Botryococcus braunii as a potential candidate for the waste water treatment and hydrocarbon accumulation

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Abstract: The micro alga Botryococcus is the green photosynthetic autotroph which is the eukaryotic primary producers of the aquatic ecosystem. The Botryococcus species are known for its own significant production of different types of hydrocarbons. The Botryococcus is the only genera of micro alga which can directly synthesize hydrocarbons and are the potent candidate for commercial biodiesel production in future. There are several reports on the implementation of Botryococcus in waste water treatment from different countries. At the same time the micro alga Botryococcus can alternatively serve as a biological source for the production of biodiesel. One such study is still pending in India, which is one of the larger producers of domestic waste water with increasing urban population. Such studies are much needed for the developing countries to fight against pollution and new emerging diseases.

From this present study, the green micro alga Botryococcus braunii strain was used to treat the domestic waste water from different regions of the Chennai district, Tamil Nadu, India and this study mainly focused on the lipid and hydrocarbon analysis from the biomass of B. braunii after the waste water treatment. All the physiochemical analysis has shown good results in comparison of untreated waste water with the treated waste water. The current study suggests the B. braunii as an effective candidate for the waste water treatment at the same time the hydrocarbon accumulation also more or less similar with the media for cultivation. However, large scale studies are required to standardize the waste water treatment followed by hydrocarbon production by the economically and environmentally useful micro alga.

Keywords: Domestic waste water treatment; Botryococcus braunii; GC-MS and FT-IR.

1. Introduction

The micro algae are one of the very old living microorganisms which have chlorophyll as their primary photosynthetic pigment. The micro algae are the fast growing unicellular organisms with the ability to utilize atmospheric CO_2 (Carbon dioxide) for the production of energy. These organisms can capture solar energy for hydrolysis with the efficiency of about 10 to 50 times higher when compared to advanced terrestrial land plants (Wang *et al*., 2008). The major edaphic factors much important for the micro algae growth are sun light, nutrients, temperatures and pH (Rousch *et al*., 2003). The micro algae have been suggested as a very important source for fuel production due to the production of high lipid content through autophototrophic mode of nutrition and they are well known for effective and high photosynthetic ability, high biomass production and faster growth when compared to any other source (Miao and Wu, 2006; Banerjee *et al*., 2002; Li *et al*., 2007). However, the micro algae can be used to produce valuable compounds like carbohydrates, hydrocarbons, pigments and natural oils. Micro algae are the only source which can be an alternate to the fossil fuel (Chisti, 2007). Thus, the micro algae producing high lipid profiles can be desirable for biodiesel production (Song *et al*., 2008).

The fossil fuels are non-renewable resources and are very limited will be exhausted one day. Adding to this, the combustion of fossil fuels has elevated the levels of green house gases (GHG) in the atmosphere which leads to global warming. Biodiesel from micro algae can be an alternate source against the non-renewable source of energy which recently pulls attention of researchers, governments and local and international traders. The biofuel from algae interestingly have more advantages than fossil fuel, because the biofuel are non-toxic, biodegradable and has very limited emission of green house gases during combustion (Demirbas, 2009). A number of micro algae have the interesting role for biofuel production based on their mass growth, high lipid content and mass cultivation (Chisti, 2007). But the potential micro algae can be selected based on their high production of triacylglycerol (TAG) content (Jones and Mayfield, 2012).

Some of the micro algae suggested by researchers for the biofuel production are *Phaeodactylum tricornutum, Nannochloropsis oculata, Botryococcus braunii, Scenedesmus dimorphus* and *Chlorella protothecoide* (Pulz and Gross, 2004; Rodolfi *et al.,* 2009; Radakovits *et al.,* 2011; Mc Donald, 2011). The micro alga *Botryococcus braunii* has attracted many researchers due to high lipid and different hydrocarbon production. The *B. braunii* is a colonial green micro alga distributed on all the continents in fresh water, brackish water, Saline lakes, reservoirs and small ponds (Aaronson *et al*., 1983). The *B. braunii* can accumulate more hydrocarbon content up to 20 % of its dry weight during their exponential growth (Brown *et al.,* 1969; Belcher, 1968; Gelpi *et al.,* 1968; Murray and Thomson, 1977; Knights *et al.,* 1970).

During stressful conditions the *B. braunii* can accumulate more amounts of carotenoid pigments along with high unsaponifiable lipids up to 80 % of their dry weight (Brown *et al.,* 1969; Belcher, 1968; Gelpi *et al.,* 1968; Murray and Thomson, 1977; Knights *et al.,* 1970).

There are many different hydrocarbons have been reported from *B. braunii* by varying in their carbon chain length from C_{27} to C_{31} and C_{34} in the yellow stage (Maxwell *et al.*, 1968). The colonial nature of *Botryococcus* has attained interest due to its astonishing ability to synthesize various types of hydrocarbons (Demetrescu, 1998). Three distinct races have been reported from *Botryococcus braunii* based on their different hydrocarbon productions (Metzger *et al.*, 1988) which are A, B and L races. The Race A type can synthesize C_{23} to C_{33} odd numbered *n*-alkadienes, mono, tri, tetra and pentaenes (Metzger *et al.*, 1990), race B can accumulate C_{30} to C_{37} unsaturated hydrocarbons known as botryococcenes and minimum amount of methyl branched squalenes (Metzger and Largeau, 2005) and the L race brings on single tetraterpenoid hydrocarbon known as lycopadiene (Metzger *et al*., 1990).

