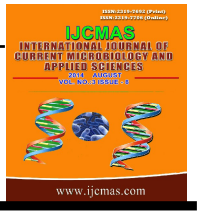
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 9 (2014) pp. 47-57**
http://www.ijcmas.com



Original Research Article

Evaluation of Oleaginous Bacteria for Potential Biofuel

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A B S T R A C T

The present work was aiming to exploit bacteria for biodiesel production, twenty two bacterial isolates were screened for lipid content by Sudan black B staining method, among those three isolates is lipid rich viz, *F. oryzihabitans*, *P. aeruginosa* and *Morococcus* sp. which have been taken for lipid extraction by Bligh and dyer method and thin layer chromatography for determination of fatty acid, which revealed that the amount of lipid was highest in *Morococcus* sp. (0.68mL/100mL) of Lauric acid (C12). Optimization of C/N ratio the amount of lipid was ranged highest in *Morococcus* sp. i.e. carbon (0.70mL/100mL) of palmitic acid (C18), nitrogen (0.65mL/100mL) of arachidonic acid (C20) and at pH 7.0 (1.96mL/100mL) of oleic acid (C18) and in strain improvement by mutagenesis *F. oryzihabitans* got improved and accumulate high lipid at 10 min (1.50mL/100mL) of linoleic acid (C16).

K e y w o r d s

Oleaginous bacteria, Optimization, Strain improvement, Thin layer chromatography.

Introduction

Biofuels are of rapidly growing interest for reasons of energy security, diversity and sustainability as well as for greenhouse gas mitigation (Kent Hoekman, 2009), according to International Energy agency. Biofuels have potential to meet more than a quarter of world demand for transportation fuels by 2050 (Volk *et al.,* 2000). Recently, with global shortage of fossil fuels, excessive increases in price of crude oil and increased environmental concerns have resulted in rapid growth in biodiesel production. In biodiesel production, the transesterification reaction which could be catalyzed either chemically or enzymatically (Akhil *et al.,* 2010), this novel approach might pave way for industrial production of biodiesel equivalents from renewable resources by employing engineered micro-organisms (Rainer *et al.,* 2006). Biodiesel refers to a vegetable oil or animal fat based diesel fuel consisting of long chain alkyl (methyl, ethyl or propyl) esters (Demisbas, 2009) which is typically made by chemically reacting lipids with an alcohol producing fatty acid esters.

In recent years, much attention has been paid to exploration of microbial oils, which might become one of the potential oil sources for biodiesel production in future (Qiang *et al.,* 2008). Not all microorganisms can be considered as abundant sources of oils and fat, those microorganisms that do

produce a high content of lipid are termed as oleaginous. The lipid which accumulates in oleaginous microorganisms is mainly triacylglycerol (Hull, 2010) and these microorganisms have been considered as an alternative to agricultural and animal oil and fat sources (Xin *et al.,* 2009).

Biodiesel production using microbial lipids which is named as single cell oils (SCO) has attracted great attention in the whole world; there are various kinds of microorganisms that accumulate lipids such as Bacteria, Fungi and Algae (Joseph, 2006), the regulation mechanism of oil accumulation in these microorganism and approach of making microbial diesel is economically competitive (Xin *et al.,* 2009).

The genus like *Lipomyces, Yarrowia, Cryptococcus, Rhodosporidium* (Li *et al.,* 2007) are known to accumulate between 40% to 70% of their biomass as lipids, the moulds of Zygomycetes like *Mortierella* and *Cunninghamella* also possess the ability of lipid accumulation (Papanikolaou *et al.,* 2007) and the major fatty acids present in single cell oil are oleic acid, linoleic acid (Chen and Liu, 1997), palmitoleic acid, arachidonic acid, palmitic acid and stearic acid (Peng and Chen, 2008). This reviews the property of oleaginous microorganism with emphasis on biotechnological strategies for increasing lipid production such as mutation technique, genetic and metabolic engineering.

Hence, with the above knowledge acquired through literature and with the aim of our research work pertaining to isolation and characterization of oleaginous bacteria from soil samples, lipid profiling from screened isolates and optimization studies in selected oleaginous microorganisms have been framed to carry out the research work.

