Arachis hypogea* L.

All the oil seeds (fresh and stored) were collected from local market and brought to the laboratory for further studies.

Isolation, screening and identification of fungal species :
Different types of oil seeds like sesame, coconut, sunflower, groundnut etc. were 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.

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Similarly some amount of each 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.

Dilutionsof each seed sample were prepared such as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. 0.5 ml of each dilution was aseptically poured in separate sterilized petriplates 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.

All the 11 fungal forms extensively grown on culture plates were observed under light microscope. On the basis of microscopic examination and morphological characteristics, all the fungal forms were identified with the help of "Manual of Soil Fungi" by Gilman [38] and found to be species of genus *Aspergillus*, *Fusarium, Alternaria, Rhizopus*and *Penicillium.* Authentication of all the isolates was carried out from Agharkar Research Institute, Pune.

Measurement of vegetable oil degradation:
The oil degrading ability of all the isolated fungal forms was tested in laboratory by employing various methods i.e. both qualitative and quantitative.

Preparation of the Inoculum:
A pure culture of each of the 11 isolates was grown for 5 days on Czapek-dox Agar supplemented with 50µg/ml of Streptomycin (to inhibit bacterial growth) in petriplates. 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 [4]. The composition of the medium is:

i) K_2HPO_4:1.0 gm
ii) NH_4NO_3:1.0 gm
iii) $MgSO_4.7H_2O$: 0.2 gm
iv) $CaCl_2$. $2H_2O$: 0.1 gm
v) NaCl: 0.1 gm
vi) $FeCl_3.6H_2O$: 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 each vegetable oil was prepared in 15 conical flasks (15 conical flasks for each vegetable oil). Each flask was seeded with a disk of respective inoculum. The inoculated flasks were kept on rotating shaker and readings were taken from day 0 to day 30 with an interval of 5days. One flask free from microorganism and one flask free from oil 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 98 ml of dichloromethane was added. 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. The residual oil left in a beaker was used for GC-MS analysis.

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. The amount of CO_2 evolved during degradation of LDPE was calculated gravimetrically and volumetrically using Sturm Test [12] Several modifications were carried out in Sturm Test during experiment. The Modified Sturm test method has been adopted by the author in the present study for CO_2 estimation.

GC-MS Analysis:
Gas Chromatography - Mass Spectrometry (GC-MS) 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.The GC-MS analysis was done at SAIF Department, IIT- Pawai, Mumbai, Maharashtra (India).

Observations and Results :
Microscopic and morphological studies were carried out for identification of isolated oil degrading fungi. In all 11 different fungal species belonging to 5 different genera were isolated from all four vegetable oil seeds. List of various fungal species obtained from respective oil seeds with their lipolytic activity on Tributyrin agar plates in the form of zone of clearance is given in the following **Table No. 01.**

Table No. 01: Isolates obtained from respective oil seeds with their lipolytic activity on tributyrin agar plate (showing zone of clearance)

Source	Isolate	Lipolytic activity
Ground nut seeds	*Aspergillus fumigates Fresenius*	+
	Aspergillus flavus Link.	++
	Fusarium oxysporum (Martius) Appel Wollenweber	+
	Fusariumoxysporum (Martius) AppelWollenweber	+
	Aspergillus glaucus Link.	+++
	Aspergillus wentiiWehmer	+
	Alternaria tenuis Nees	+
	Aspergillus parasiticusSpeare	++
Sunflower seeds	*Aspergillus flavus Link.*	++
	Aspergillus fumigates Fresenius (Syn. Aspergillus oryzae (Ahlburg.) Cohn.)	+
	Penicilliumchrysogenum Thom	+
	RhizopusnigricansEhrenberg	+
Coconut	*Aspergillus flavus Link.*	++
	Aspergillus niger gr.	++
	Penicillium chrysogenum Thom	+
Sesame seeds	*Aspergillus flavus Link.*	++
	Aspergillus niger gr.	++
	Penicillium chrysogenum Thom	+
	Aspergillus glaucus Link.	++

Qualitative Analysis:

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Fungal isolates that gave good results during Sturm test were further subjected to qualitative analysis. Qualitative analysis is attributed to comparative study of GC-MS analysis of degraded and non-degraded oil samples by respective fungal species. GC-MS analysis could not be carried out for sesame oil due to inability of the fungus to degrade oil during Sturm test.

