COMPARATIVE STUDY ON CULTIVATION OF MICROALGAE USING FRESH WATER AND INDUSTRIAL EFFLUENT (MATCH INDUSTRY) AS MEDIUM FOR BIOFUEL PRODUCTION

Lakshmi Praba S[1]*, Dhivya K[1], Subashini R[1], Rajadurai Jesudoss R P[1], Gnanasaraswathi M[1]
[1]*Department of Biotechnology, PSR Engineering College, Sivakasi-626140*
*Corresponding author: E.Mail: lakshmipraba@psr.edu.in; Mobile: 8148636440

ABSTRACT

Today's petroleum fuels are unsustainable because of depleting supplies and the contribution of fuels to the accumulation of carbon dioxide in the environment. We need alternative transport fuels to prevent carbon dioxide accumulation in the environment. Biofuel is biodegradable, less CO_2 and NOx emissions. Biofuel produced from microalgae is a potential renewable and alternative to petroleum fuels. Microalgae, which are one of the best producing oil crops, convert carbon dioxide to biofuel as well as involved in bioremediation process. The match industry effluent contains both organic, inorganic substances and color to the water. In this present study, the microalgae was isolated from drainage areas and cultivated in two different conditions, fresh water and industrial effluent (Match industry, Sivakasi) as medium. Lipid extraction from microalgae was studied with various solvents as maximum extraction of lipid as target. This study results evidences that microalgae cultivated in industry effluent yields 1/3 fold of cell growth as compared with commercial medium and solvent extraction of lipids from microalgae was maximum with acetonitrile followed by cyclohexane extraction. Thus the extracted lipids were confirmed by TLC. Functional components in Lipids were examined through HPLC and FT-IR. We conclude that this studies proved to be a effective tool for useful utilisation of industrial effluent for microalagae cultivation which will lead to the production of environment-friendly biodiesel.

KEYWORDS: Microalgae, Match industry effluent, Solvent extraction, HPLC and FT-IR

1. INTRODUCTION

Today's continued use of fossil source fuels such as coal, petroleum products, for domestic and industrial purposes have led to the continuous emission of greenhouse gases (GHG) and the depletion of the available natural energy sources. Renewable carbon neutral transport fuels are necessary for environmental sustainability. A renewable resource of power is that derived from biologically based fuels such as biomass, methane and ethanol which lead to phenomenal decrease in the greenhouse gas (GHG) emission. The match industry effluent contains both organic, inorganic substances and color to the water bodies (Selvaraj, 2013). Microalgae are one of the most promising sources available in wide range and have high oil content and effectively involved in waste water treatment process.

Whereas the first generation biofuel from feed stock like sugarcane, cereal grains and oil seeds (Wu, 2007) are used to produce bioethanol and biodiesel, the second generation biofuel includes bioethanol and biobutanol from lignocellulosic crops, straw wastes and jatropha (Tiwari, 2007).The third generation biofuel from non-feed stock such as microbial sources such as cyanobacteria, algae and diatoms. These first and second generation fuels compete for agricultural land and hike food consumption. To reduce the cost of biodiesel production, low cost waste materials need to be used as feed stocks which will produce environment friendly fuel as well as reduce pollution of the wastes. (Nigam, 2011).The limitations of first and second generation biofuel from feed stock sources are overcome by third generation biofuel from microalgae.

Microalgae can also be used for the waste water treatment and cultivation combined with biological cleaning process. Wastewater contains a variety of organic and inorganic compounds of anthropogenic and natural origin. The organic compounds found in the waste water have been utilized by the microalgae as its nutrient. Several medium has been reported to study the efficient growth of microalgae. The effects of sewage effluent and industrial sources that contains the nitrogenous waste that are arising from the water treatment or fish aquaculture and thereby uphold the biodiversity was reported by (Teresa, 2009).The nitrogen and phosphorous removed from the waste water by the microalgae with an average removal efficiency of 72% for nitrogen and 28% for phosphorous (Aslan, 2006).