Materials and Methods

Present investigation has been carried out to evaluate the oleaginous bacteria for lipid production. Experiments comprises of sample collection from fields and laboratory studies. All experiments were conducted in triplicates.

Soil sample: Soil samples like decomposed, house backyard, farm yard soil, Kolar gold field soil, University campus soil were collected for isolation of oleaginous bacteria (Aneja, 2006).

Serial dilution method: It was performed according to standard protocol.

Plating: It was done in both pour plate method and streak plate method**,** discrete colonies from a mixed culture was done by selection of individual cells that were picked up with a sterile needle and transferred to separate nutrient agar slants to obtain pure culture of single bacterial species (Cappuccino and Sherman, 2004), followed with Gram's staining and Sudan staining (Thakur *et al.,* 1988).

General and colony characteristics studies: Morphological characteristics of purified isolated bacterial colonies were studied as per Bergey's manual of determinative bacteriology 1994.

Bio chemical tests: Mannitol motility, Triple sugar iron agar, Simmon's citrate utilization, urease, starch hydrolysis, gelatin liquefaction, catalase, oxidase, methyl red, Voges-proskauer, Carbohydrate fermentation test are performed according to standard protocol (Cappuccino and Sherman, 2004).

Scale up media preparation: Scale up media was inoculated by identified organism for increasing colony number to obtain high lipid yield.

Quantitative and Qualitative analysis of Lipids and Fatty acids

Extraction of Lipids: Extraction of lipids from bacterial isolates was done by Bligh and Dyer (1959).

Thin Layer Chromatography (TLC): In order to separate lipids solvents containing lipids were spotted on silica TLC plate with control myristic acid- C14, palmitic acid - c16, stearic acid - c18 carbon atoms (Mantel *et al.*, 1975), following drying process the plate was developed in mobile phase petroleum ether / diethyl ether / glacial acetic acid (90:10:1, v/v), plate was removed from chromatography chamber when front size was 1cm from upper edge and dried with warm air, then 10% sulphuric acid was sprayed in a horizontal position until a layer of fluid is visible on the plate and it was placed in an oven for 20 min at 140^0C.

Optimization Studies

Effect of different carbon and nitrogen source: All constituents were dissolved in distilled water with altering glucose as carbon and nitrate as nitrogen sources, media was sterilized after cooling about 1.0 mL of identified pure suspension was inoculated into each flask and then they were incubated in a shaker at 120 rpm for 5 days at room temperature (Venkata Rao *et al.*, 1993).

Effect of pH: Medium was prepared, pH was adjusted to 5.5, 7 and 10, autoclaved, after cooling about 1.0 mL of identified pure suspension was inoculated into each flask and then they were incubated in a shaker at 120 rpm for 5 days at room temperature (Aneja, 2006).

Strain improvement by mutation: Bacteria were exposed to (280–310nm) UV rays at different time intervals of 10, 15 and 30 minutes. Survivors were grown by spreading 0.1 mL of treated culture on media and incubating at 30^oC for 24hrs (Kuhad *et al.*, 1994). The obtained colonies were inoculated to scale up media and incubated for 7 days at 180 rpm for 48 hours to allow inoculated organism to grow and accumulate high lipids content.

Results and Discussion

Isolation of bacterial species from soil sample: Twenty two different bacterial strains were isolated from different soil samples. In preliminary screening by Sudan black B staining shows different intensity in color uptake of dye based on their lipid content. Out of this, three isolates from Kolar gold field soil, university campus soil and decomposed soil are high lipid accumulating.

Identification of oleaginous bacteria: The results obtained from staining, colony, general and biochemical characteristics were correlated with Bergy's manual of determinative bacteriology and the organisms were identified as *Flavimonas oryzihabitans* (from decomposed soil 10^{-1}), *Pseudomonas aeruginosa* (from university campus soil 10^{-4}*)* and *Morococcus* sp. (from Kolar gold field soil, 10^{-3}) (Table 1, 2 and 3)

Lipid extraction

Lipid concentration is higher in scale up media comparatively to nutrient broth in all three isolates, which revealed that amount of lipid were ranged highest in *Morococcus* sp (0.68mL/100mL), which is comparatively less in *P. aeruginosa* (0.51mL/100mL) and *F. oryzihabitans* (0.30mL/100mL) respectively (Table 4).

