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SINGLE CELL OIL PRODUCTION FROM *Morococcus cerebrosus* ISOLATED FROM MARINE SEDIMENTS

S. Brammavidhya* and V. Karthika,
Department of Microbiology, A.V.C College (Autonomous), Mannampandhal, Tamil Nadu, India.

Abstract

In the present study, the density of oleaginous microbes isolated from Vellar estuary area at Parangipettai was found to be 1.0×10^4 cfu/g. The oleaginous bacteria were stained with Sudan Black B and observed under an on oil immersion microscope for the presence of blue or grayish colored fat globules within the cells. The bacterial strains showing fat globules within the cells were selected for further studies. The optimized parameters for mass scale production were done to get maximum, growth and lipid accumulation. The use of cheaper substrate for the higher lipid production wheat bran gave high quantity of lipid yield (6.2 g/L) followed by rice straw (5.8 g/L). The fatty acids present in the bacterial culture was analysed by GCMS. The results revealed that the major fatty acids and derivatives were found with carbon backbone of C_{16} to C_{37} were produced.

Key words: Single cell oil, *Morococcus cerebrosus*, Marine sediments and GC-MS.

1. Introduction

The use of fossil fuels has caused immediate environmental and ecological effects as well as contributed to a vast increase in green house gas carbon dioxide levels. The search for renewable forms of energy to displace fossil fuels is a constant and ongoing process. The field of alternative energy is brimming with new technologies driven by the desire to produce energy that is not constrained by diminishing resources and the corresponding effects on the environment. The United Nations (UN) has recently outlined an initiative to limit global warming temperatures to 2 ^oC that could prevent major climate change and its consequences. This proposal would require energy consumption derived from 30 % renewable and carbon-free sources by 2030 (Knothe, 2005).

Biodiesel production using microbial lipids which is named as single cell oils (SCO) has attracted worldwide attention and 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 seems to be economically competitive (Xin *et al.*,2009).

All microorganisms synthesize lipids for essential functions of their membranous structures; however, few of them can accumulate lipids more than 20 % of their dry cell weight and are called "oleaginous organisms" (Ratledge and Wynn, 2002). These oleaginous organisms store lipids in oil vacuole in the form of triacylglycerols. 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

Corresponding author*: **S. Brammavidhya
E. mail: brammav@yahoo.com
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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).

2. Materials and Methods

Sample collection

Marine Sediment samples collected from Vellar estuary area. The Vellar estuary opens into the Bay of Bengal at Parangipettai and links with the Coleroon River, which is the distributary of River Cauvery. Samples were aseptically transferred in to a sterile polythene bags and samples were immediately processed for the isolation of oleaginous bacteria.

Isolation of oleaginous bacteria from sediment samples

Sediment samples were plated directly on sterilized carbon rich and nitrogen limited medium (g/L): (Glucose – 60 g/L, Yeast extract – 1 g/L, KH_2PO_4- 2 g/L, $MgSO_4.7H_2O$ - 0.75 g/L, Na_2HPO_4 – 1 g/L, $CaCl_2.2H_2O$ - 0.2 g/L, $FeCl_3$ - 0.01 g/L, $ZnCl_2$ - 0.1 g/L, pH - 7.0). The plates were incubated at 28 °C±2 ^oC for 24 – 48 hrs (Li *et al.,* 2012).

Sudan Black B staining method

The oleaginous bacteria were stained with Sudan Black B and observed under an on oil immersion microscope for the presence of blue or grayish colored fat globules within the cells. The bacterial strains showing fat globules within the cells were selected for further studies. The most potential strain was identified using biochemical tests as per Bergey's manual (Thakur, 1989).

Optimization of growth of oleaginous *M. cerebrosus*

Optimization of growth and lipid accumulation, the parameters such as pH, temperature, salinity, carbon sources (Maltose, Sucrose, Glucose, Starch and Cellulose) and nitrogen sources (Peptone, Ammonium nitrate, Beef extract and Yeast extract) were done in shake flask. To these optimized parameters mass scale production was done to get maximum, growth and lipid accumulation.

Extraction of Lipids

The total lipid was extracted from pelletes by adding chloroform/methanol (1:1) into the tube, shaked vigorously with a vortex oscillator and then centrifuged at 5,000 rpm for 10 min. The lipid containing chloroform layer (the lower layer) and the concentration of the total lipid content was measured **(**Bligh and Dyer, 1959).

