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VISCOSITY CHANGES IN NEEM OIL AND BIOSURFACTANT PRODUCTION BY BACTERIA

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Abstract

Depletion of fossil fuels has created a necessity to find out alternative sources of non-conventional energy. Plant species evaluated for their potential as alternative energy from secondary metabolites such as oil, hydrocarbons and chemical sources. The present investigation has attempted to understand the importance of the alternative source of energy. Neem oil extracted from neem press cake used by the solvent of hexane and aqueous ethanol from the soxhlet's apparatus oil was separated and used for further studies. The extracted neem oil reduced the viscosity by microbial activity. The composition of hexane soluble fractions of neem oil, neem powder and non-degraded and degraded neem oil by microbes analyzed using Gas chromatography. The oil-degrading bacterial species such as *pseudomonas putida* and *pseudomonas aeruginosa* produced more growth in stone medium among the four different medium. The soluble fraction of water and hexane extracts of neem oil used as a carbon source. In the present experiments, three controls maintained simultaneously to verify whether the extract enhances the growth of the microbes. Control I was only medium with uninoculated microbes and no energy sources, control II was medium with extract without microbial inoculants. The control III flasks contained an inoculated medium with neem oil extract and nitrogen sources maintained at different temperature and pH level. The bacterial species *pseudomonas putida* produce better biomass and biosurfactant in the presence of nitrogen source (NH4)2HPO4 and most of the components degraded. Hence, Higher biomass and biosurfactant correlated with the viscosity of the oil. The result was *pseudomonas putida* degraded the oil and reduced the density from 200 to 60, which suggested that possibility of using of this microbe can enhance the fuel efficiency of neem oil for engines.

Key words: Biomass, Biosurfactant, Oil degradation, Neem oil, Hydrocarbon degradation

Introduction

Utilization of whole plant oils as an alternative source of conventional and non–conventional oils and primary industrial feedstocks is obtaining greater importance throughout the world (Goering et al.,1987). Curcus oil is a potential substitute for biodiesel, providing the country a cheap and renewable source of energy for transport and power and can affect sizable savings on the foreign exchange (Peter, Vedamuthu and Rangasamy. 2003). The various sources identified for alternative fuel, edible oil, non-edible oils considered to be ideal because of compatible properties concerning diesel (Rangaraju. K, 2005). Use of vegetable oils as an engine fuel is one such concept which dates back to 1900 when Rudolf diesel developed the first engine to run on peanut oil and demonstrated it at the world exhibition in Paris. The scientific investigation and experiments in recent years have established that this renewable source is as efficient as petroleum diesel in powering diesel engines without any substantial modification to the existing design (Dilip Biswas, 2003). Though, the utilization of edible plants, oil for fuel may endanger the food supply (Buchanan et al.,1978).

Moreover, vegetable oils cannot be used directly as fuel. They consist of glycerol esters, fatty acids with varying carbon chain length and double and triple bonds giving rise to molecular weight with a range of 650-970 and higher viscosity which decreases their flow property. Therefore, vegetable oils transesterified to get a lower viscosity and are known as biodiesel. India has about 86 different oil tree species. The studies indicate that vegetable oils could be mixed with petrodiesel up to 25% and if esterified then the proportion could go beyond 75%. The

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process involves the maintenance of continuous contact with enzymes and fresh fat or oil.

It is also possible to use the biotransformation method to reduce the viscosity by the microbial activity. Hence the ability of microbes which synthesis the enzymes have transesterification properties. Many studies reported that Immobilized lipase from *Mucor* or *Rhizomucor Michie* (Hayes, DG and Kleiman .R. 1996, Hallberg M.L.,1999). The synthetic of idol and polyol esters have also employed. In the chemical and enzymatic transesterification of the rapeseed methyl ester with TMP, the maximum conversion obtained at 3:3:1 and 3:5:1, respectively (Uoskainen et al., 1998). During the process, microbes possess the potential to produce biosurfactants. The interest in microbial surfactants has been steadily increasing due to their diversity, environmentally friendly characteristics. Therefore, the possibility of production, fermentation and their potential applications in such areas as the environmental production, crude oil recovery, health care and food processing industry.