Thin layer chromatography

Thin layer chromatography result for three

isolates grown in nutrient broth and scale up media showed that *F. orizihabitans* sample may contain myristic acid(C14) in scale up media, in nutrient broth arachidonic acid (C20). *P. aeruginosa* contains lauric acid (C12) in scale up, where as in nutrient broth eicosapentaenoic acid (C20) and *Morococcus* sp. sample may contain lauric acid (C12) in scale up media and in nutrient broth stearic acid (C18). Comparison with standards C14 (myristic acid), C16 (palmitic acid) and C18 (stearic acid) are shown in Fig.1 and Plate 1.

Optimization with C/N ratio and pH

In optimization studies glucose as carbon source *Morococcus* sp. accumulate more amount of lipid (0.70mL/100mL) compared to *F. oryzihabitans* and *P. aeruginosa* (0.60mL/100mL), in nitrate as nitrogen source which revealed that amount of lipid were ranged highest in *Morococcus* sp. (0.65mL/100mL) than *F. oryzihabitans* (0.55mL/100mL) and *P. aeruginosa* (0.40mL/100mL) respectively. A comparison to growth at pH 5.5, 7.0 and 10. *Morococcus sp* accumulates high amount of lipid at pH 7.0 (1.96mL/100mL) than *F. oryzihabitans* and *P. aeruginosa* (Table 5)

Thin layer chromatography: TLC results for C/N and pH shows that sample was run along with standards C14, C16 and C18. *F. orizihabitans* in carbon source contains oleic acid (C18), in nitrate arachidonic acid (C20) and at pH 5.5 Myristic acid (C14) and at pH 10 capric acid (C10). *P.aeruginosa* sample may contain linoleic acid (C20) in carbon source, nitrogen source and at pH 5.5 and at 7.0 may contain eicosapentaenoic acid (C20) and *Morococcus* spin carbon source contain palmitic acid (C16) in carbon source, in nitrate arachidonic acid (C20), pH 7.0 oleic acid (C18) and at pH 10 may found to be capric acid (C10) (Fig.2 and plate 2)

Strain improvement by mutagenesis: Strain improvement by mutagenesis at different time exposure to UV. *F. oryzihabitans* accumulates more amount of lipid (01.50mL/100mL) at 10 minutes, compared to *P. aeruginosa* (0.65mL/100mL) and *Morococcus* sp. (0.80mL/100mL) (Table 6)

Thin layer chromatography: TLC for strain improvement by mutagenesis shows that *F. oryzihabitans* accumulates palmitic acid (C18) at 15 minutes and linolic acid (C18) at 10min. *P. aeruginosa* may contain arachidonic acid (C20) at 15 min and eicosapentaenoic acid (C20) at 10 minutes, where *Morococcus* sp may contain arachidonic acid (C20) at 10 minutes (Fig.3 Plate 2).

Biodiesel is a monoalkyl ester of fatty acids presently produced by catalytically transesterification (Ma and Hanna, 1999). Present investigation was carried on evaluation of oleaginous bacteria for biofuels production, instead of using vegetable oil. Soil bacteria can use for suitable oil because of their high oil productivity, the isolation and preliminary screening of soil bacteria by Sudan black B staining, reviews that three isolates are rich in lipids these observations concurs with Michael Hupfer *et al.,* (2008) who has shown that certain bacteria are rich in lipids later they identified based on gram's staining, colony, general and biochemical characteristics which were correlated with Bergy's manual of determinative bacteriology and identified as *Flavimonas oryzihabitans, Pseudomonas aeruginosa* and *Morococcus* sp.