Determination of SCO Productivity, Growth Yield Efficiency and SCO Yield Efficiency

Lipid content in each trial condition was determined by the following equation.SCO productivity (Lipid content) = SCO Weight (g L^{-1})/Cell dry weight (g L^{-1}) × 100. The supernatant was analyzed for glucose consumption by dinitrosalicylic acid (DNS) solution (Kraisintu*et al.,* 2010).

Growth yield efficiency = Cell dry weight (g L^{-1})/Sugar consumed × 100

SCO yield efficiency = SCO Weight (g L^{-1})/Sugar consumed × 100

Lipid profile analysis using Gas chromatography

Lipid profile analysis was done by using gas chromatography. An Agilent 6890 gas chromatograph was equipped with a straight deactivated 2 mm direct injector liner and a 15 m Alltech EC-5 column (250 µ I.D., 0.25 µ film thickness). A split injector was used for sample introduction and the split ratio was set to 10:1. The oven temperature program was programmed to start at 35^oC, hold for 2 min and ramp at 20 ^oC per min. to 300 ^oC and then hold for 5 min. The helium carrier gas was set to 2 ml/minute as flow rate (constant flow mode).

Mass Spectrometry

A JEOL GC mate II bench top double - focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-20001 software was used for all analyses. Low resolution mass spectra were acquired at a resolving power of 1000 (20 % height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were acquired at a resolving power of 5000 (20 % height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan.

Mass spectrometry library search

Identification of the components of the purified compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 pr.

3. Result and Discussion

Oleaginous bacterial density

The density of oleaginous microbes was found to be 1.0×10^4 CFU/g.

Sudan black staining

Intensity of Sudan Black staining was used as a criterion for lipid accumulation. Kitcha and Cheirsilp (2011) used Sudan black staining to screen the oleaginous yeast for lipid accumulation. Shruthi *et al.* (2014) used Sudan black B staining to screened lipid content of 18 oleaginous bacterial isolates.

Screening for oleaginous microbe

The bacterial strains showing fat globules within the cells were selected for further studies. The present study based on morphology and physico-chemical methods the potent oleaginous bacterial isolate was identified as *Micrococcus cerebrosus.* Shruthi *et al.* (2014) identified oleaginous *Flavimonas oryzihabitans* and *Pseudomonas aeruginosa* as potential forms based on staining, colony, general and biochemical characteristics using Bergy's manual of determinative bacteriology.

Optimization of growth of oleaginous microbe

Many factors including medium components, such as carbon source, nitrogen source and C/N molar ratio etc. as well as culture conditions (Temperature and pH) have significant influences on cell growth and lipid accumulation of oleaginous microorganism (Papanikolaou *et al.*, 2007). Regarding optimization of growth and lipid accumulation, the parameters the produciton such as pH (4, 6, 7.4, 8 and 10), temperature (30 ^oC, 35 ^oC, 40 ^oC and 45 ^oC), salinity (0.5 - 2.5 %), carbon sources (Maltose, sucrose, glucose, starch and cellulose) and nitrogen sources (Peptone, ammonium nitrate, beef extract and yeast extract) were done in shake flask. To these optimized parameters mass scale production was done to get maximum, growth and lipid accumulation.

In the present study, pH 6 to pH 10 was tested, maximum growth was observed at pH - 9. Sriwongchai *et al.* (2012) used 5 different pH (i.e.) pH 4, 6, 7.4, 8 and 10, where at pH 7.4 maximum cellular lipid was found (1.83 g/L). In the present study, regarding temperature, range of 30 ^oC – 45 ^oC with an interval of 5 ^oC was tested. Maximum growth of 1.146 OD was obtained at 35 ^oC whereas minimum growth was observed at 30 ^oC. Sriwongchai *et al.* (2012) found 30 ^oC as favourable. Regarding salinity, 2.5 % salinity resulted in maximum growth (0.292 OD) and the minimum growth was observed at 0.5 % NaCl concentration.