Biosurfactants are biological surface-active compounds released by the microorganisms (Salihu A.,2009). are lower the surface tension of water and other solvents, and have potential applications in environmental uses such as organic pollutants treatment and oil recovery. Biosurfactants have also been reported to be produced by many fungi and bacteria during the degradation of hydrocarbons (Neves et al., 2007). The production of biosurfactants is often related to the consumption of hydrocarbons, including oily residues, and occurs during the exponential cellular growth (Cirigliano MC and Carman GM .1984). But they can also be produced from the sugars (sucrose, glucose and lactose), glycerol, vegetable oils, or starch as carbon sources (Robert et al.,1989). Candida *lipolytica* has been reported to produce biosurfactant using corn oil as substrate. Many microorganisms synthesize biosurfactants using different carbon sources. Many studies reported that the yield of a biosurfactant varies depending on the carbon source and the nutrient medium (Bodour et al.,2003; Soberón-Chávez et al.,2005; Guerra-Santoset al.,1984). Crude oil, glucose, sucrose, and glycerol have been reported as good carbon sources for biosurfactant production (Desai JO, Banat IM.1997; Banat et al., 2010).But, the composition and yield depends on the culture conditions as well as the producer strain. There are a number of studies in the literature about effects of various factors on biosurfactant production. The carbon, nitrogen source, and the culture conditions, such as pH, temperature, influence the quality and quantity of biosurfactant. Additionally, recombinant and mutant producer strains may give high yields of biosurfactants and can be an important step to their economical production (Naganishi, K.1975).

With the above background, the present investigation has been attempted to use microbes as such to reduce the viscosity of vegetable oil and to find out the possibility of obtaining biosurfactant during the process. Neem oil used as substrate for the production of biosurfactant. Neem has been reported to contain several biologically active constituents such as Azadiractin (Warthen et al.,1978), (salanin Lavie et al., 1965), Meliantriol (Shin-foon C,1984), as well as nimbin and nimbidin (Figure 1,2&3). The microbes such as *pseudomonas putida* and *pseudomonas aeruginosa* known to degrade hydrocarbons and produced biosurfactants. The oil degrading bacteria of *P. putida*, called "multi-plasmid hydrocarbon-degrading Pseudomonas," Different studies have identified numerous oil degrading bacteria capable of utilizing crude oil as a source of carbon and energy. Moreover, *Pseudomonas* species are able to grow in simple media and the nutritional versatility of a number of them includes the ability to utilize many compounds as substrates.

Therefore, the objective of the present work is,

1. To study the possibility of using microbes *Pseudomonas putida* and *Pseudomonas aeruginosa* for reducing the viscosity of neem oil and its possible use as biodiesel.

2. To find out production of biosurfactant by the above microbes using neem oil.

3.Analysis of Gas chromatographyTherefore, the objective of the present work is,

Materials and Methods

Extraction of oil from neem press cake :For the present study, 25gm of neem press cake powder has -

placed in butt tubes of the soxhlet's apparatus. The solvent hexane was taken in the extraction chamber to extract the oil. The condenser over the extraction chamber was connected to tap for water circulation, and the temperature maintained at 60°C. The condensed vapour collected in the extraction chamber, where it dissolves the fat present in the sample. Then the setup was kept undisturbed for approximately 6 hours. Then the extract was poured into the petri dish. After pouring, the petri dish containing extract of hexane incubated at 60°C for 6 hours. After the incubation period is over, the evaporated solutions collected by using the same solvents used at the beginning (Hexane). To that, hexane (12.5 ml) and aqueous ethanol (1:11:5) added. Then the extract and oil formed the immiscible layer. By using the separating funnel, the oil was separated and used for further studies.

Inoculum and media

For further study, Neem press cake, Extracted Neem oil used as a substrate. The bacterial species *Pseudomonas putida*, and *Pseudomonas aeruginosa* obtained from laboratory. The strain was cultured in 50 ml of four different medium, 10ml of distilled water, acetone and hexane extracts of neem oil amended in the medium as an energy source.

Control and growth conditions

Un Inoculated medium with the carbon sources (extract of neem oil) maintained at room temperature with neutral pH served as control. Another control maintained with inoculated stone medium with carbon sources. The culture conditions are as follows pH4 – pH9, temperature 25°C ,35°C, 40°C in BOD incubator, and 20mg of Nitrogen sources viz., (NH4) 2HPO4 (di-ammonium hydrogen orthophosphate), (NH4)2SO4 (Ammonium sulphate), NH4Cl (Ammonium chloride) and KNO3(Potassium nitrate) per 50ml of stone medium with neutral pH amended medium.