Table.1 Colony characteristics

	Colony characteristics						
Soil sample	**Whole colony**	**Edge**	**Surface**	**Elevation**	**Color**	**Size**	**Gram's staining**
Decomposed soil	Circular	Entire	Smooth	Low convex	Yellow	Medium (1mm)	Gram's negative rods
University campus soil	Circular	Entire	Smooth	Convex	Orange	Medium (1 mm)	Gram's negative coccobacillus
Kolar gold field soil	Irregular	Entire	Smooth	Convex	White	Medium (1 mm)	Gram's positive cocci

Table.2 General Characteristics

General characteristics	*F. oryzihabitans*	*P. aeruginosa*	*Morococcus* sp.
1.No. of Flagella	1	1	1
2.PHB accumulation	+	+	+
3.Lipid inclusions by Sudan B staining	+	+	+
4.Pigments on :			
a. Nutrient agar	+	+	+
b.EMB agar	+	+	-
5.Growth at 4^0C	-	-	-
6.Growth at 38^0C	+	+	+
7.Disc diffusion assay of antibiotic susceptibility:			
a.Chloramphenicol	+	+	+
b.Streptomycin	+	-	+
c.Penicillin	-	+	+
d.Gentamycin	+	+	+

Table.3 Biochemical characteristics

Biochemical tests	*F. oryzihabitans*	*P. aeruginosa*	*Morococcus* sp.
Catalase test	+	+	+
Oxidase test	-	+	-
Carbohydrate fermentation	+	-	+
Gas production	+	-	+
Mannitol motility test	+	+	-
Simmon's citrate agar test	+	+	-
Starch hydrolysis test	-	-	-
Methyl red test	+	-	+
Voges proskauer test	-	+	-
Urease test	-	-	-
Triple sugar iron test :			
Fermentation	+	-	+
Oxidative decarboxylation	+	-	+
Gas Production	+	-	-
H_2S production	-	-	-
Gelatin liquefaction test	-	-	-

Table.4 Effect of different media on lipid concentration

Different Media	*F. oryzihabitans*		*P. aeruginosa*		*Morococcus* sp.	
	OD at 660nm	Lipid concentration in mL/100mL	OD at 660nm	Lipid concentration in mL/100mL	OD at 660nm	Lipid concentration in mL/100mL
Nutrient broth	0.426	0.29	0.90	0.32	0.43	0.42
Scale up media	0.736	0.30	1.32	0.51	0.70	0.68

Table.5 Optimization of microbes in C/N ratio and different pH

C/N ratio with different pH	Carbon source		Nitrogen source		At Different pH					
	OD at 660nm	**Lipid concentration mL/100mL**	**OD at 660nm**	**Lipid concentration mL/100mL**	**OD at 660nm**			**Lipid concentration mL/100mL**		
					5.5	**7.0**	**10**	**5.5**	**7.0**	**10**
F. oryzihabitans	1.66	0.60	1.33	0.40	0.75	0.00	1.23	0.21	0.00	0.16
P. aeruginosa	1.62	0.60	1.50	0.55	0.17	1.26	0.19	0.04	0.58	0.14
Morococcus sp.	1.60	0.70	1.70	0.65	0.00	0.71	0.00	0.00	1.96	0.00

Table.6 Strain improvement by mutagenesis for lipid concentration

Organisms	UV exposed time					
	OD 660nm			**Lipid concentration mL/100mL**		
	10min	**15min**	**30min**	**10min**	**15min**	**30min**
F. oryzihabitans	0.47	0.45	0.00	1.50	1.73	0.00
P. aeruginosa	1.93	0.42	0.01	0.65	0.04	0.00
Morococcus sp.	0.39	0.00	0.00	0.80	0.00	0.00

