CHANGES IN VISCOSITY OF NEEM OIL AND BIOSURFACTANT PRODUCTION BY HYDROCARBON DEGRADING MICROBES

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ABSTRACT

Depletion of fossil fuels has created a necessity to find out alternative sources of non-conventional energy. Plants containing secondary metabolites such as, oil and hydrocarbons that are attractive alternate energy and chemical sources. Realizing this important, the present investigation has been attempted, neem oil was extracted from neem press cake by using hexane and aqueous ethanol form the soxhlet's apparatus. Oil was separated and used for further studies. To reduce the viscosity of extracted neem oil used by microbes to find the possibility of obtaining biosurfactant during the process. The oil degrading fungal species such as *Aspergillus oryzae* and *Penicillium chrysogenum* could grow well in stone medium among the four different medium. Soluble fraction of water and hexane extracts of neem oil used as carbon source. In this experiments two controls were maintained simultaneously to verify whether the growth of the microbes is enhanced by the extract. Control I was only medium with uninoculated microbes and no energy sources, control II was medium with extract without microbial inoculants. The control III flask contained inoculated medium with neem oil extract and nitrogen sources maintained at different temperature and pH level. The fungal species *Aspergillus oryzae* produce better biomass and biosurfactant in the presence of nitrogen source $(NH_4)_2HPO_4$. Higher biomass and biosurfactant could be correlated with viscosity of the oil. The result was Aspergillus *oryzae* degraded the oil and reduced the viscosity from 200 to 50 which suggested the possibility of uses of this microbe for enhancing the fuel efficiency of neem oil for engines.

KEY WORDS

Biomass, Biosurfactant, Oil degrading, Neem press cake, Neem oil, Hydrocarbon-degrading.

INTRODUCTION

Utilization of whole plant oils as an alternative source of conventional and non –conventional oils and major industrial feed stocks is gaining greater importance throughout the world (Goering and Schwab et al, 1987). Plants containing secondary metabolites such as, oil and hydrocarbons that are attractive alternate energy and chemical sources. There are several reports of plant species evaluated for their potential as an alternate source of energy and hydrocarbons. (Buchanan et al, 1978).

Use of vegetable oils as 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).

But in a technical point of view, vegetable oils cannot be used directly as fuel. They consists of glycerol esters, fatty acids with varying carbon chain length and double and triple bonds giving rise to molecular weight with range of 650-970 and higher viscosity which decreases their flow property. Therefore, vegetable oils are 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 petro diesel up to 25% and if esterified then the proportion could go beyond 75%. Transesterification may be done either by using acid or alkali. The process involves maintenance of continuous contact with enzyme and fresh fat or oil.

NEEM PRESS CAKE

NEEM OIL

It is also possible to use the biotransformation ability of microbes which synthesis the enzymes having transesterification properties. It is also known that few microbes' possess the potential to produce biosurfactant, the surface active molecules formed during the process of utilization of oil by fermentation. *Candida lipolytica* has been reported to produce biosurfactant using corn oil as substrate. Biosurfactants have also been reported to be produced by many fungi and bacteria during the degradation of hydrocarbons. The interest in microbial surfactants has characteristics, the possibility of their production, fermentation and their potential applications in such areas as environmental protection, crude oil recovery, health care and the food processing industry.

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 by microbes such as *Aspergillus oryzae and Penicillium chrysogenum* known to degrade hydrocarbons and produced biosurfactants. Fungi are known to be one of the best oil-degrading organisms (Batelle CD. 2000), (Ojo OA. 2006). Different studies have identified numerous fungal genera capable of utilizing crude oil as a source of carbon and energy. The ability of these microbes to reduce the viscosity of neem oil which was used as substrate has also been tested. Since, neem oil is highly viscous and contains several terpenoids, steroids, alkaloids, flavonoids and glycosides etc. Neem has been reported to contain several biologically active constituents such as Azadirachtin (Naganishi, 1975), Meliantriol (Lavie et al, 1965), Salanin (Warthen et al., 1978) as well as nimbin and nimbidin (Shin-foon, 1984).

Therefore the objective of the present work is, 1. To study the possibility of using microbes A*spergillus oryzae* and *Penicillium chrysogenum* 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.

MATERIALS AND METHODS

EXTRACTION OF OIL FROM NEEM PRESS CAKE

To study the oil compounds in press cake, 25gm of neem press cake powder has been 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 was maintained at 60°C. The condensed vapour is 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 petri dish. After pouring, the petri dish containing extract of hexane was incubated at 60°C for 6 hours. After incubation period is over, the evaporated solutions were collected by using the same solvents which was used at the beginning (Hexane). To that hexane (12.5 ml) and aqueous ethanol (1:11:5) was 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 substrate. The fungal species *Aspergillus oryzae*, *Penicillium chrysogenum* were obtained from laboratory. The strain was cultured in 50 ml of stone medium, 10ml of distilled water, acetone and hexane extracts of neem oil was amended in the medium as 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 was 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., $(NH_4)_2HPO_4$ (di-ammonium hydrogen orthophosphate), $(NH_4)_2SO_4$ (Ammonium sulphate), NH_4Cl(Ammonium chloride) and KNO_3(Potassium nitrate) per 50ml of stone medium with neutral pH was amended medium.