In this present study, the microalgae was isolated from drainage areas and cultivated in two different conditions like fresh water and industrial effluent (Match industry, Sivakasi) as medium. Lipid extraction from microalgae was studied with various solvents as maximum extraction of lipid as target. The extracted lipids were confirmed by TLC. Functional components in Lipids were examined through HPLC and FT-IR

2. MATERIALS AND METHODS

2.1. Isolation of Culture and pre-inoculum Preparation: Microalgae were collected from the drainage areas and cultivated in 250 mL Erlenmeyer flasks containing 100mL of CFTRI medium. The composition of the CFTRI medium was as follows (per liter):4.5g $NaHCO_3$, 0.5g K_2HPO_4, 1.5g $NaNO_3$, 1.0g K_2SO_4, 1.0 NaCl, 0.2g MgSO4.7H_2O, 0.04g $CaCl_2$, and 0.01g FeSO4.The flasks were subjected to direct sunlight and optimum temperature until they reached sufficient growth of microalgae for inoculation in further studies.

2.2. Estimation of microalgae growth: 1 mL of the mother culture was inoculated in 100 mL of two different 250 mL Erlenmeyer flasks containing 100 mL of fresh water and match industry effluent. The chamber was used to give regular light/dark cycle and the optical density was measured for every 24hours (daily basis) at 540nm.

2.3. Analysis of Growth: Further, it was investigated which (light/dark) cycle causes the changes in microalgae populations. From that, the cell growth was compared through growth curve analysis. Briefly, 1 mL of 6th day inoculum was inoculated in 100 mL of two different medium, fresh water and match industrial effluent. The microalgae populations were measured for every 1 hour at 540 nm by spectrophotometer.

2.4. Optimization of solvent mixture: Best solvent for lipid extraction was analyzed. Solvents were chosen with polar, partial polar and non-polar characterized. Chosen solvents were Acetone, Proponal, Methanol and Acetonitrile. The extraction procedure was repeated triplicate on the same microalgae, workable solvent mixture with lipids to be detected. Non-Polar solvents cyclohexane was added to the lipid solubilization in further analysis.

2.5. Extraction of lipids: Extraction of lipid was done by following protocol of modified Bligh and dyer (1959).The match industry effluent grown cells were harvested on 6th day by centrifugation at 10,000rpm for 15minutes at 4 °C. The supernatant was discarded and pellet was subjected to wet weight estimation and then dried in oven for 1 hour at 80 °C. Acetonitrile were added in equal ratio to biomass and transfer to separating funnel and leave it for 24hours at room temperature. After 24 hours the layers were separated and lower organic layer containing lipids was transferred to a clean fresh pre-weighed vial (W_1). Evaporation was carried out in hot air oven at 80 °C for 40 minutes. The weight of the vial was again recorded (W_2). Lipid content was calculated by subtracting W_1 from W_2 and was expressed as % dry cell weight.

Lipid content = $(W_2-W_1) \times 100$

The lipid productivity was calculated by the equation given below (Yanqun Li, 2008):

$$P_{Liquid}\ g\ L^{-1}\ day^{-1} = \frac{C_{Lipid}\ (g/g) \times DCW\ (g/L)}{Time\ (day)}$$

Where, P_{Liquid} is lipid productivity, C_{Lipid} is lipid content of cells, DCW is dry cell weight and Time is the cultivation period in days. The same extraction procedure was used for above mentioned solvents for selection of solvents.

2.6. Chromatography Analysis of Lipid Content

2.6.1. Thin Layer Chromatography: The oil was fractionated qualitatively and quantitatively on 0.25mm and 0.5mm thick silica gel chromatoplates. Thin layer chromatograms of 0.25mm thickness were prepared by using 25 gm silica gel and 50 ml water. These plates were activated at 105^{o}C for two hours. A known weight of oil was loaded in a straight line about 3cm above the lower edge of chromate plate. The developing media for neutral and polar lipids were heptane: acetic acid: petroleum ether in the ratio of 75:15:15.The presence of lipids was detected with iodine by appearance of yellow/blue violet spots on a pink background TLC plate (Lowsenstein, 1969).The Retention Factor (R_f) was calculated using formula,

R_f = Distance moved by the solute/Distance moved by the solvent (cm)

The same procedure was followed in above mentioned solvents for estimation of R_f.