Fig.1 Rf value of different microbes in different media

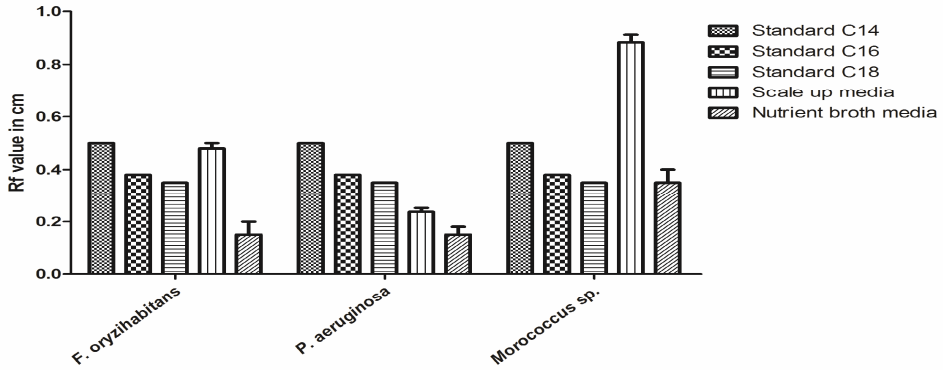


Fig.2 Rf value of Optimization of microbes in C/N ratio and different pH

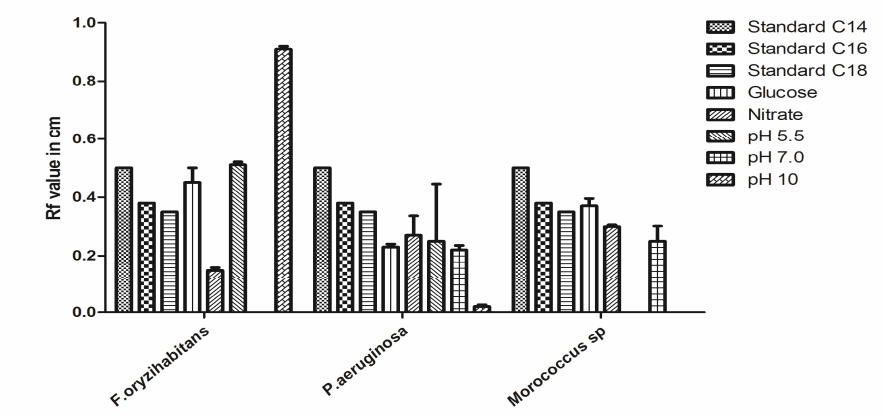


Fig.3 Effect of UV on lipid concentration

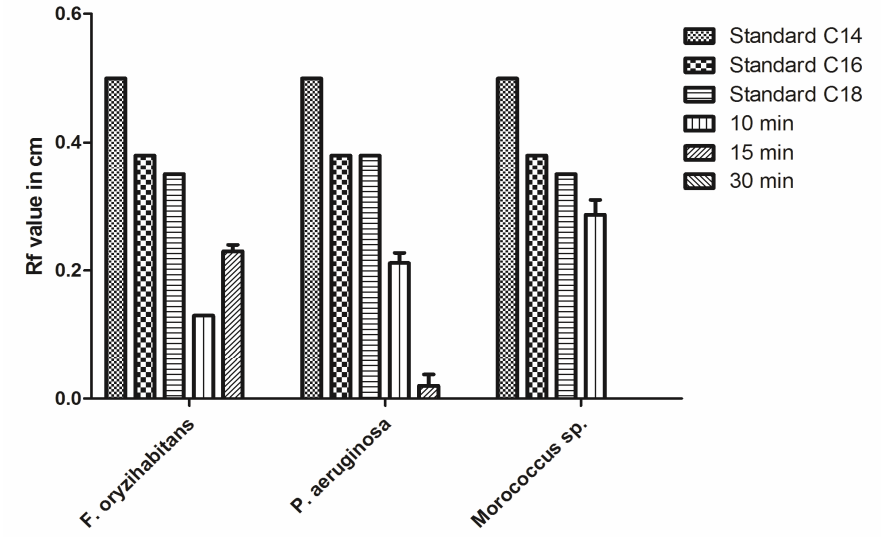


Plate-1 *F.orizihabitans*(VO) *P.aeruginosa*(US) *and Morococcus* sp.(KGF)showing separation of lipids on TLC plate with standard lipids C14, C16, C18, along with other bacterial lipid samples which have less amount of lipid.

