

# Investigation on Tannery Wastewater as Feedstock for Marine Microalgae in biofuel production

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## Abstract

Tannery wastewater high in salinity makes the treatment process perplexed. The oleaginous marine microalgae *Nannochloropsis marina*, *Chlorella marina*, *Thalassiosira sp.* and *Dunaliella saline* were studied for their lipid producing ability grown in secondary effluent from tannery wastewater treatment plant. Among the strains used *Chlorella marina* showed maximum biomass and lipid yield of 1.92g/L and 0.7 g/L respectively, with resulting lipid content of 42%. Also, 78% of COD removal efficiency by the species *C. marina* made it a significant organism in the treatment of tannery effluent. Also the biodiesel obtained by direct transesterification was found to be promising with the biodiesel yield of 0.59 g/L. Hence the study signifies that the tannery effluent could be a better feedstock for marine microalgae in biofuel production.

**Key words:** Marine microalgae, secondary effluent, direct transesterification, biodiesel.

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## 1. Introduction

Tannery waste water emerge from leather tanning process, where the hides of animals are transformed to leather. Tanning can be done in two ways – chrome tanning and vegetable tanning, where chrome tanning is widely followed in many industries. The waste water from tanneries constitutes high amounts of BOD<sub>5</sub>, COD, sulphur, chromium, salinity, carbon, nitrogen and phosphorous contents, which require higher degree of treatment to meet the permissible limits to discharge. This makes the treatment process high-priced and require large area of land. The annual cost including capital and maintainance cost of activated sludge treatment process in a common effluent treatment plant in India measured Rs.3.36 million/million liters per day and the land occupied was 0.95 hectares/MLD [16], but this was almost fifteen years ago, and now the cost would be much higher. The effluent from secondary sedimentation tank must undergo microfiltration or reverse osmosis to reduce the salinity, which add up to the cost. To overcome this problem, a cost effective and efficient method of treatment is required.

On the otherhand the researches are engrossed in finding an alternate fuel source to meet the energy demand. Biofuels produced from crops involve occupancy of land, competing with the food crops. Microorganisms capable of producing oil more than 20% of its cell dry weight are termed as oleaginous microbes [17] and such microbes can be used in producing biodiesel by transesterification of triacylglycerols produced by the cells. In general, microorganism utilise the nutrients and organics present in various waste [3] which results in prominent advantage of waste management. The oleaginous microbes can be algae, fungi, bacteria and yeast, which are capable of producing single cell oil (SCO) measuring 20 to 80% of its dry weight [18]. The use of microalgae GRAS (generally regarded as safe) and easy to cultivate has certain advantages in biodiesel production compared to other energy crops: their higher photosynthetic activity with respect to terrestrial plants; easy adaptability to different growing conditions; possibility of growing either in fresh-waters or marine-waters, avoiding use of land; microalgae can also produce valuable by-products such as lipids, vitamins, polysaccharides, pigments, bioactive compounds, proteins, and antioxidants [13]. Moreover, from an environmental point of view, the use of microalgae as raw material for biodiesel production gives a solution to the treatment of a waste collected. Along with other advantages, the biodiesel produced by microalgae measure up to 250 times higher the amount of oil per acre as soybeans produces [22], which is more efficient than the conventional biofuels from food crops.

This study focus on the utilisation of secondary effluent from tannery wastewater treatment plant, as a culture medium for marine microalgae in production of SCO. As the salinity of tannery waste water exceeds 16s it was preferred to use marine microalgae to treat the wastewater. The microalgae apart from being used in wastewater treatment process it can also help in carbon sequestration, which leads to reduced air pollution. Four marine algae were chosen for the study – *Nannochloropsis marina*, *Chlorella marina*, *Thalassiosira sp.* and *Dunaliella salina*. These species have become very popular for studies on biofuels and the production of long-chain polyunsaturated fatty acids. [5,23]

## 2. Methodology

### 2.1 Characterisation of tannery wastewater

Tannery waste water was collected from secondary sedimentation tank in treatment plant at EKM Tanneries Ltd., Erode. Samples were charecterised for their hardness, Organic carbon content, Inorganic carbon content, Total phosphates, Total nitrates. Total organic carbon, and Total Nitrogen were quantified using Shimadzu TOC analyzer, phosphates were quantified using UV – Vis Spectrophotometer (Perkinelmer). BOD, COD, TSS, pH and hardness were analysed by standard procedure. All the chemicals used in the study were of analytical grade and study was done in triplicates.