Table No. 1.2: Analysis of GC-MS peaks for untreated and fungal treated

Sunflower oil

Sr. No.	Retention Time (min)	Untreated Sunflower oil	Treated Oil		
			Fungal Forms		
			Rhizopus-nigricans	*Aspergillus flavus*	*Penicilliumchrysogenum*
1	12.5			√	√
2	14.8	√		√	
3	14.9	√		√	
4	15.0				√
5	15.3		√		
6	18.1		√		
7	20.0				
8	20.3				√
9	23.8		√		
10	34.1	√			
11	34.8		√		
12	35.0	√			
13	35.4				√
14	36.3				√
15	36.4		√		

Table no. 1.2 shows different components of untreated and fungal treated sunflower oil eluted during GC-MS analysis at different retention time. From this comparative table it was observed that oil treated with ***Rhizopusnigricans*** and ***Penicilliumchrysogenum*** formed different components than untreated oil which may be formed because of degradation of oil. Oil treated with ***Aspergillus flavus*** showed only one degradation product.

Table No. 1.3: Analysis of GC-MS peaks for untreated and fungal treated **coconut oil**

Sr. No.	Retention Time (min)	Untreated Coconut oil	Treated Oil		
			Fungal Forms		
			Aspergillus niger	*Penicilliumchrysogenum*	*Aspergillus flavus*
1	4.8		√		
2	6.1	√			
3	8.0	√			
4	12.4	√			
5	12.5		√		
6	12.6			√	
7	12.7				√
8	15.1	√			
9	15.3			√	
10	15.4				√
11	27.0		√		
12	27.1	√			√
13	28.5			√	
14	28.6				√
15	29.3			√	
16	30.6		√		
17	30.8			√	
18	31.0				√
19	31.8		√		
20	32.1		√		√

Table No. 1.3 shows that coconut oil treated with all the three fungi degraded it and resulted in production of different components which were absent in untreated one.

Table No. 1.4: Analysis of GC-MS peaks for untreated and fungal treated

Ground nut oil

Sr. No.	Retention Time (min)	Untreated Groundnut oil	Treated Oil		
			Fungal Forms		
			Aspergillus flavus	*Aspergillus glaucus*	*Aspergillus parasiticus*
1	4.8			√	
2	5.0			√	√
3	5.5		√		
4	16.1				√
5	16.5			√	
6	17.5		√		
7	23.6			√	
8	28.8		√		
9	29.0	√			
10	31.0				√
11	32.6	√			
12	34.6		√		
13	34.7				√

From the above table it is evident that all the peaks of components that are produced from the fungal treated ground nut oil are different from untreated oil and are also eluted at different retention time.

Table No. 1.5: GC-MS analysis of the byproducts from non-degraded and degraded **sunflower oil**

Sunflower Oil	Retention Time (min)	Name of the product
Non-Degraded	14.8	Oleic acid
	14.9	9,12-Octadecadienoic Acid
	34.1	2,6-Bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo octane
	35.0	Dodecanoic acid, 1,2,3-propanetriyl ester.
Degraded by Rhizopusnigricans	15.3	9-Octadecenoic acid
	18.1	Decanoic acid, 1,2,3-propanetriyl ester
	23.8	Glycerol2-acetate 1,3-dipalmitate
	34.8	2,6-Bis(3,4-methylenedioxyphenyl)-3,7dioxabicyclo octane
	36.4	Dodecanoic acid, 1,2,3-propanetriyl ester
Degraded by Aspergillus flavus	12.5	n-Hexadecanoic acid
	14.8	9,12-Octadecadienoic acid
	14.9	cis-Vaccenic acid
Degraded by Penicilliumchrysogenum	12.5	n-Hexadecanoic acid
	15.0	cis-Vaccenic acid
	20.0	Sitosterol
	35.4	Dodecanoic acid, 1,2,3-propanetriyl ester
	36.3	Dodecanoic acid, 1,2,3-propanetriyl ester

Table no. 1.6: GC-MS analysis of the byproducts from non-degraded and degraded **coconut oil**

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Coconut oil	Retention Time (min)	Name of the product
Non-degraded	6.1	Octanoic acid
	8.0	2-undecanone
	12.4	Dodecanoic acid
	15.1	Tetradecanoic acid
	27.1	Dodecanoic acid, 2,3-dihydroxypropyl ester
	30.1	Dodecanoic acid, 2,3-dihydroxypropyl ester
Degraded by Aspergillus niger	4.8	2-Nonanone
	12.5	Dodecanoic acid
	27.0	2,2-Bis[4'-cyanooxyphenyl] propane
	30.6	Dodecanoic acid, 2,3-dihydroxypropyl ester
	31.8	Dodecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester
	32.1	Dodecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester
Degraded by Penicilliumchrysogenum	12.6	Dodecanoic acid
	15.3	Tetradecanoic acid
	28.5	Dodecanoic acid, 1,2,3-propanetriyl ester
	29.3	Dodecanoic acid, 1,2,3-propanetriyl ester
	30.8	Dodecanoic acid, 2,3-dihydroxypropyl ester
Degraded by Aspergillus flavus	12.7	Dodecanoic acid
	15.4	Tetradecanoic acid
	27.1	Dodecanoic acid, 1,2,3-propanetriyl ester
	28.6	Dodecanoic acid, 1,2,3-propanetriyl ester
	31.0	Dodecanoic acid, 2,3-dihydroxypropyl ester
	32.1	Dodecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester