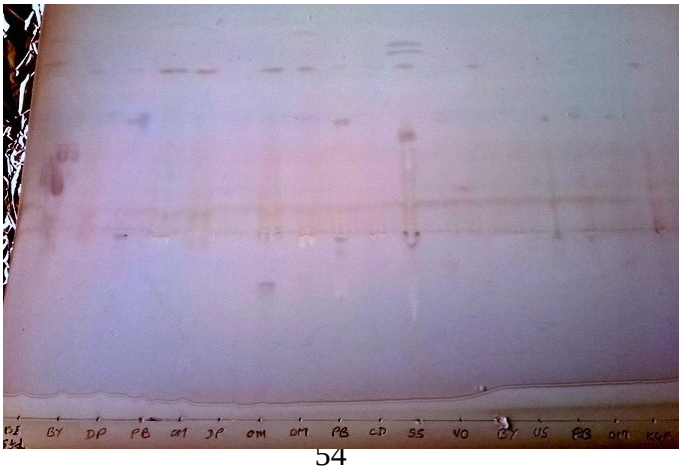
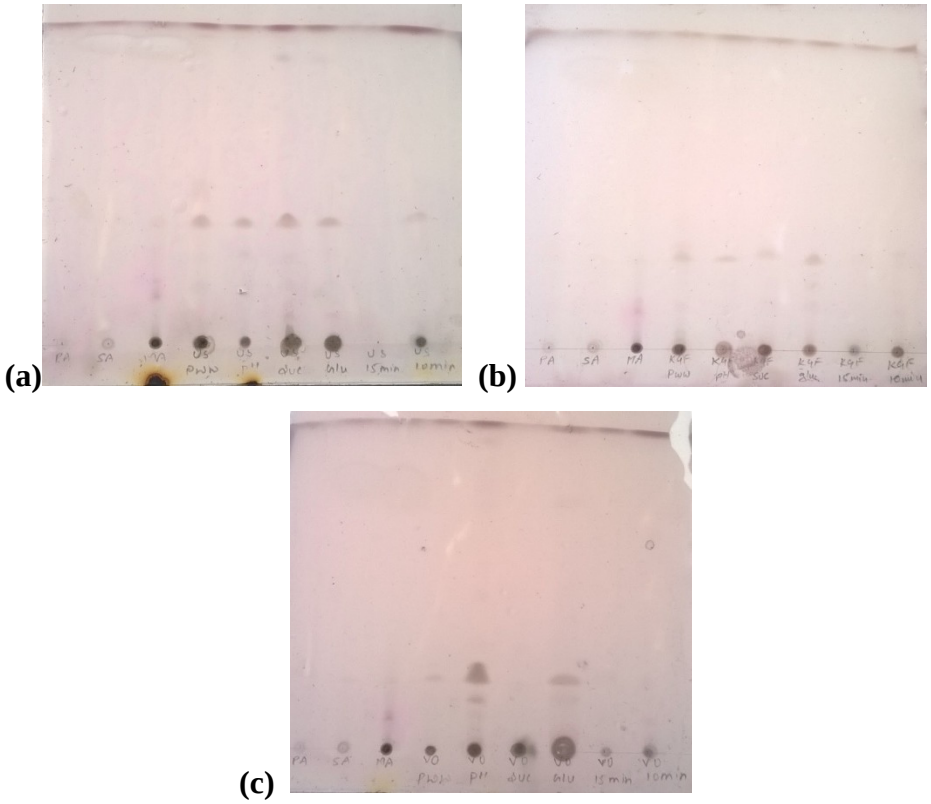


Plate-2 *P. aeruginosa* (a), *Morococcus* sp. (b) and *F. orizihabitans* (c) showing separation of lipid after optimization and strain improvement by mutagenesis on TLC plate with standard lipids C14, C16 and C18.



These results of present work imply more detail studies of Bayliss and Adams (1972). The bacterial cultures were then grown in scale up media to enhance the lipid content which confirms the observation of Gopinathan (2011). The result shows that *Morococcus* sp. revealed more lipid than *Flavimonas oryzihabitans* and *Pseudomonas aeruginosa.* Concurs to the study of Mantel *et al.,* (1975) the present study has shown that the TLC for lipid extracted from these isolates *Morococcus* sp. accumulates lauric acid in scale up and stearic acid in nutrient broth. In optimization studies of C/N ratio there is more accumulation of lipid in *Morococcus* sp. which confirms the observation of Di Russo and Black (1999), where different pH also influenced on lipid enhancement according to Edward *et al.,* (1994). Alexander *et al.,* (2007) reported that by mutagenesis it is possible to improve strain which accumulates more lipids which confirms in present study that *F. oryzyhabitans* revealed high lipid content than *Morococcus* sp. and *Pseudomonas aeruginosa* which are improved by mutagenesis. Hence the soil bacteria can be grown in low cost cultivation in the laboratory, growth can be optimized and strains can be improved to provide good source of biomass to meet requirement of alternate fuel production in future.

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