BIOMASS AND BIOSURFACTANT PRODUCTION BY FUNGI USING NEEM OIL

Distilled water, acetone and hexane extracts of 5 ml of oil was obtained using 10ml of extractives. The extract was centrifuged and the supernatant was taken in conical flask. This extract served as carbon source. To each of these extracts 50ml of stone medium was added and inoculated with fungal species *Aspergillus oryzae*, and *Penicillium chrysogenum* are incubated for 6 days.

ISOLATION OF BIOSURFACTANT (Swaranjit Cameotra, 1995)

After separating the biomass, the culture filtrate was centrifuged at 10,000rpm for 30 minutes to remove any debris. The clear supernatant was then treated with 3 volumes of ice cold acetone .The precipitate formed is collected by centrifugation at 5,000rpm for 30 minutes.

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 room temperature 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

EXPERIMENTAL RESULT

SUITABLE MEDIUM FOR THE GROWTH OF OIL DEGRADING MICROBES USING AS ENERGY SOURCE

In this experiment, using four different liquid medium such as Bushnell and Hass, Nutrient broth, Mineral medium and Stone medium were tested for their efficiency in supporting the growth of the oil degrading microbes with water, acetone, and hexane extract of neem oil as carbon sources, under normal laboratory conditions. Two controls were maintained simultaneously to verify whether the growth of the microbes enhanced by the extract. Control I was only medium with uninoculated microbes with no energy source and control II was medium with extract without microbial inoculants. The experimental flask contained inoculated medium with extract. Optical density of the cultures were measured at 420nm at the end of the log phase and the results were recorded. The results are presented in table 1. The results suggested that stone medium is suitable for the present study.

SUITABLE MEDIUM FOR THE GROWTH OF OIL DEGRADING MICROBES- Table -1.

MEDIUM (O.D at420nm)	ORGANISM	CONTROL-I (M+I)	CONTROL-II (M+E)			CONTROL-III (M+E+I)		
			WATER	ACETONE	HEXANE	WATER	ACETONE	HEXANE
BUSHNELL& HASS MEDIUM	A.O	0.13	–	–	–	0.30	0.22	0.35
	P.C	0.12				0.25	0.19	0.21
MINERAL MEDIUM	A.O	0.11	–	–	–	1.0	0.47	0.85
	P.C	0.09				0.90	0.40	0.81
NUTRIENT BROTH MEDIUM	A.O	0.55	–	–	–	1.4	0.56	1.3
	P.C	0.40				1.3	0.85	1.2
STONE MEDIUM	A.O	0.80	–	–	–	1.7	0.90	1.5
	P.C	0.72				1.5	0.85	1.3

A.O-*Aspergillus oryzae*, P.C -*Penicillium chrysogenum*

M+I-MEDIUM+INOCULAN,

M+E-MEDIUM+EXTRACT,

M+E+I – MEDIUM+EXTRACT+INOCULANT

BIOMASS PRODUCTION BY THE FUNGI *ASPERGILLUS ORYZAE* AND *PENICILLIUM CHRYSOGENUM* USING THE NEEM OIL

To find out whether oil degrading microbes have the ability to produce biomass and biosurfactant utilizing neem oil. The fungi *Aspergillus oryzae* and *Penicillium chrysogenum* were inoculated in stone medium with extracted neem oil as an energy source. At the end of log phase the biomass was 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.

BIOMASS PRODUCTION

Aspergillus oryzae produced significant amount of biomass in the presence of $(NH_4)_2HPO_4$ AND $(NH_4)_2SO_4$. Alkaline conditions and room temperature (30°C) also favoured growth of *Aspergillus oryzae*. Acidic condition did not show much influence. All the other factors had no favourable effect. The result showed in table2.

BIOMASS PRODUCTION OF OIL DEGRADING MICROBES-Table-2.

FACTORS	FRESH WEIGHT Mg/50ml of medium		DRY WEIGHT Mg/50ml of medium	
	I	II	I	II
CONTROL I	1.98	0.89	0.88	0.76
CONTROL II	-	-	-	-
$(NH_4)_2HPO_4$	5.16	4.39	2.09	1.38
$(NH_4)_2SO_4$	4.92	3.85	1.96	0.97
$(NH_4)CL$	4.11	4.11	1.76	0.82
KNO_3	2.34	2.19	1.76	0.82
pH-4	2.02	2.89	0.78	0.63
pH-9	3.29	3.96	1.15	1.01
25°C	3.23	3.01	1.18	1.02
30°C	4.96	4.26	2.23	1.08
35°C	2.04	2.08	1.07	0.56
40°C	1.92	1.08	0.99	0.48

I-*ASPERGILLUS ORYZAE*, II-*PENICILLIUM CHRYSOGENUM*

The nitrogenous source $(NH_4)_2HPO_4$ enhanced the biomass production by *Penicillium chrysogenum*. *(*$(NH_4)_2SO_4$ also enhanced better biomass production. Other factors had influencing effect on this fungi excepting KNO_3 and higher temperature.