In this study, sucrose was found to be the best C source for maximum growth (0.942 OD). Regarding the concentration sucrose as the most potent carbon source which was tested from 0.5 % to 2.5 %, maximum growth was found at 6% sucrose which resulted in 0.91 OD. Most of the oleaginous microorganisms reported to utilize glucose to produce lipids (Li *et al.*, 2007; Loffhagen *et al.*, 2006 and Ratledge, 2002). Enshaeieh *et al.* (2015) used xylose as carbon source. Sriwongchai *et al.* (2012) used glycerol as

c source (30 g/L) at which *R. erythropolis* produced cellular lipid of 47.72 % of their dry cell weight. In this study, five different nitrogen sources were tested in which peptone resulted in maximum growth of 1.25 OD at 42 hrs. Regarding the concentration of peptone as the ideal nitrogen source tested in different concentration, it was ranged from 0.2 – 1.0 % with an interval of 0.2 %, maximum growth of 0.421 OD was observed with 0.6 % peptone. In the present study when compared to inorganic nitrogen sources, organic nitrogen sources were more beneficial to growth and lipid production by the selected oleaginous strain. Kitcha and Cheirsilp (2011) reported that among organic nitrogen sources tested, a mixture of yeast extract and peptone (1:1) gave the best biomass (17.05 g/L for *T. spathulata* and 11.1 g/L for *K. ohmeri*) and the maximum lipid production (10.43 g/L for *T. spathulata* and 4.53 g/L for *K. ohmeri*). For *M. cerebrosus* 100 rpm resulted in maximum growth and lipid yield. Sriwongchai *et al.,* 2012 found 150 rpm as ideal for *Rhodococcus erythropolis*. In the present study 42 hrs of incubation resulted in maximum yield and growth. Sriwongchai *et al.* (2012) found 48 hrs as optimum incubation period.

Mass scale growth of oleaginous microbe in shake flask

In the present study mass scale production in shake flasks was done using pH 9, 35 ^{o}C, 2 % NaCl, 6 % sucrose, 0.6 % peptone and 42 hrs of incubation period were found to be optimum parameters. The observed growth and lipid accumulation under these optimized conditions by *M. cerebrosus* was 1.632 OD and 7.6 g/L of lipid in the culture medium. Regarding use of cheaper substrate for the higher lipid production wheat bran gave high quantity of lipid yield (6.2 g/L) followed by rice straw (5.8 g/L).

Shruthi *et al.* (2014) observed highest lipid (Lauric acid) content (0.68 ml/100 ml) in *Morococcus* sp. They studied the optimization of C/N ratio for the amount of lipid was ranged highest in *Morococcus* sp. They extracted lipid from oleaginous *Flavimonas oryzihabitans, Pseudomonas aeruginosa* and *Morococcus* sp. at a concentration of 0.30 ml/100 ml, 0.51 ml/100 ml and 0.68 ml/100 ml respectively. Compared to these strains, the present strain used seemed to be more potent. The advantages of microbial lipids include the short life cycle of microorganisms, less labor requirement, little affection by season and climate and also the relevant ease in scaling them up (Syed *et al.,* 2006; Li *et al.,* 2008; Leesing and Baojungharn, 2011).

Strains of *Rhodotorula* are pigmented yeasts that have high potential of lipid production and some of their strains can accumulate lipid on xylose and lignocellulosic substrate as sole carbon sources. This ability is important in using these strains in industrial processes (Postgate, 1994; Dai *et al.,* 2007). Lipid accumulation occurs when one of the nutrients (usually nitrogen) was exhausted. At the same time, there are excess amounts of carbon sources such as glucose in the medium. The cell's response to exhaustion of nutrients is that they cease to grow or multiply, but they continue to take up glucose from the medium. The surplus sugar is used for lipid biosynthesis. Under nitrogen limited condition, the first requirement for the cells is to cease energy production i.e. ATP, as it is no longer required for the synthesis of macromolecules such as proteins and nucleic acids. This is because the cells have stopped to grow or divide. In addition to nitrogen limitation, phosphate limitation can also improve lipid accumulation in oleaginous microorganisms too (Muniraj *et al.,* 2013).

Kraisintu *et al.* (2010) evaluated different chemical and physical parameters on lipid production in *Rhodosporidium toruloides* DMKU3-TK16 and obtained 9.26 g L^{-1} lipid production in nitrogen-limited medium containing 70 g L^{-1} glucose, 0.55 g L^{-1} $(NH_4)_2SO_4$ with a pH of 5.5 at 150 rpm and 28 ^{o}C.