The *B. braunii* sometimes in mass scale blooms in natural ponds or lakes or in shoreline deposits yield highly aliphatic oils (Wake and Hillen, 1981). It has been resulted that the crude oil extracted from Sumatran region consists of 1.4 % of botryococcane content and which is fully hydrogenated (Moldowan and Seifert, 1980). The *B. braunii* can dominate the water bodies against other micro flora with spectular blooms (Aaronson *et al*., 1983) and produce rubbery deposits on the shores (Wake and Hillen, 1981). Thus, it can produce high lipid content in large scale of about 86 % from their dry weight (Brown *et al*., 1969). The early studies on *Botryococcus* concerned the implication of this micro alga due to the formation of some fossil materials in the age of Ordovician to the present (Cane, 1969; Cane and Alion, 1973) which have high petroleum potentials. The fossil chemical compounds from *B. braunii* were found high in some crude oil (Moldowan and Seifert, 1980; Brassell *et al.,* 1986; McKirdy *et al.,* 1986) or in recent sediments (Huang *et al*., 1996). The *Botryococcus* is the only living micro alga in the world can synthesize high amount of hydrocarbons (Swain and Gilby, 1964; Maxwell *et al.*, 1968), before that they were considered as colonies consisted with essential fatty acids (Zalessky, 1926 and Blackburn, 1936).

The micro algae have been reported to grow well in domestic waste water high in carbon, nitrogen and phosphorous conditions. The reduction of nitrogen, phosphorous and some toxic metals from the waste water using the micro algae and their importance in the waste water treatment have been studied well (Pittman *et al*., 2011). The implementation of micro algae towards waste water treatment has been drawing much attention from all over the world due to many advantages including removal of pollutants, environmental friendly treatment and the biomass can be utilized for many applications. The micro alga *Botryococcus* sp. can grow well in the domestic sewage which was pretreated by activated sludge treatment and will be a very good source of hydrocarbon production. The secondary treated sewage was found to obtain large amount of nitrogen and phosphorous, by mass cultivation of *B. braunii* on the secondary treated sewage aerated with 1 % of CO_2 at 25°C without changes in pH. The results found that the nitrate content has been reduced from 7.67 g m^{-3} to a very low level below detection, the phosphate content was reduced up to 0.02 g m^{-3} and the ammonia content was not utilized and the biomass yielded about 0.35 kg m^{-3} for a week with 53 % of hydrocarbon content from dry biomass when compared to the Chu-13 medium where 58 % of hydrocarbon was accumulated from dry biomass (Sawayama *et al*., 1992).

This current research study deals with the treatment of domestic waste water collected around the Chennai city, Tamil Nadu, India using the high hydrocarbon producing micro alga *Botryococcus braunii* and the analysis of different fatty acid profile and hydrocarbon by GC-MS and FT-IR techniques.

2. Materials and methods

2.1.Collection and biological treatment of domestic waste water

About 4 *l* of domestic waste water samples were collected in a clean 5 *l* can from different sites around the Chennai city. The domestic waste water collection sites are 1) Aminjikarai (AK), 2) Ethiraj College (EC), 3) Adyar (AR) and 4) Napier's bridge (NB). From 4 *l*, about 2 *l* of the four samples were filtered to remove large solid particles and autoclaved at 15 psi for 15 min. in 5 *l* flasks. Then all the four samples were inoculated and treated with *Botryococcus braunii* for 23 days under light illumination for the period of 12: 12 hours of light and dark at room temperature (Fig. 1 a and b).

2.2. Comparison of physiochemical parameters between untreated and treated waste water

The physiochemical parameters such as Total alkalinity, Total dissolved solids (TDS), Turbidity, Electrical conductivity, Total hardness, Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K), Iron (Fe), Manganese (Mn), Free ammonia (NH_3), Nitrate (NO_2), Chloride (Cl), Fluoride (F), Sulphate (SO_4), Phosphate (PO_4) Biological oxygen demand (BOD) and Chemical oxygen demand (COD) were analyzed for both untreated and treated waste water samples and recorded. The physiochemical parameters were analyzed by TWAD (Tamil Nadu Water and Drainage Department laboratory, ISO: 9001), Chennai, Tamil Nadu, India.

2.3. Biomass yield and extraction of lipid

The biomass was obtained by centrifugation at 8000 rpm for 5 min. and the wet biomasses were recorded gravimetrically and kept to dry in shade. The dry biomasses from the four different samples were recorded gravimetrically and further subjected to extraction of lipid. For lipid extraction, about 100 mg of dry biomasses were taken in a clean vial with methanol and chloroform in the ratio of 2:1 and kept overnight. Then the next day the extract was centrifuged at 8000 rpm for 5 min. to remove cell debris and supernatant consists of lipid which was then subjected for further analysis.

2.4. Gas chromatography mass spectrometry (GC-MS)

Fatty acid methyl esters (FAME) were quantified using GC-MS-QP 2010 (SHIMADZU) in VF-5ms column with 30.0 m × 0.25 mm and film thickness of 0.25 µm. GC Conditions such as oven temperature 70°C for 3 min., Injector temperature of 240°C with split ratio 10, Helium (99.9995 % purity) as carrier gas with column flow of 1.51 ml/min. and injection volume of 1 µl. Column temperature was raised to 300°C for 9 min.

Mass spectrum conditions such as Ion source temperature 200°C, Interface temperature-240°C, scan range from 40-1000 m/z, MS start time: 5 min and end time: 35 min, Ionization: EI (-70ev) and scan speed of 2000 were concerned. NISTO8s, WILEY8 and FAME softwares were used as MS library for FAME identification and analysis.

2.5.Fourier transmission infra red spectrometry (FT-IR)

FT-IR analysis involves spectral range of analysis from 450-4000 cm^{-1} with a resolution of 1 cm^{-1}. FT-IR was analysed using Perkin Elmer Spectrum-1. Dry samples were made ready to analyze lipid components.