2.6.2. High performance liquid chromatography: The separated lipids were subjected to HPLC analysis for ratification. Extracted lipid sample from microalgae were suspended in polar solvents such as methanol. Flow rate of the column was fixed as 1ml/min. Mobile phase was chosen as optimum proportion of methanol and water of HPLC grade. Mobile phase and sample were pre-filtered in a wattman No1 filter paper. C18 column was made as stationary phase.

2.6.3. Fourier Transform Infrared Spectroscopy: Extracted algal biomass was compressed as pellet with KBr and dried at 100 °C for 4 hour.The dried sample was subjected to FT-IR spectrum using Fourier transform IR spectrometer (Kansiz, 1999).

3. RESULTS AND DISCUSSION

3.1. Estimation of biomass: The cells were harvested from 0th to 6th day culture grown under two different medium. When compared to fresh water medium, match industry effluent medium shows higher biomass 0.063 g/L,0.165g/L,0.247g/L,0.353g/L,0.472g/L,0.551g/L and 0.603 g/L.The results were given in Table 1 (Fig:1&2). The microalgae utilize organic and inorganic substance in match industry effluent as nutrients. Microalgae cultivation of match industry effluent shows a significant that, there is no accumulation of algae in medium. And also has ability to remove toxic chemicals from effluent on its growth. In case of fresh water medium the growth has been low that, may be due to absence of organic substances. (Selvaraj, 2013) study clearly shows that the green algae used *i.e. Gracillaria corticata* can efficiently remove the toxicity from effluent. Hence they strongly suggest that *Gracillaria corticata* can be used as a bioadsorbant to remove the toxicity of effluent polluted environment for sustainable agriculture. Therefore match industry effluent has been chosen for microalgae cultivation in further studies.

3.2. Estimation of Lipid Content: The high lipid content at 6th day culture of match industry effluent shows 68% because of microalgae consumed all nutrients sufficiently. In case of fresh water medium the lipid productivity to be low due to insufficient utilization of nutrients (Yujie Feng, 2010). The results were tabulated in Table 2.

Table 1: Growth of Microalgae in different medium

Time (Days)	Optical Density at 540 nm	
	Fresh water medium	Match Industry Effluent
0	0.061	0.063
1	0.093	0.165
2	0.142	0.247
3	0.198	0.353
4	0.221	0.472
5	0.279	0.551
6	0.369	0.603

Table 2: Estimation of lipid content on 6th day by harvesting microalgae

Time (Days)	Lipid content			
	Fresh water medium		Match Industry Effluent	
6	DCW (g/L)	P_{lipid} (%)	DCW (g/L)	P_{lipid} (%)
	0.37	2.89	0.68	4.69

Table 3: Growth curve analysis on 6th day culture as seed inoculum

Time (Hours)	Fresh Water medium Optical Density at 540 nm	Industrial Effluent medium Optical Density at 540 nm
0	0.056	0.061
1	0.071	0.099
2	0.081	0.125
3	0.099	0.146
4	0.109	0.165
5	0.115	0.183
6	0.126	0.225
7	0.138	0.256
8	0.149	0.262
9	0.161	0.284
10	0.178	0.307
11	0.184	0.327
12	0.19	0.343
13	0.196	0.363
14	0.209	0.382
15	0.217	0.401
16	0.226	0.423
17	0.239	0.438
18	0.247	0.451
19	0.258	0.469
20	0.269	0.482
21	0.271	0.499
22	0.282	0.511
23	0.293	0.523
24	0.304	0.535
25	0.311	0.542
26	0.324	0.561
27	0.332	0.573
28	0.346	0.584
29	0.355	0.596
30	0.362	0.609