Table No. 1.7: GC-MS analysis of the byproducts from non-degraded and degraded **ground nut oil**

Coconut oil	Retention Time (min)	Name of the product
Non-degraded	29.0	Sarcosine, N-(1-naphthoyl) octyl ester
	32.6	Dodecanoic acid, 1,2,3-propanetriyl ester
Degraded by Aspergillus flavus	5.5	2,4-Decadienal
	17.5	9,12-Octadecadienoic acid
	28.8	Dodecanoic acid, 1,2,3-propanetriyl ester
	34.6	Dodecanoic acid, 1,2,3-propanetriyl ester
Degraded by Aspergillus glaucus	4.8	2,4-Decadienal
	5.0	2,4-Decadienal
	16.5	9,12-Octadecadienoic acid
	23.6	Quinazolin-4(3H)-one, 2-[2-(4-methoxyphenyl) ethyenyl]
Degraded by Aspergillus parasiticus	5.0	2,4-Decadienal
	16.1	9,12-Octadecadienoic acid
	31.0	Dodecanoic acid, 1,2,3-propanetriyl ester
	34.7	Dodecanoic acid, 1,2,3-propanetriyl ester

Different components from untreated and fungal treated oil samples are well represented in Table No. 1.5, 1.6 and 1.7. Maximum number of products are observed in coconut oil degrade by *Aspergillus niger*. All the products are uncommon to untreated coconut oil. Sunflower oil treated with *Aspergillus flavus* shows only two new components.

Discussion :

The isolated fungal species were screened to check their lipolytic activity on tributyrin agar plate. The rate of degradative ability of all 11 fungal forms is given in table no. 01. For all the four types of oil, *Aspergillus flavus* was found to be a common degradative isolate giving optimum zone of clearance on tributyrin agar plates except for the ground nut oil where *Aspergillus glaucus* showed maximum lipolytic activity producing maximum zone of clearance among the all 11 isolates.

When the fungi were allowed to grow on oil from MSM, in the absence of any sugar, these fungi secreted several lipolytic enzymes that degraded the triglycerides of in to short or medium chain fatty acids, glycerols, esters or ketones. That was indicated by increased percentage of FFA, increased value of peroxides and decreased iodine number. Degraded compounds were utilized by fungi to make their biomass. Thus, those compounds that were observed in the chromatogram of treated oil samples and not observed in non-treated chromatogram could be formed because of biodegradation and thus eluted out at different time interval.

The chromatogram of untreated sunflower oil showed 3 peaks at different retention time **(Table No. 1.2).** Those peaks that were observed could be as a result of the microorganisms acting on the substrate which must have broken down some of these components into smaller molecules that are easily consumed. The appearance of the component except 3rd, 10th and 12th could therefore be a product of the biodegradation.

For sunflower oil *Rhizopus nigricans showed* formation of five different compounds than non-treated oil. Thus all the three fungi brought about degradation of sunflower oil. **(Table no. 1.2 and Table no. 1.5)**

The chromatogram of degraded coconut oil also showed difference in the components. From the oil degraded by *Aspergillus niger* and *Aspergillus flavus*, seven different components were detected **(Table no. 1.3 and Table no. 1.6)**. Dodecanoic acid observed as a common component for all the three degraded coconut oil samples.

The untreated ground nut oil showed only two components, whereas all the degraded oil samples showed four components which were found to be totally different than the components of non-degraded ground nut oil. 2, 4-Decadienal and 9,12-Octadecadienoic acid were common components observed in all the three degraded oil samples which were not detected in non-degraded oil sample **(Table No. 1.4 and Table No. 1.8)**. From this it can be concluded that the ground nut oil could stand for biodegradation by isolated fungal forms.

More or less similar results were observed by Dass[12] from degraded Baobab seed oil by *Aspergillus niger*. Seven components were detected at the end of 5th week of incubation. The microbes were noticed to have acted on the unsaturated fatty acids to form 9-Octadecanoic acid and 2-hydroxy-1, 3-propanediyl ester.

This could be as a result of the growth of the microorganisms as they act on the substrate (oil) possibly by the se-

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cretion of enzymes to facilitate the breaking down process [2]. Biodegradation is expected to have been initiated by the enzymatic hydrolysis of triglycerides to glycerol and long chain fatty acids (LCFAs), which serve as the growth substrates for fungal mycelium.

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