3. Results

3.1. Domestic waste water treatment

The physiochemical parameters such as TDS, turbidity, electrical conductivity, COD, BOD, ammonia were reduced in the treated water sample of all the sites. Among that, 67 % of TDS were removed in the sample AR followed by only 27 % in the sample NB. The electrical conductivity and ammonia were drastically reduced when compared with untreated water sample up to 70 % (Table 1).

3.2. Gas chromatography mass spectrometry (GC-MS) analysis of lipid

Fatty acid methyl ester analysis by GC-MS of all the four samples reveals the presence of saturated fatty acids which found highly in NB (64.71 %) and very least amount of saturated fatty acids recorded in sample EC (9.37 %). Samples AK and AR engulf about 33.1 and 32.03 % of saturated fatty acids respectively. Rest of them was covered by Unsaturated fatty acids especially monounsaturated fatty acids (MUFA) likely high in sample EC of about 37.81 %, samples AR and AK showed about 36.68 and 32.59 % respectively (Fig. 2 to 5). Monounsaturated fatty acids found to occur very low in sample NB 3.28 %. When comparing Polyunsaturated fatty acids (PUFA) from all the treated samples, which was very low in the samples AK, NB and AR of 15.23, 18.86 and 18.91 respectively but accumulated high in the sample EC (33 %).

Hexadecanoic acid (C: 16:0), Octadecenoic acid (C: 18:1) and Octadienoic acid (C:18:2) are the major fatty acid methyl esters abundant in all the four extracted lipid samples. Hexadecanoic acid (C:16:0) is found abundant in sample NB of 56.11 % almost half of the lipid extract but absent in sample AR and present about 29.07 and 27.9 % in the samples AK and AR samples. On analyzing Octadecanoic acid (C: 18:1) from all the samples comparatively high in samples EC and AR of 33.4 and 34.69 respectively. Very least amount recorded in case of NB.

Samples EC showed accumulation of high amount of Octadienoic acid (C: 18:2) of 30.06 % and comparatively low in other three samples.

3.3. Fourier transmission infra red spectrometry (FT-IR) analysis of lipid

The FT-IR spectrum of the dried treated micro algal biomass showed the presence of hydroxyl groups (O-H) and Methyl (Alkanes) groups (C-H) ranges from 3399 to 3900 cm^{-1} and 2851 to 2932 cm^{-1} respectively in all the treated samples (Fig. 6 to 9). Especially wave number from 612 to 945 cm^{-1} along with 729 cm^{-1} in both the samples AK and NB found the presence of Aromatic bends in methyl groups (C-H bends) of all the samples which prove the presence of benzene rings as in hydrocarbons. Presence of carboxylic acid (C=O) is seen in aliphatic chain of fatty acids (1852 cm^{-1}) only in AK sample when compared to other samples. Aliphatic chains in the esters of fatty acids present due to the occurrence of Alkanes, methylene groups (C-H) from wave numbers 1425 to 1456 cm^{-1}. Aromatic carbon and carbon double bonds (C=C) prove the occurrence of aromatic rings as in hydrocarbons in all the treated samples. Especially the presence of ether groups in samples AK, EC and NB can be seen in their fatty acids. The FT-IR results possibly showed the presence of hydrocarbons and esters of fatty acids by analyzing the FT-IR spectrum.

4. Discussion

The most important structure among lipids is fatty acids which have a long hydrophobic hydrocarbon tail attached to a hydrophilic carboxyl group and are built in other types of lipids (Mathews *et al.,* 2000). The components and amounts of lipids differ between species and classes (Huang *et al.,* 2009) and the growth phase and environmental conditions affect the composition and productivity (Huerlimann *et al.,* 2010). Lipids within algal cells are divided into neutral lipids (triglycerides and cholesterols) and polar lipids (phospholipids and galactolipids) (Huang *et al.,* 2009). Triglycerides are the most desirable product for biodiesel production (Mehrotra *et al.,* 2010). Triglycerides are often the form of which fatty acids are stored within organisms (Mathews *et al.,* 2000).

Hydrocarbon oils extracted from *B. braunii* produces a distillate comprising of 67% gasoline fraction, 15% aviation turbine fuel, 15% diesel fuel and 3% residual fuel after their hydro-cracking (Hillen *et al.,* 1982). The fuel fractions so obtained are free from oxides of sulphur and nitrogen (SOX and NOX) after their combustion. Furthermore, algal lipids have been suggested as a potential diesel fuel substitute with an emphasis on the neutral lipids due to their lower degree of unsaturation and their accumulation in algal cell at the end of growth stage (Casadevall *et al.*, 1985; McGinnis *et al.*, 1997). Micro algae are considered as an efficient microbe for fuel production due to their fast growth, high biomass production and high lipid accumulation when compared to other energy crops (Miao and Wu, 2006 and Banerjee *et al.*, 2002). Proportion of palmitic acid C: 16:0 was greater than 43 % among total fatty acids in *Synechocystis* sp. PCC6803 (Tran *et al.*, 2009).