Pan *et al.* (2009) isolated oleaginous yeasts with assimilating capacity of xylose and the best yeast strain could produce 5.8 g L^{-1} lipid using 40 g L^{-1} xylose. Li *et al.* (2005) reported that lipid production in *Rhodosporidium toroloides*

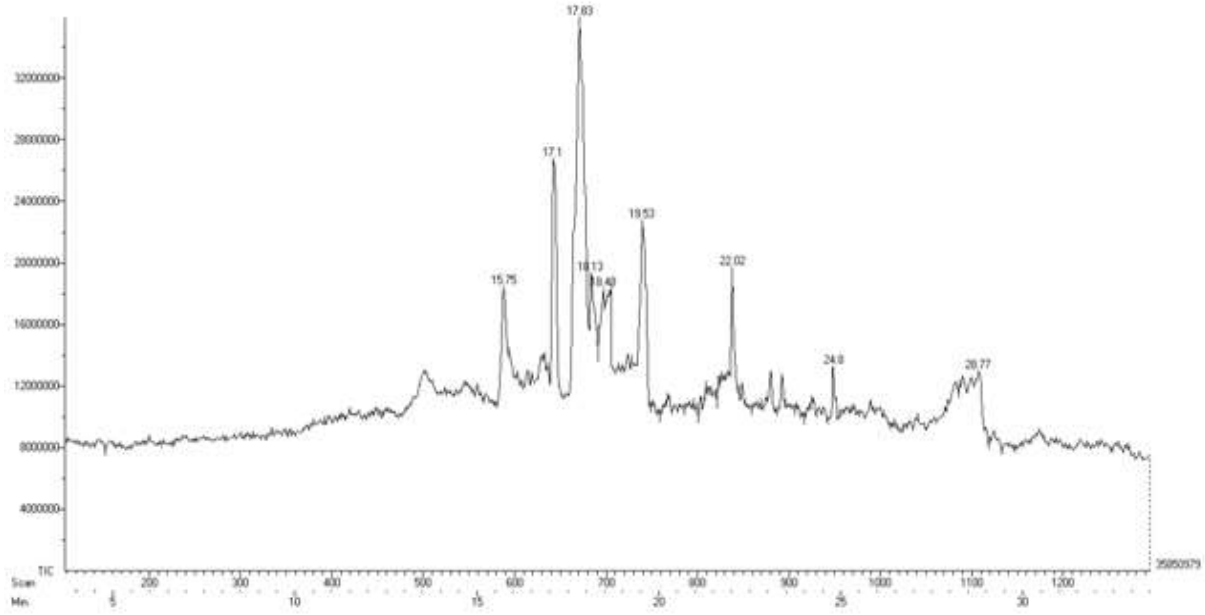

Fig - 1: GCMS analysis of single cell oil

AS2.1389 was 10.6 g L^{-1} when using 100 g xylose. In their study, lipid production on xylose with *Rhodotorula mucilaginosa*, was 6.98 g/L. Lignocellulosic biomasses are the most abundant organic sources and various oleaginous microorganisms, especially yeasts have attracted attentions for converting them to microbial oil (Xu *et al.,* 2012).

Table - 1: GCMS analysis of single cell oil

Peak No.	RT (Min.)	Compound Name	Peak area (%)
1	15.75	Tetradecanoic acid	10.00
2	17.10	Pentadecanoic acid	15.25
3	17.83	n-hexadecanoic acid	21.00
4	18.13	hexadecanoic acid	10.50
5	18.48	n-hexadecane	12.50
6	19.53	Oxacyclotetradecane,2-11-dione	12.15
7	22.02	9-octadecanoic acid	11.00
8	24.80	9-octadecanoic acid (Z) Hexyl ester	7.6
Total			**100**