Fig 1: Growth of Microalgae in different medium

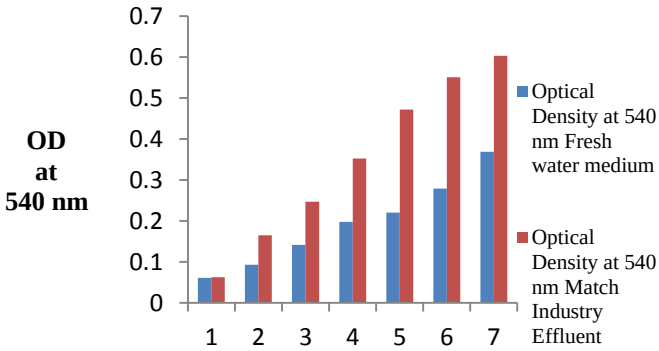

Fig 2:Growth of Microalgae in different medium

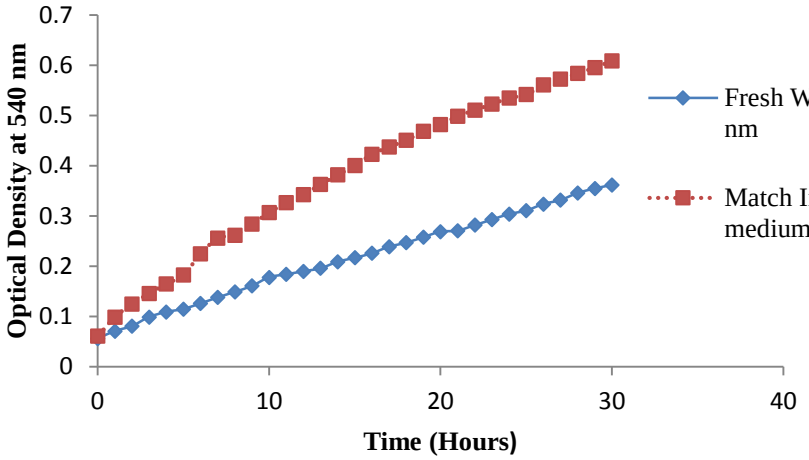

Fig 3: Growth curve analysis on 6th day culture as seed inoculum

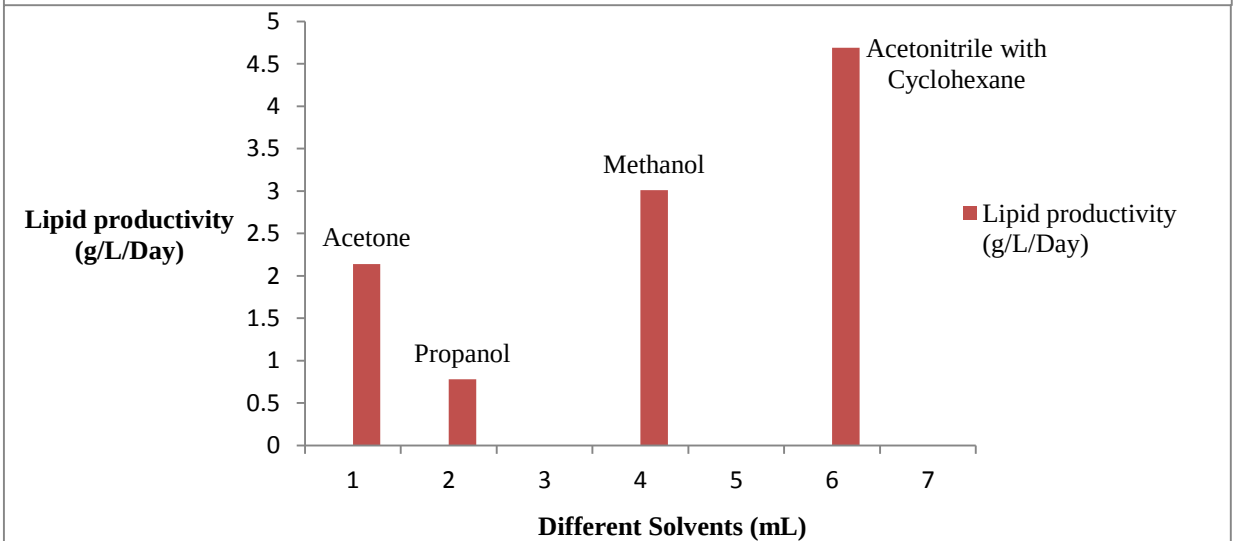

Fig 4:Effect of solvent in lipid extraction

3.3. Growth curve analysis: The growth was tracked for 30hours at regular light-dark cycle due to slow increasing of cell growth and microalgae have still growing after stationary point. (Sostarie, 2009) reported growth curves of microalgae *Chlorella vulgaris* cultured at different medium. In our results, the match industry effluent shows fast growth when compared to fresh water medium that may be due to absence of organic substances, microalgae growth started slowly.During dark cycle the algal growth has been increased 1/3 fold compared to dark cycle were Tabulated in Table 3 (Fig 3).The cell growth was lower under light cycle due to respiration. (Kwangyong Lee, 2001)

Table 4: Effect of solvent in lipid extraction

Different Solvents (mL)	Lipid productivity (g/L/Day)
Acetone	2.14
Propanol	0.78
Methanol	3.01
Acetonitrile	4.69

3.4. Effect of solvent in lipid extraction: The extraction of lipids mostly depend on solvent mixtures used (Fig 4).From given acetonitrile followed by cyclohexane provided high lipid extract shows lipid productivity of 4.69 g/L/day compared to other (Eline Ryckebosch et al.,2012).Solvent mixtures containing a polar and a non-polar solvent extracts high amount of lipids. In these results, the mid-polar solvent (Acetonitrile) releases the lipids from their lipid- protein complexes of microalgae and the released lipids continuously dissolved in the non-polar solvent (Cyclohexane).