Biomass and biosurfactant production by bacteria using neem oil

The 5ml oil extracts (distilled water, acetone and hexane) obtained using 10ml of extractives. The extract centrifuged and the supernatant taken in the conical flask. This extract served as a carbon source. In each extract, 50ml of stone medium added into the flask and inoculated with bacterial species pseudomonas putida and pseudomonas aeruginosa incubated for six days.

Isolation of biosurfactant[20]

After separating the biomass, the culture filtrate was centrifuged at 10,000rpm for 30 minutes to remove any debris. The clear supernatant treated with three volumes of ice-cold acetone. The precipitate formed is collected by centrifugation at 5,000rpm for 30 minutes.

Gas chromatography analysis

Working standard of 2µl was prepared from the stock solution by dilution and used in finding of the retention times and qualification of the compounds in GC-ECD.

End analysis

Oil was estimated by gas chromatography model varian Cp 3800 equipped with electron capture detector (ECD) fitted with capillary column. The following were the operating parameters.

Detector	ECD
Temperature C	Column – 250C Injector – 250C Detected – 250C
Column	1/8 inch 55 Packing – OV17
Nitrogen flow rate	30ml/min
Threshold	10 µv
Volume injected	2µl

Analytical methodology

Sample (2µl) oil was injected into the injection port by using the injection needle. In GC unit carrier gas used will ensure the migration of the components of the sample. The column used was 1/8 inch 55 packing OV-17. The ECD detector was used to

observe free electrons entered by radioactive sources. The current produced by free electrons were detected by detector, when current is decreased. The response of the detector is plotted by the recorder which furnishes the chromatogram. Then the physical measurement like retention time, peak height and areas were measured.

Determination of relative and absolute viscosity method:

The viscometer was first thoroughly cleaned and dried. A definite quantity of water (15ml) was introduced into the bulb and sucked up through the capillary into the smaller bulb. Water was allowed to touch the mark and it was held there by placing the finger at the top of the narrow limb. Finger was removed as soon as it reached the mark and the time for water to reach the mark below the bulb was noted. Process was repeated 3-4 times and took the mean value. Before noting the time, the viscometer was kept in a thermostat for 15 minutes, so that the roomtemperature is acquired.

Viscometer was dried and filled with 15ml of non-degraded oil/ degraded oil/ hexane or acetone extract of oil and kept in the thermostat for 15 minutes. So that the liquid obtained the room temperature. Time was noted during the flow of liquid between the 2 marks above and below the bulb. The process was repeated 3-4 times and the mean value was taken. Washed, dried and empty pyknometer (specific gravity bottle) was weighed. It was filled with water and finally with 15ml non -degraded oil/ degraded oil/hexane or acetone extract of oil weighed. Noted the room temperature by recording the temperature of water in the thermostat.

Calculation

Density of water		
Weight of empty pyknometer	=	gms
Weight of pyknometer with water	=	gms
Weight of water	=	gms
Volume of water	=	15cc
Density of water (d1)	=	weight/volume
Density of oil:		
Weight of empty pyknometer	=	gms
Weight of pyknometer with oil	=	gms
Weight of oil	=	gms
Volume of oil	=	15cc
Density of oil (d2)	=	/15
Time taken for the flow of 15ml of water (t1)	=	sec
Time taken for the flow of 15ml of oil (t2)	=	sec
Room temperature	=	300K
Viscosity of water (n1)	=	0.8545
Viscosity of oil (n2)	=	d2t2/d1t1×n1

Result and discussion

Suitable medium

Four different liquid medium used for further study such as Bushnell and Hass, Nutrient broth, Mineral medium and Stone medium to find out the efficiency in supporting the growth of the oil-degrading microbes with water, acetone, and hexane extract of neem oil as carbon sources, under standard laboratory conditions. The optical density of the culture measured at 420nm at the end of the log phase. The results suggested that the stone medium is suitable for the present study (Table 1). The composition of the stone medium is Calcium carbonate, Ammonium nitrate, Di-sodium hydrogen phosphate, Potassium dihydrogen phosphate, magnesium sulphate, Manganous chloride, Calcium, zinc and ferrous. The oil-degrading bacterial species *Pseudomonas putida* and *Pseudomonas aeruginosa* utilized with acetone, water and hexane extract of carbon source and produced better growth in the stone medium.

Two controls were maintained simultaneously to verify whether the growth of the microbes enhanced by the extract. Control I was only medium, uninoculated microbes and no energy source, and control II was medium, extract and without microbial inoculants. The experimental flask contained an inoculated medium with the extract.