When compared to this present study, *B. braunii* treatment yielded about 56.11, 29.07 and 27.9 % of C: 16:0 fatty acids in samples Napier's bridge, Aminjikarai and Adyar respectively and absent in sample Ethiraj College. And C: 16:0 is the major fatty acids accumulated among other fatty acids in the samples mentioned above. Oleic acid content (C:18:1) are the precursors of n-alkadienes (Banerjee *et al.*, 2002) on analyzing in this present study, the presence of C:18:1 oleic acid was seen in all the samples but accumulated high in sample Adyar (34.69 %) when compared to other samples. Accumulating C: 16:0, C: 18:1 and C: 18:2 as principle fatty acids in *Tetraselmis suecica* which seems to have required fatty acid profile for high quality biodiesel conversion. Similar results obtained in this study from all the *B. braunii* treated fatty acid profiles. The present GC-MS results were in agreement with the former results of *B. braunii* where palmitic acid (C: 16:0), oleic acid (C: 18:1) and linoleic acid (C: 18:3) were the major fatty acids recorded (Tran *et al.*, 2009). The pattern of fatty acid profile from this study seems to be completely related with chlorococcales as exemplified by Ahlgren *et al*. (1992). Palmitic acid and Oleic acid are the major constituents of fatty acids present in fatty acid profile of *B. braunii* reported by Dayananda *et al.*, 2007. The present GC-MS results of domestic treated *B. braunii* also support strongly the above results in which Palmitic acid (C:16:0) and Oleic acid (C:18:1) are the major fatty acids.

Methylated aldehydes are found in the cells of *B. braunii* (Metzger *et al.*, 1991). Those aldehydes synthesized from fatty acids methylation converted into saturated hydrocarbons (alkanes) (Banerjee *et al.*, 2002). The present IR results reveal the accumulation of methyl groups in the form of alkanes ranges from 3400 to 3900 cm^{-1}. Presence of hydrocarbons can be supported by the presence of aromatic bends in the methyl groups in all the treated samples.

5. Conclusion

The domestic waste water treatment using the micro alga *Botryococcus braunii* has shown potential results in the reduction of ammonia, potassium, electrical conductivity and TDS etc. The *Botryococcus* is one of the economically important micro algae for the production of hydrocarbons; in this current study such strain was implemented for the treatment of domestic waste water. The GC-MS and FT-IR analysis showed the presence of high lipid contents and precursors of hydrocarbons which are specific characteristic features of *B. braunii* and thus it can also be used for biodiesel. In this study, the micro alga *Botryococcus* has been proved as a potent candidate for biological treatment of domestic waste water. Furthermore, future studies are still essential in the large scale treatment to explore more results related to phycoremediation as well as high biomass production of economically important micro alga.

6. Acknowledgement

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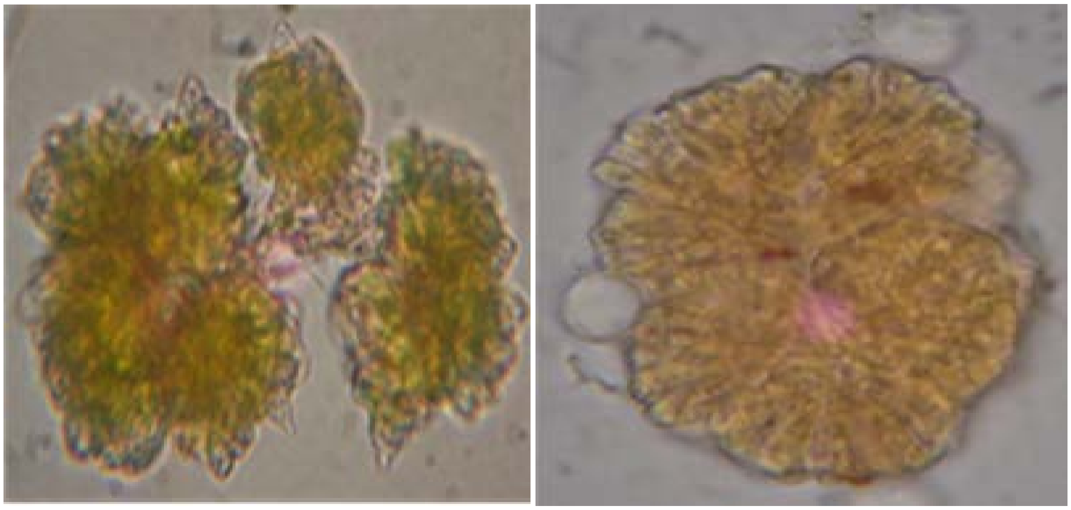

Fig. 1 a: Microscopic image of *Botryococcus braunii* seen from the fresh water sample in the temple pond sites Saidapet and Triplicane

Fig. 1 b: Domestic waste water treatment using *Botryococcus braunii* collected from different sites of the Chennai city 1) Aminjikarai (AK), 2) Ethiraj College (EC), 3) Adyar (AR) and 4) Napier's bridge (NB)