3.5. Chromatography Analysis for Extracted lipids

3.5.1. Thin layer chromatography: The presence of lipids was detected with iodine vapor. Appearance of yellowish spots on a pink background TLC plate indicates lipids. R_f values of extracted lipids could be compared with standard lipids as 0.678 and 0.756 shown in fig 5. Patil et al. calculated the R_f values of triglycerides ($R_{f.TG}$) and Methyl ester ($R_{f.ME}$) as 0.564cm and 0.645cm.Hence the extract of acetonitrile evidences to lipids by coupling their R_f values with standard.

Table 5: R_f value (TLC) for lipids extracted from different solvents

Lipids Extracted from Different Solvents	TLC -R_f (cm)
Acetone	0.467
Propanol	0.269
Methanol	0.531
Acetonitrile	0.678
Coconut oil	0.756

3.5.2. High Performance Liquid Chromatography: Chromatogram of standard commercial coconut oil shows about 5 peaks various retention times. On that, peaks of retention time 2.473 (peak 4), 2.180 (peak 2) and (peak 1) 1.310 (Jessica Jones, 2012) evidences the coconut oil confirmed (Fig 7). Chromatogram of lipids extracted from microalgae also evidences following peaks, (Peak1) 1.257, (peak 2) 2.173, (peak 3) 2.457.Based on density the sample peak differs from the standard peaks only with its area and height. Thus, results strongly confirmed that, the component extracted from microalgae were lipids of slightly low density. (Peak 4) 2.707 may be an impurities or undesired product during extraction procedure (Fig 6).