Biomass and Biosurfactant production

To find out whether oil-degrading microbes can produce biomass and biosurfactant utilizing neem oil. The bacteria *pseudomonas putida* and *pseudomonas aeruginosa* inoculated into the stone medium and extracted neem oil as an energy source. At the end of the log phase, the biomass separated, and culture filtrate tested for the presence of biosurfactant. Effect of various environmental parameters such as nitrogenous source, pH, temperature and nutritional conditions of the medium were also studied (Table 2)

Table 1 Suitable medium for the growth of oil degrading microbes

Medium O.D@420nm	Organism	Control-I (M+I) nm	Control-II (M+E) nm			Control-III (M+E+I) nm		
			Water	Acetone	Hexane	Water	Acetone	Hexane
Bushnell & Hass Medium	P.P	0.15	_	_	_	0.35	0.28	0.40
	P.A	0.12				0.27	0.19	0.29
Mineral Medium	P.P	0.12	_	_	_	1.0	0.50	0.89
	P.A	0.09				0.90	0.40	0.81
Nutrient Broth Meduim	P.P	0.56	_	_	_	1.5	0.59	1.4
	P.A	0.47				1.3	0.85	1.2
Stone Medium	P.P	0.80	_	_	_	1.8	0.95	1.7
	P.A	0.75				1.7	0.89	1.5

P.P-Pseudomonas putida, P.A –Pseudomonas aeruginosa, M+I - Medium+Inoculant, M+E - Medium+Extract, M+E+I - Medium+Extract+Inoculant

Table 2 Biomass and Biosurfactant production of Oil Degrading Microbes

Factors	Water Extract (nm)				Acetone Extract (nm)				Hexane Extract (nm)			
	P.putida		P.aeruginosa		P.putida		P.aeruginosa		P.putida		P.aeruginosa	
	BM	BS	BM	BS	BM	BS	BM	BS	BM	BS	BM	BS
C1	-	-	-	-	-	-	-	-	-	-	-	-
C2	-	-	-	-	-	-	-	-	-	-	-	-
C3	0.72	0.48	0.58	0.32	0.56	0.25	0.27	0.12	0.80	0.53	0.50	0.27
(NH4) 2HPO4	1.27	0.59	0.80	0.45	0.59	0.28	0.52	0.31	1.38	0.55	0.92	0.43
(NH4) 2SO4	0.94	0.30	0.27	0.13	0.30	0.16	0.28	0.09	1.02	0.48	0.63	0.38
(NH4) Cl	1.24	0.52	0.68	0.34	0.25	0.10	0.43	0.20	0.47	0.25	0.28	0.12
KNO3	-	-	-	-	-	-	-	-	-	-	-	-
pH4	-	-	-	-	-	-	-	-	-	-	-	-
pH9	0.26	0.13	0.32	0.16	0.15	0.07	0.33	0.17	0.24	0.12	0.32	0.18
25°C	0.31	0.10	0.23	0.11	-	-	-	-	0.32	0.15	-	-
30°C	0.76	0.46	0.58	0.32	0.56	0.25	0.29	0.13	0.80	0.53	0.51	0.24
35°C	-	-	-	-	-	-	-	-	-	-	-	-
40°C	-	-	-	-	-	-	-	-	-	-	-	-

Biomass and Biosurfactant production

To find out whether oil-degrading microbes can produce biomass and biosurfactant utilizing neem oil. The bacteria *pseudomonas putida* and *pseudomonas aeruginosa* inoculated into the stone medium and extracted neem oil as an energy source. At the end of the log phase, the biomass separated, and culture filtrate tested for the presence of biosurfactant. Effect of various environmental parameters such as nitrogenous source, pH, temperature and nutritional conditions of the medium were also studied (Table 2).. *Pseudomonas putida* produced a significant amount of biomass and biosurfactant in the presence of $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$. Alkaline conditions and room temperature (30°C) also favoured the growth of *pseudomonas putida.* The acidic condition did not show much influence. All the other factors had no favourable effect. The nitrogenous source $(NH_4)_2HPO_4$ enhanced the biomass and biosurfactant production by pseudomonas aeruginosa. $(NH_4)_2SO_4$ also enhanced better production. Other factors had an influencing effect on this bacteria excepting KNO_3 and higher temperature.]