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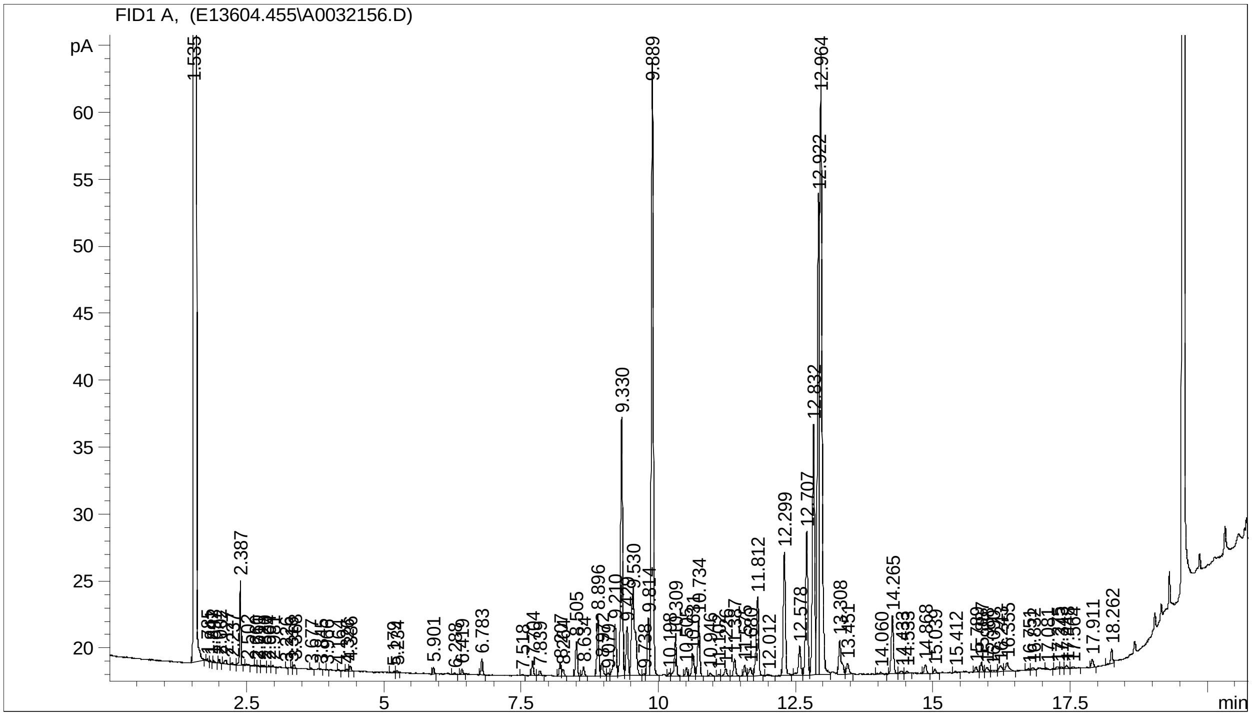

Fig. 2: Gas Chromatogram of *B. braunii* from Aminjikarai waste water treated biomass

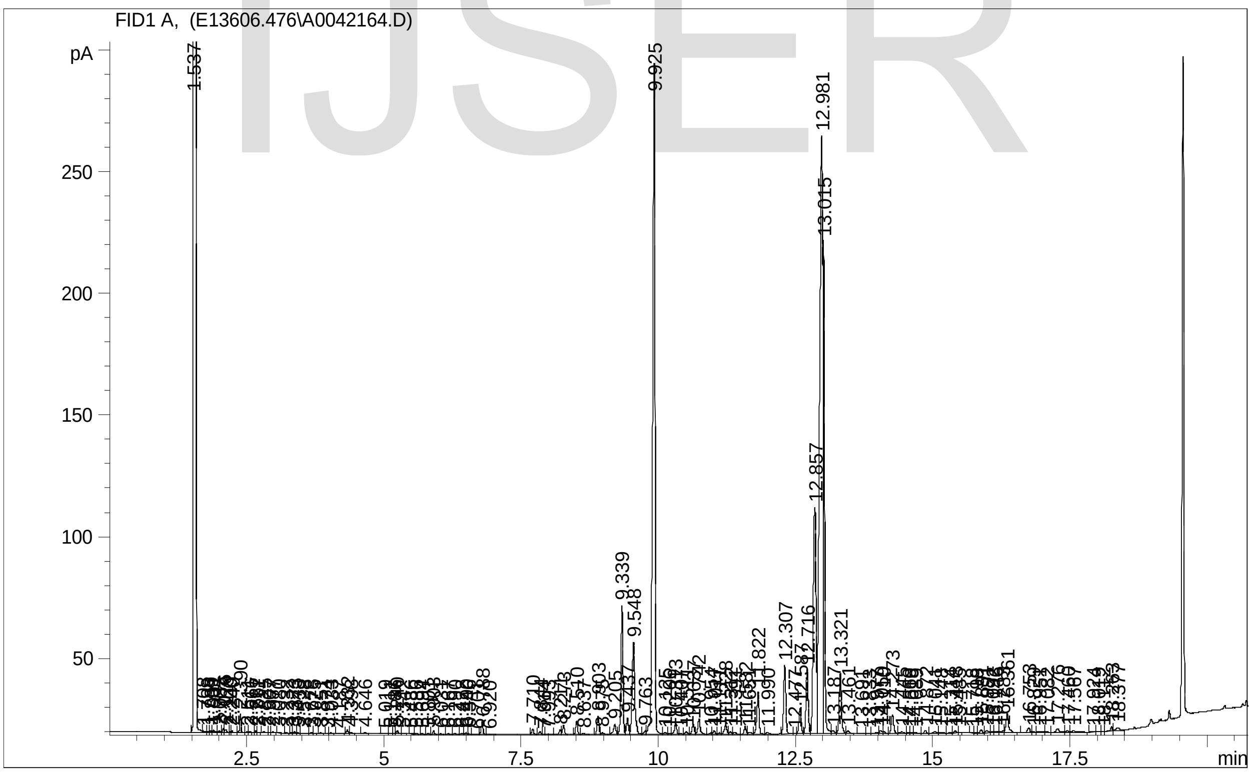

Fig. 3: Gas Chromatogram of *B. braunii* from Ethiraj College wate water treated biomass