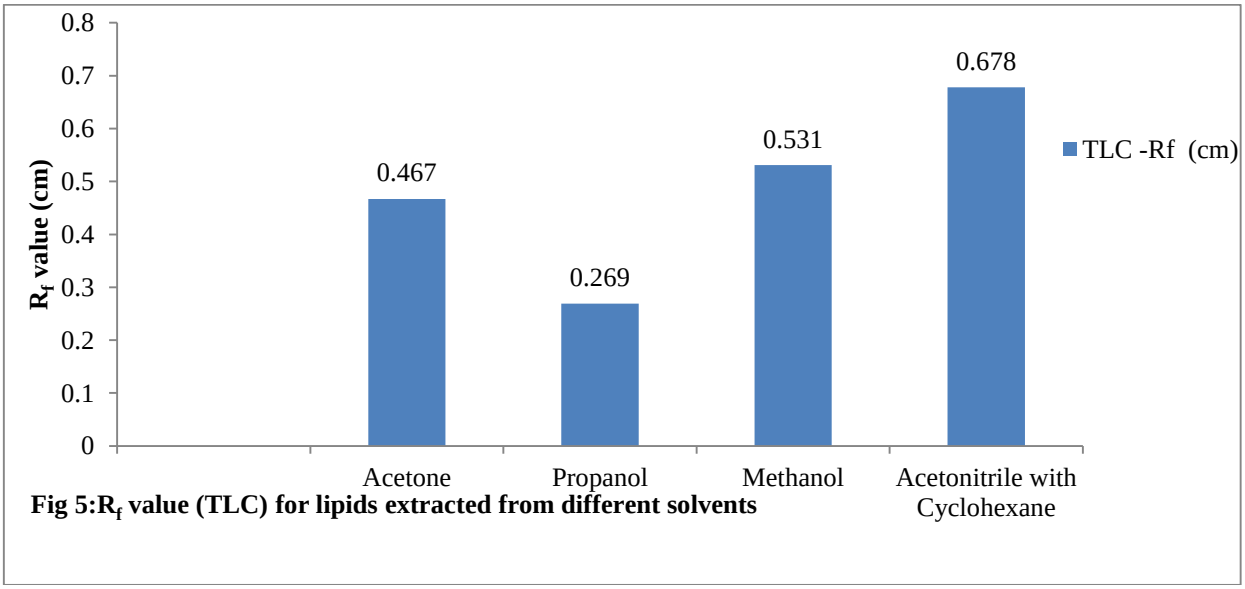

Fig 5:R_f value (TLC) for lipids extracted from different solvents

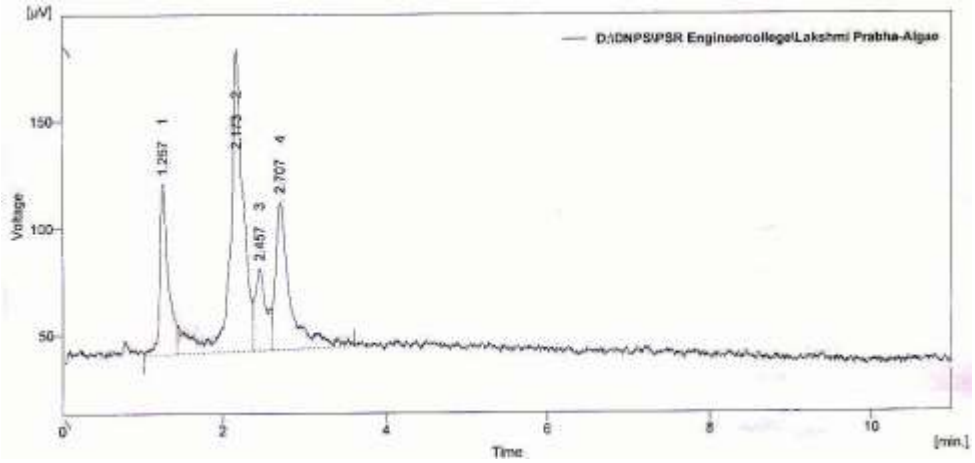

Fig: 6 HPLC Analysis for Extracted lipids from Microalgae

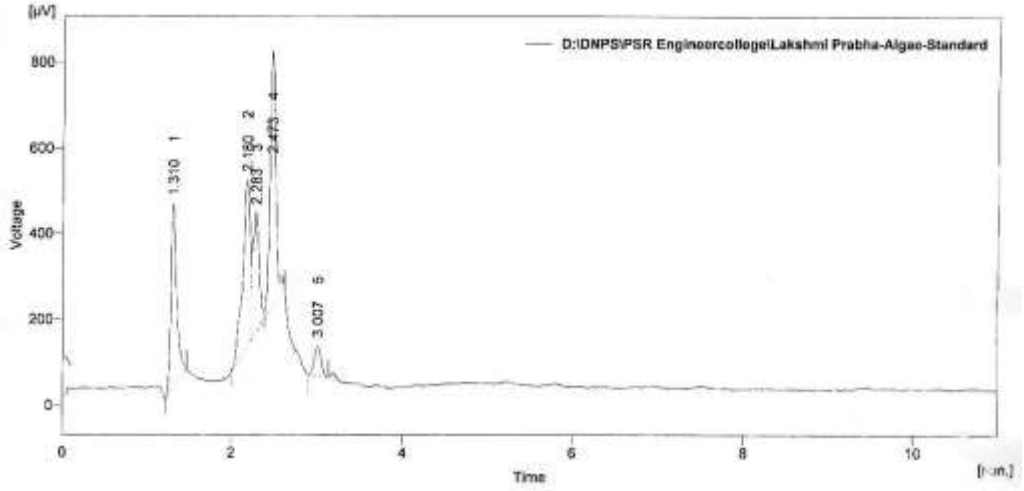

Fig: 7 HPLC Analysis for standard Lipid (Coconut oil)

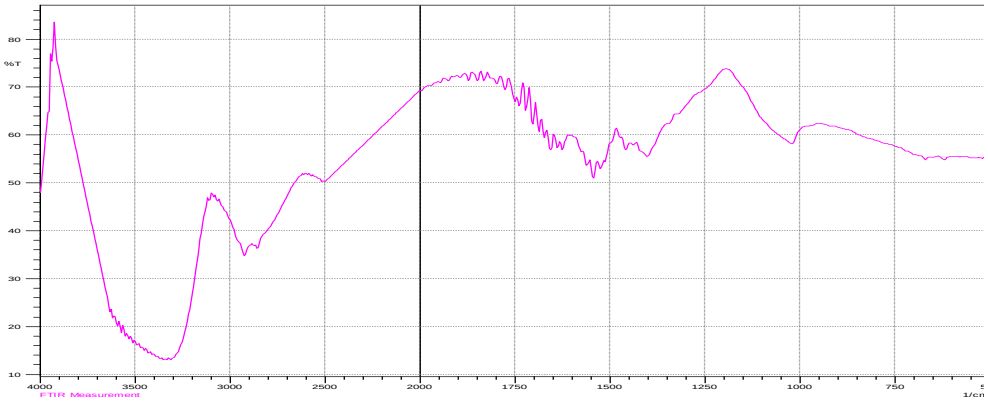

Fig: 8 FT-IR Analysis for extracted lipid from microalgae

3.5.3. FT-IR analysis: The result showed C═O: The main characteristic of the IR spectra of carboxylic compounds is the strong C═O stretching absorption band in the region of 1–1690 cm^{-1}. In the case of esters, this band appears in the 1750–1500 cm^{-1}. C─O─C: corresponding to ethers. These stretching vibrations produce a strong band in the 1320–1000 cm^{-1} region. C─H: absorption bands found in region of 2900-2850 cm^{-1} shows methyl group, IR spectra. In microalgae, C─H stretching absorption band in the region 2924 and 2854 cm-1 (Fig 8).O-H bands stretched in region of 3300cm^{-1} indicated carboxylic acids.CO_2: they produce strong bands in between 2900-2500 cm^{-1} as well as in 700 cm-1 region. H_2O: the adsorption bands of water can be observed in the range of 3500-3300 cm^{-1}. As many algal species have been found to grow rapidly and produce substantial amounts of lipids and are thus belongs to as microalgae (Sanniyasi Elumalai, 2011).

4. CONCLUSION

The present study evidences, the match industry effluent act as best medium for microalgae cultivation and their biomass has been increased 1/3 fold time when compared with fresh water medium. Solvent mixtures were optimized for extracting high lipids from microalgae. From this results, Acetonitrile followed by Cyclohexane provided high lipid content and thus the preferred solvent mixture for further analysis. The extracted lipid algal cells were confirmed by Thin Layer Chromatography, High Performance Liquid Chromatography and extracted algal biomass of functional groups was analyzed by FT-IR. In our results strongly suggested that microalgae can utilize waste water (match industry effluent) as medium for their growth and produced high lipid content. However, using microalgae the extracted lipids converted to oil through transesterification and removal of toxic chemicals from match industry effluent will be goal of our future research.