GC Analysis:

To find out the components present in the non degraded neem oil and degraded neem oilanalysed to compare the components.

Table-3: Changes in viscosity of neem oil

Sl.No	Nature of Oil	viscosity
1	Actone(Neemoil) extract	2.64 centipoise
2	Hexane (neem oil) extract	2.30 centipoise
3	Non-degraded neem oil	200 centipoise
4	Neem oil degraded I *pseudomonas putida* Ii *pseudomonas aeruginosa*	65.16 centipoise 117.4 centipoise

Figure 1. Neem tree

The results showed (Figure 4) 14 peaks representing the presence of number of 14 components of which 4 peaks appeared to be significant. All the peaks in the neem oil could be recorded by 15 mins.

GC Analysis of hexane extract of neem oil degraded by pseudomonas putida showed that almost all the components were progressively utilized by the microbes and therefore only one peaks appeared in the GC Analysis. The degraded peaks appeared for about 23 minutes. (Figure 5)

Changes in viscosity of neem oil degraded by bacteria

Previous experiments indicated that the bacterial species could grow well using neem oil. To find out whether this ability of the microbes could be reducing the viscosity of neem oil. In the present experiment viscosity is an essential factor that decides the flow properties of oil and uses as biodiesel in engines. Bacteria were allowed to grow on the oil, and changes in viscosity tested. Hexane, acetone extracts of neem oil, non-degraded neem oil and degraded neem oil tested for this purpose. Viscosity was very much less in hexane and acetone extracts of the oil. But, the higher viscosity showed in non-degraded neem oil, which is reduced 75% viscosity by inoculated pseudomonas*putida*. The results presented in Table3.

Conclusion

As the results suggest, *Pseudomonas putida* degraded most of the components as well as reduce the viscosity by 60%. The use of *pseudomonas putida* enhancing the fuel efficiency of neem oil by decreasing the viscosity and other properties as per recommended standards could be explored further. Moreover, Surfactants have several applications in agriculture and agrochemical industries. This studies will help in replacing the harsh chemical surfactants with green ones. Several researchers indicate that a variety of environmental niches such as soil, water, and leaf surface explored for biosurfactant producing bacteria. Hence, *pseudomonas putida* is the best and safest oil -degrading organism.

Figure 2. Neem press cake

Figure 3. Neem oil

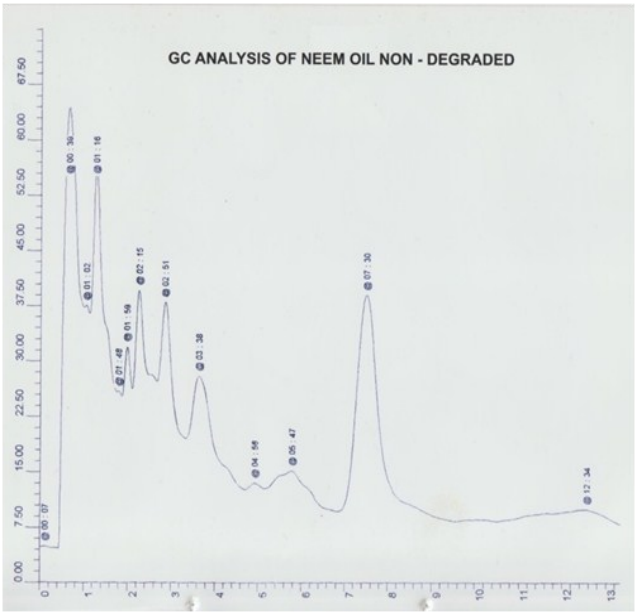

Figure 4. GC Analysis of neem oil non-degraded

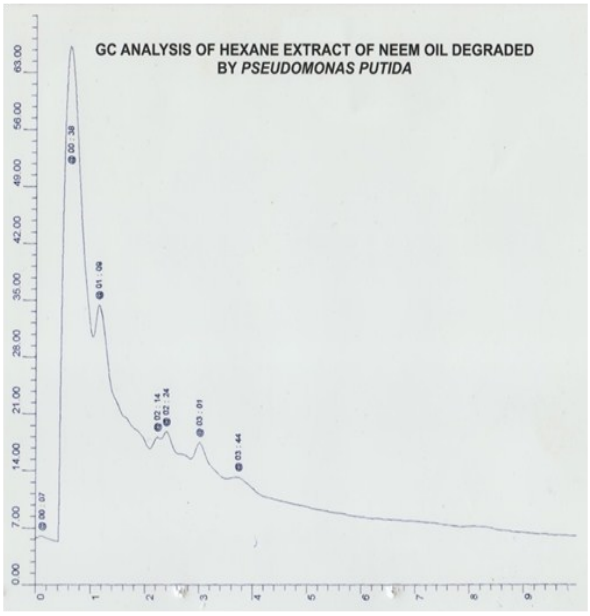

Figure 5. GC Analysis of neem oil non-degraded

References

Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R. 2010.Microbial biosurfactants production, applications and future potential, ApplMicrobiolBiotechnol, 87:.427-444.