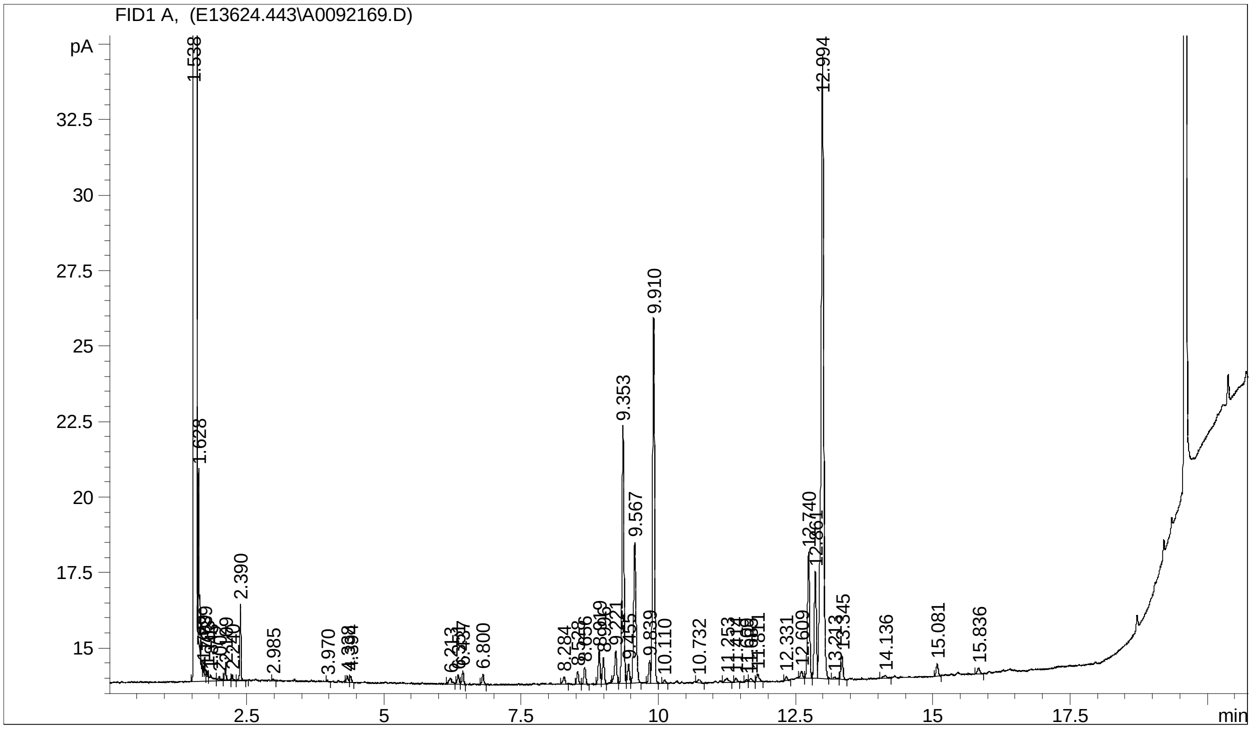

Fig. 4: Gas Chromatogram of *B. braunii* from Adyar waste water treated biomass

Fig. 5: Gas Chromatogram of *B. braunii* from Napier's bridge waste water treated biomass

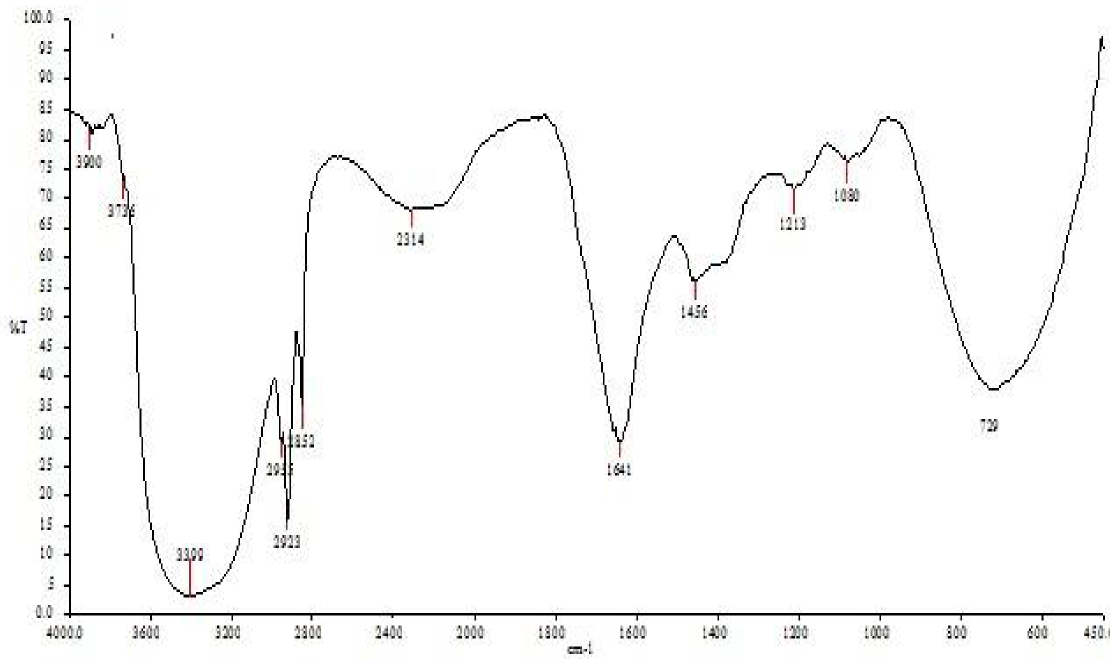

Fig. 6: FT-IR spectrum of sewage treated *Botryococcus braunii* biomass from sample Aminjikarai