The SCO production from lignocellulosic materials containing xylose has been carried out by several investigators (Meester *et al.*, 1996; Zhao, 2005; Papanikolaou, 2008; Gong *et al.*, 2013; Enshaeieh *et al.*, 2013). Yeast strains that can use xylose in lignocellulosic hydrolysate have potential for industrial application (Dai *et al.*, 2007; Huang *et al.*, 2012). Yu *et al*. (2011) used *Cryptococcus curvatus* on wheat straw as carbon source and the lipid content was reported as 33.5 %. Huang *et al.* (2009) reported 40.1 % lipid content by using *Tricosporon fermentens* and rice straw as the sole carbon source. Therefore, it was suggested that lignocellulosic materials are a good substrate for microbial oil production due to their low cost and abundance (Zheng *et al.,* 2012 and Khot *et al.,* 2012). Enshaeieh *et al.* (2012) used *Rhodotorula mucilaginosa* and a novel carbon source, i.e. grass hydrolysate, and reported 55 % lipid content, which showed high potential of this strain in lipid production. The study done by Eishaeiels *et al.* (2015) showed that the *Rhodotorula mucilaginosa* had lipid content of 41.81 and 36.91 % on wheat straw and corn stalk, respectively.

Table - 2: Biochemical identification of *Morococcus cerebrosus*

Biochemical characters	Results
Gram staining	- ve
Shape	Cocci-cluster
Motility	-
Growth on	
Nutrient agar	+
Blood agar	+
Cholate agar	+
Sucrose agar	+
McConkey agar	-
Oxidase	+
Catalase	-
Hemolysis	+
Nitrate nitrite	+
Nitrate to gas	-
H_2S	-
Urease	+
Decorboxylase test	
Arginine	-
Lysine	-
Ornithine	-
Esculinhydolysin	+
Gelatin	-
Starch	-
Tween 20	-
Carbohydrate fermentation	
Fructose	+
Glucose	+
Maltose	+
Sucrose	+
Arabinose	-
Inulin	-
Lactose	-

Table - 3: Lipid production using agricultural residues on growth of *M.cerebrosus*

Agricultural residue	Lipid production (g/L)	Biomass (g/L)	SCO efficiency (%)
Sucrose	7.6	17.27	51.5
Wheat bran	6.2	16.48	48.4
Corn straw	5.2	12.22	34.3
Rice straw	5.8	13.73	38.6
Corn cobs	4.3	11.85	31.2

GC-MS analysis

In the present study, the fatty acid present in the bacterial culture was analyzed by GC-MS analysis. The results revealed that the major fatty acids and derivatives were Tetradecanoic acid, Pentadecanoic acid, n-hexadecanoic acid, hexadecanoic acid, n-hexadecane, Oxacyclotetradecane, 2 - 11-dione, 9 - octadecanoic acid and 9 - octadecanoic acid (Z) Hexyl ester etc., (i.e.) Fatty acids, alcohol, carboxylic acids, aldehydes, alkanes, alkenes, eposide, antibiotics, steroid and a hormone precursor were produced by *M. crebrosus*. Various compounds with carbon backbone of C_{16} to C_{37} were produced. Microbial oil has a potential to substitute the plant oil in the market. Microorganisms that produce lipid more than 20 % of their biomass are called oleaginous (Wynn and Ratledge, 2005; Amaretti *et al.*, 2010). The great part of microbial lipid is TAG, which contains long chain fatty acids and is comparable to conventional plant oil (Pan *et al.*, 2009; Kosa and Ragauskas, 2010; Fei *et al.*, 2010). Some oleaginous yeast can metabolize pentoses. This shows the ability of TAG production from lignocelluloses substrates and other low cost materials (Li *et al.*, 2007; Sabirova *et al.*, 2010; Zheng *et al.*, 2012).The advantages of microbial lipids include the short life cycle of microorganisms, less labor requirement, little affection by season and climate and also the relevant ease in scaling them up (Syed *et al.*, 2006; Li *et al.*, 2008; Leesing and Baojungharn, 2011).

4. Conclusion

In recent years, much attention has been paid to the exploration of microbial oils, which might become one of the potential oil sources for biodiesel production in future. Microbial lipids are viewed as a possible alternative for industrial production because their fatty acid composition is similar to that of vegetable oils, as well as the fact that they are rich in polyunsaturated fatty acids. The economic values of microbial lipid production become more favorable when waste materials with

zero or negative economic value are utilized as carbon source. Using bioprocesses such as microbial lipid production from waste materials, the problem of shortage of energy resources, and also air pollutions caused by fossil fuels, could be eliminated.

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