Bodour AA, Drees KP, Maier RM.2003. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils, Appl. Environ. Microbiol, 69(6): 3280-3287. [Links]

Buchanan, R.A, Oleyfit, Russel C.R, Cull, I.M. 1978. Whole plant oil, potential new industrial raw materials. J.Am. oil chem. Soc. 55:657-62.

Cirigliano MC, Carman GM .1984. Isolation of a Bioemulsifier from Cândidalipolytic. Appl Environ Microbiol. 48: 747-750.

Desai JO, Banat IM.1997. Microbial production of Biosurfactants and their commercial potential. Microbial.Mo.Rev.61:47-64.

Dilip Biswas, 2003. Development of Biofuels, CPCN, New Delhi from http:/WWW.Cpcb.nic.in

Goering C.E., Schwab A.W., Daugherty M.J., Pryde E.H., Heakin A.J. 1987. Fuel properties of eleven vegetable oils. Transesterification of the ASAE.25:1472-7.

Guerra-Santos LH, Kappeli OR, Fiechter A.1984. Pseudomonas aeruginosabiosurfactant production in continuous culture with glucose as carbon source, Appl. Environ. Microbiol, 48 (2): 301-305

Hallberg, M.L; Wang .D and Harrod,1999. Enzymatic -

synthesis of wax esters from rape sedd fatty acid methyl esters and fatty alcohol . J.Amer.Chem.Soc.76:183-189.

Hayes, DG and Kleiman .R. 1996. Lipase catalyzed synthesis of lesquerolic acid wax and diol esters and their properties. J Amer. Oil.Chem.Soc. 73:1385-1392.

Lavie. D, Jain MK, Shapan-GabrielithSR(1965). A locust phagorepellent from two Melia species. Chem.Commun.910-911.

Naganishi,K.(1975).Structure of the insect antifeedantaza-dirachtin.In:Recent advance in phyto chemistry, V C Runeckles (ed.), Plenum,Newyork,N.Y.Vol.5 pp.283-298.

Neves LCM, Oliveira KS, Kobayashi MJ, Penna TCV, Converti A.2007. Biosurfactant Production by Cultivation of Bacillus atrophaeus ATCC 9372 in Semidefined Glucose/Casein-Based Media. ApplBiochemBiotechnol. 137-140: 539-54.

Peter, Vedamuthu and Rangasamy. 2003.Jatropha crucas oil-A green fuel with immense future.MNES project report pp.110-114.

Rangaraju. K, 2005.Manufacturing of Bio-diesel. Production of biodiesel.

Robert M, Mercade ME, Bosch MP, Para J, Espiny MJ, Manresa MA, Guinea J. 1989.Effect of the carbon source on biosurfactant production on Pseudomonas aeruginosa 44 T1, BiotechnolLett, 11: 871-874. [Links]

Salihu A, Abdulkadir I, Almustapha MN. An investigation for potential development on biosurfactants. BiotechnolMolBiol Rev. 2009; 4: 111-117.

Soberón-Chávez G, Lépine F, Déziel E. 2005.Production of rhamnolipids by Pseudomonas aeruginosa, ApplMicrobiolBiotechnol, 68: 718-725. [Links]

Uoskainen, E,;Linko, Y.Y.; Lamsa, M.; Tervakan-GAS,1998.T and rapeseed oil methyl ester to environmentally acceptable lubricants. J.Amer.Oil.Chem.Soc.75:1557-1563.

Warthen JD Jr, Uebel EC, Dutky SR, Lusby WR, Finegold H (1978).An adult housefly feeding deterrent from neem seeds. UD Agric. Res. Results RR-NE2

Shin-foon C (1984). The active principles and insecticidal properties of some Chinese plants, with special reference to Meliaceae. Pp. 255-262. Ibid.