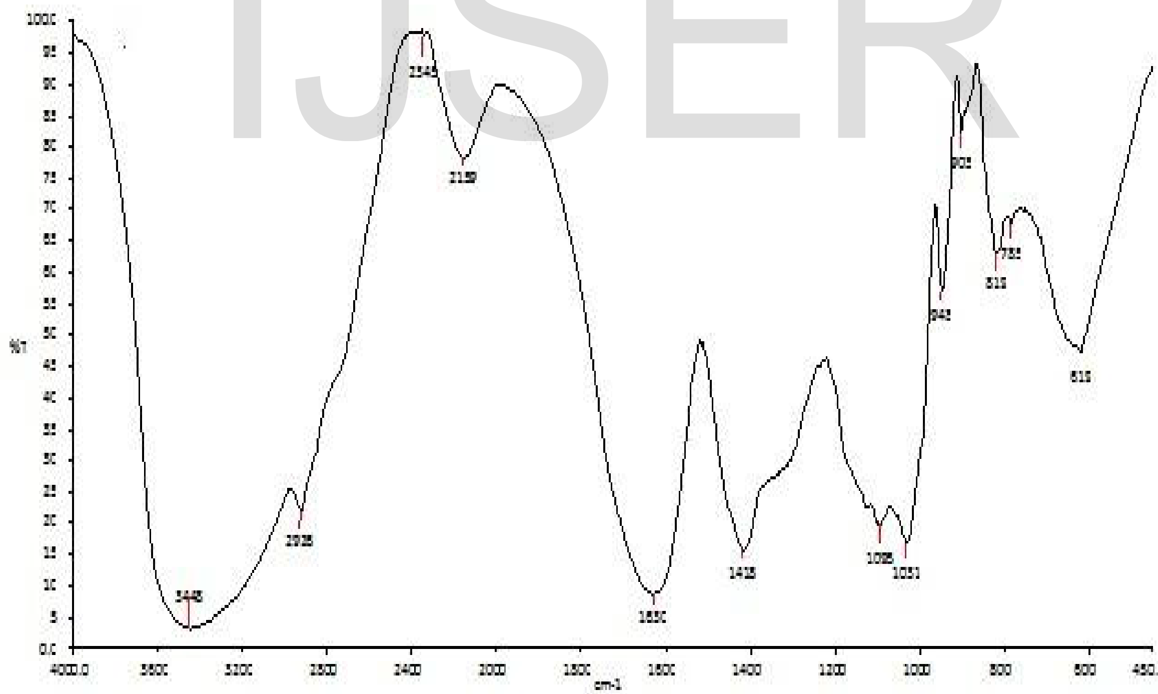

Fig. 7: FT-IR spectrum of sewage treated *Botryococcus braunii* biomass from sample Ethiraj College

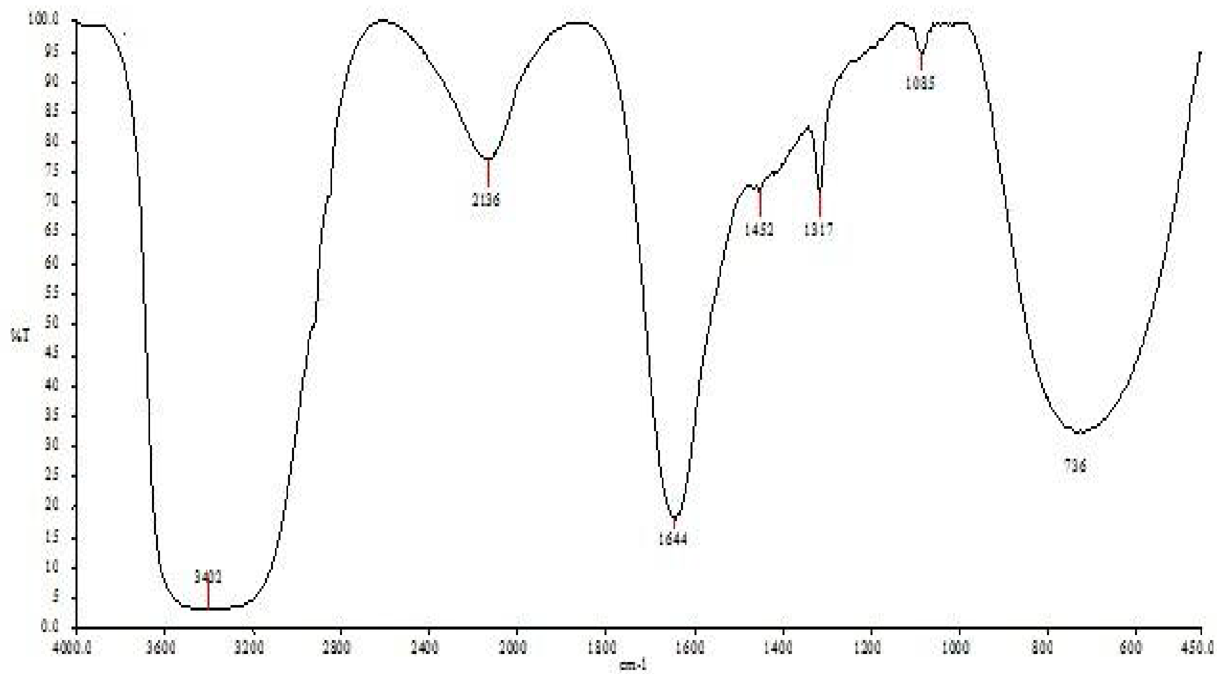

Fig. 8: FT-IR spectrum of sewage treated *Botryococcus braunii* biomass from sample Adyar