BIOSURFACTANT PRODUCTION BY THIS FUNGI *ASPERGILLUS ORYZAE* AND *PENICILLIUM CHRYSOGENUM* USING THE NEEM OIL

Both the fungi studied produced biosurfactant during the degradation process. Normal laboratory temperature 30°C and the presence of nitrogenous sources excepting KNO_3 favoured biosurfactant production.

BIOSURFACTANT PRODUCTION BY FUNGI USING THE NEEM OIL-TABLE -3

FACTORS	BIOSURFACTANT Mg/50ml of medium	
	I	II
CONTROL I	0.03	0.025
CONTROL II	-	-
$(NH_4)_2HPO_4$	0.40	0.32
$(NH_4)_2SO_4$	0.28	0.21
(NH4)CI	0.37	0.28
KNO_3	0.08	0.04
pH-4	0.06	0.03
pH-9	0.13	0.11
25°C	0.16	0.10
30°C	0.18	0.14
35°C	0.9	0.4
40°C	0.4	0.09

I-ASPERGILLUS ORYZAE, II- PENICILLIUM CHRYSOGENUM

The best results were obtained in the presence of $(NH_4)_2HPO_4$ for both *Aspergillus oryzae* and *Penicillium chrysogenum.*

CHANGES IN VISCOSITY OF NEEM OIL DEGRADED BY FUNGI

Previous experiments indicated that, the fungal species could grow using neem oil. To find out whether this ability of the microbes could be reduce viscosity of neem oil. The present experiment was done l since viscosity is an important factor that decides the flow properties of oil. To be used as biodiesel in engines. Fungi were allowed to grow on the oil and changes in viscosity was tested. Hexane, acetone extracts of neem oil, non-degraded neem oil and degraded neem oil were tested for this purpose. Viscosity was very much less in hexane and acetone extracts of the oil. Non degraded pure neem oil showed higher viscosity and when it was allowed for degradation by microbes, 75% of viscosity was reduced, when *Aspergillus oryzae* was inoculated. *Penicillium chrysogenum* decreased the viscosity only by 50%.The results are presented in table-4.

CHANGES IN VISCOSITY OF NEEM OIL-Table-4.

S.NO	NATURE OF OIL	VISCOSITY
1	ACETONE(NEEM OIL) EXTRACT	2.64 centipoise
2	HEXANE (NEEM OIL)EXTRACT	2.30 centipoise
3	NON-DEGRADED NEEM OIL	200 centipoise
4	NEEM OIL DEGRADED I *ASPERGILLUS ORYZAE* II *PENICILLIUM CHRYSOGENUM*	 54.84 centipoise 90.86 centipoise

DISCUSSION

Non edible oils offer excellent scope for conversion into Biodiesel. Pongamia oil and jatropha oil have already established their credentials admirably in this field. Though cleaned, dried and filtered raw oil can be directly used in diesel engines with or without modifications in engines, transesterification of these oils seems too beneficial as it reduces the exhaust emission. However the process of transesterification of oils involved in the production of methyl esters is proving to be costlier. Therefore, there is a need for development of new technologies for converting bio-oils to fuels. It is also known that apart from transesterification biofuel from vegetable oils can be made efficient by micro emulsification which is nothing but the dispersions of oil, water

and surfactant and often a small amphiphilic molecule called co surfactant. A micro emulsion of methanol with vegetable oil can perform nearly as efficient as diesel fuels.

In the present investigation efficiency of microbes is enhancing the fuel capacity of neem oil has been tested. The experiment performed to find out the suitable medium suggested that in stone medium with water and hexane extracts of neem oil as energy sources, the oil degrading microbes could produce appreciable amount of biomass and biosurfactant. Presence of nitrogenous sources in the medium such as $(NH_4)_2HPO_4$ seemed to play a significant role in biomass and biosurfactant production under laboratory conditions and neutral pH. Higher biomass and biosurfactant production could be correlated with viscosity changes of oil.

In the transesterification of vegetable oils, the process is catalysed by either acid (sulphuric acid) or a base such as sodium hydroxide. Although the enzyme catalysed transesterification process are not yet commercially developed, new results have been reported in recent articles and patents. The common aspects of these studies consist in optimizing the reaction conditions (solvent, temperature, pH, type of microorganism which generates the enzymes etc.) in order to establish suitable characteristics for an industrial application (Posorske, 1984).

Viscosity changes tested for hexane and acetone soluble fractions of neem oil, degraded and non-degraded neem oil suggested that hexane and acetone soluble fraction of neem oil has less viscosity and the oil degrade by *Aspergillus oryzae* decreased the viscosity of oil by 75% i.e. from 200 to 54.84. It is to be noted that viscosity of transesterified biodiesel of *jatropha curcus* was found to be 50.73, for madhuca and pongamia oil it is 45.36 and 48.48 respectively (Naresh Kaushik and sushil kumar). Therefore, the use of *Aspergillus oryzae* for enhancing the fuel efficiency of neem oil by reducing the viscosity and other properties as per recommended standards could be explored further.

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