ACKNOWLEDGEMENT

The authors are very much thankful to Management, Principal, Head and all faculty members of Biotechnology Department, P.S.R.Engineering College for providing all the support and required facilities in the department to complete this project successfully. The authors also extend our loving thanks to parents, friends and well-wishers for their constant support and encouragement to this project.

REFERENCES

Bligh EG, Dyer WJ, A Rapid method of total lipid extraction and purification, Canadian Journal of Biochemistry and physiology, 37 (8), 1959, PP.911-917.

Eline Ryckebosch, Koenraad Muylaert, Imogen Foubert, Optimization of an Analytical Procedure for Extraction of Lipids from Microalgae, J Am Oil Chem Soc, 89, 2012, 189–198.

Ilknur AK, Semra Cirik, Tolga Goksan, Effect of Light-intensity, Salinity and Temperature growth in Camalti strain of Dunaliella Viridis Teodoresco from Turkey, Journal of Biological sciences, 8, 2008, 1356-1359.

Jessica Jones, Schonna Manning, Morela Montoya, Karin Keller, Martin Poenie, Extraction of Algal Lipids and Their Analysis by HPLC and Mass Spectrometry, J Am Oil Chem Soc, 89, 2012, 1371–138.

Kwangyong Lee, choul-gyan Lee, Effect of light and dark cycles on waste water treatments by microalgae, Biotechnology Bioprocess Engineering, 6, 2001, 194-199.

Laila Al,Balushi, Nitin Rout, member, IAENG, Sahar Talebi, Ahmed Al Darmaki, Maryam Al Qasmi, Removal of Nitrate from Wastewater Using Trentepohlia Aurea, Microalgae Proceedings of the World Congress on Engineering, Vol I WCE ,2012, 4 - 6.

Sanniyasi Elumalai, Velu Prakasam, Ramganesh Selvarajan, 'Optimization of abiotic conditions suitable for the production of biodiesel from *Chlorella vulgaris,* Indian Journal of Science and Technology, Vol. 4, 2011, No. 2

Selvaraj K, Sevugaperumal R, Ramasubramanian V, Impact of Match Industry Effluent on growth and biochemical characteristics of *cyamopsis tetragonoloba* taub and amelioration of the stress by seaweed treatment, Indian Journal of Fundamental and Applied Life Sciences, Vol. 3 (1), 2013, pp.192-197.

Senthil Chinnasamy , Balasubramanian, Ramakrishnan , Ashish Bhatnagar, Keshav C, Das, Biomass Production Potential of a Wastewater Alga *Chlorella vulgaris* ARC 1 under Elevated Levels of CO2 and Temperature, International Journal of Molecular Sciences, Vol 10, 2009, 518-532.

Sostarie M , Golob J, Bricelj M, Klinar D, Pivec A, Studies on the Growth of *Chlorella vulgaris* in Culture Media with different carbon sources, Chem Biochem Eng Q, 23 (4), 2009, 471–477.

Subhasha Nigam, Monika Prakash Rai, Rupali Sharma,Effect of Nitrogen on Growth and Lipid Content of *Chlorella pyrenoidosa*, African Journal of Biotechnology, Vol. 9 (54), 2010, pp. 9227-9236, 27.

Teresa M , Mata, Antonio A, Martins Nidia S,Caetano, Microalgae for biodiesel production and other applications, Renewable energy and sustainable energy reviews 14, 217-232, 2010.

Wan Omar WNN, Nordin N, Mohamed M, Amin NAS, A two-step biodiesel production from waste cooking oil Optimization of pre-treatment step, Journal of applied sciences, 9 2009, 3098-3103.

Xiaoling Miao, Qingyu Wu, Biodiesel production from heterotrophic microalgal oil, Bioresource Technology, 97, 2006, 841–846.

Yanna Liang, Nicolas Sarkany, Yi cui, Biomass and lipid productivities of Chlorella vulgaris under autotrophic heterotrophic and mixotrophic growth conditions, Biotechnology Lett 31, 1043-1049, 2009.

Yujie Feng, Chao Li, Dawei Zhang, Lipid production of *Chlorella vulgaris* cultured in artificial wastewater medium, 2010.