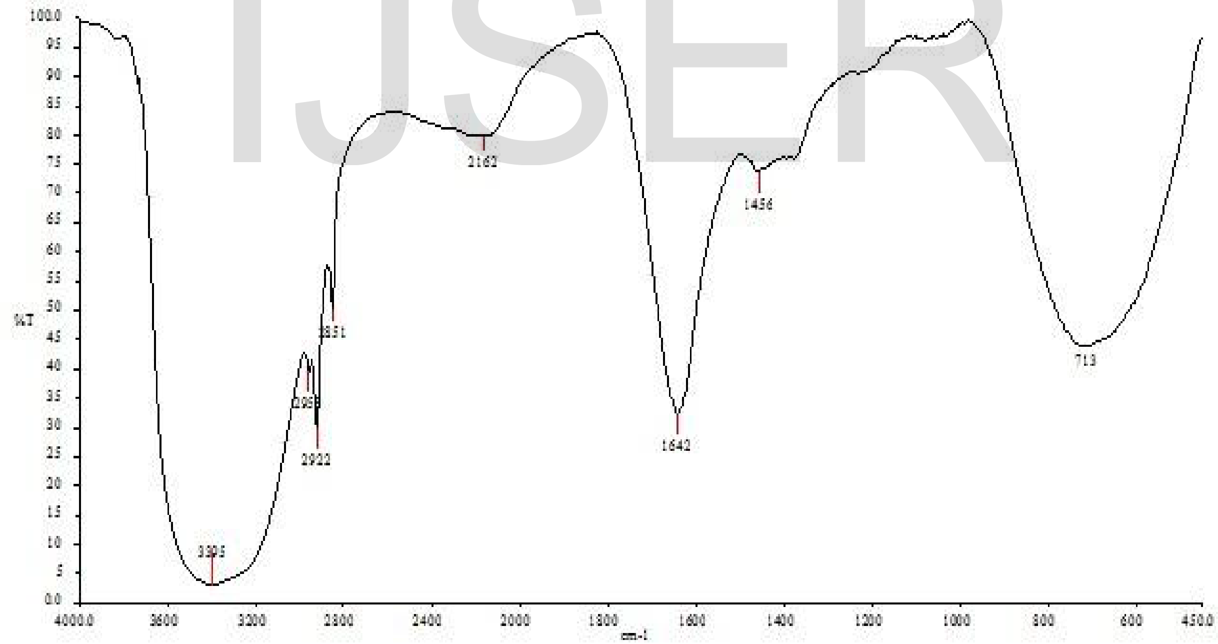

Fig. 9: FT-IR spectrum of sewage treated *Botryococcus braunii* biomass from sample Napier's bridge

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Table 1: Analysis of different Physio-chemical parameters from four various sewage pre and post treated water

		Pre-treatment	**Post-treatment**	**Pre-treatment**	**Post-treatment**	**Pre-treatment**	**Post-treatment**	**Pre-treatment**	**Post-treatment**
PHYSICAL EXAMINATION	**Control**	**Aminjikarai**	**Aminjikarai**	**Ethiraj college**	**Ethiraj college**	**Adyar**	**Adyar**	**Napier's Bridge**	**Napier's Bridge**
Appearance	Clear	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	Turbid	**Clear**
Colour (pt.co-scale)	Colourless	Black	**Green**	Black	**Green**	Black	**Green**	Colourless	Colourless
Odour	None	Foul smell	**None**	Foul smell	**None**	Foul smell	**None**	None	None
Turbidity NT Units	3.2	98.2	**30**	78.6	**73.2**	36.8	**24.4**	7.1	**1.2**
Electrical Conductivity Micro mho/cm	729	5300	**2240**	2510	**2200**	10400	**2401**	45100	**43200**
Total dissolved Solids mg/L	729	3710	**1568**	1757	**1540**	7280	**2401**	43200	31570
CHEMICAL EXAMINATION									
pH	7.99	7.52	**8.28**	7.94	**8.23**	7.7	**8.26**	6.92	**7.19**
Total Alkalinity as $CaCo_3$ mg/L	160	608	**340**	512	**336**	472	**356**	120	172
Total Haardness as $CaCo_3$ mg/L	126	600	**510**	380	480	1000	**880**	2500	10200
Calciun as Ca mg/L	38	176	**156**	108	140	280	**248**	800	3120
Magnesium as Mg mg/L	8	38	**29**	26	31	72	**62**	120	576

Table 2 cont….

Sodium as Na mg/L	101	870	**259**	375	**236**	1770	**114**	8400	**4640**
Potassium as K mg/L	9	65	**17**	30	**17**	110	**13**	550	**230**
Iron as Fe mg/L	0.34	0.51	0.81	0.63	1.09	0.44	0.92	0.41	0.53
Free Ammonia as NH_3 mg/L	0.28	12.9	**3.52**	11.32	**2.79**	10.26	**1.75**	6.77	**0.52**
Nitrate as NO_2 mg/L	1.13	0.41	**0.09**	0.02	0.28	0.04	0.05	0.72	**0.05**
Nitrate as NO_3 mg/L	8	20	32	14	26	14	16	14	**7**
Chloride as CL mg/L	98	1275	**495**	450	500	3300	**565**	14500	**13800**
Fluoride as F mg/L	0.47	0.97	**0.78**	0.84	**0.82**	0.64	**0.61**	0.61	**0.39**
Sulphate as SO_4 mg/L	24	107	**36**	62	**37**	158	**47**	221	**132**
Phosphate as PO_4 mg/L	0.13	0.22	**0.21**	0.32	**0.25**	0.05	0.22	0	0.2
Tidys Test 4 hrs.as O_2 mg/L	1	16.2	**6.8**	12.3	**6.1**	11.8	**3.7**	7.9	**1.9**
C.O.D -mg/L	6.6	198	**92.5**	162	**86.6**	140	**40.3**	89	**18.2**
B.O.D -mg/L	3	90	**38**	60	**33**	54	**18**	34	**8**