## 2.2 Microalgae growth

Crude cultures were purchased from CMFRI, Kochi. Primary seed cultures of microalgae were grown in F2 medium in individual flasks, at 27 °C and 120 rpm under illuminance at night time. The 500 ml of wastewater sample was taken in five separate Erlenmeyer flask and were sterilized at 121 °C for 15 min in fully automated autoclave. 10% (v/v) of seed culture of *Nannochloropsis marina*, *Chlorella marina*, *Thalassiosira sp.* And *Dunaliella Salina* was added to the individual flasks under sterile condition. Fifth flask was seeded with 10 % (v/v) of consortium containing *Nannochloropsis marina*, and *Chlorella marina* 5% (v/v) each. The culture was maintained at room temperature with 200 rpm, providing a light intensity of 2500 Lux, with 12 hrs of illuminance and 12 hrs dark condition. The growth of the microbial species was monitored every 24 hours by collecting 10 ml of sample, centrifuged at 10000 rpm for 5 minutes. The pellets were washed with deionised water, filtered, dried at 50 °C till constant weight was obtained and measured gravimetrically.

## 2.3 Substrate utilization and lipid analysis

The samples were collected every 24 hrs and centrifuged at 10000 rpm for 5 min. The supernatant was analysed for the COD present in the sample by standard protocol [1]. The rate of substrate utilisation was measured in terms of efficiency of COD removal by the algal species which gave maximum biomass yield. Lipid extraction was carried out with dried biomass by solvent extraction method [8], with minor modifications. It was suggested that no extraction method is 100% efficient in lipid recovery from a biological cell (4,11). This difficulty may arise due to the presence of cell wall which hinders the penetration of organic solvents into the cell [12]. Here glass beads and 4M HCl were employed for cell wall disruption. The centrifuged biomass were washed and dried at 50 °C till completely dried. The dried cells were disrupted and homogenized in 4 M HCl for 30 minutes. The cell lysate and methanol: chloroform mixture (1:2 volume ratio) was mixed for 30 minutes. It was centrifuged at 10000 rpm for 10 min. Top aqueous layer was removed and organic layer containing lipid was evaporated under nitrogen atmosphere and lipid measured gravimetrically. The lipid was confirmed using Fourier Transform Infra-Red (FTIR) spectrophotometric (PerkinElmer Spectrum, HATR) analysis. A horizontal ATR sampling accessory (PerkinElmer L1600248) with ZnSe cell was employed. The analysis was done in 5 min with spectra range from 4000-450  $\text{cm}^{-1}$  with 4  $\text{cm}^{-1}$  resolution. The result was obtained from average of 32 scans.

## 2.4 Insitue transesterification

Transesterification can be done in many ways where direct transesterification was found to be promising [2]. Transesterification is the reaction of oil with an alcohol to obtain esters and glycerol. This reaction can be carried out in absence or presence of a catalyst. It is a reversible reaction, thus it is necessary to use excess of alcohol to shift the equilibrium to the product side [14].

To the dried biomass 20 ml of methanol containing 0.2 mol/L of concentrated sulphuric acid was added. The temperature was raised to 70 °C with continuous mixing for 1 hour using thermomixture. The mixture was cooled down to room temperature using ice bath. To this reaction mixture, hexane was added and mixed vigorously for 5 min and the upper organic layer was separated and allowed to evaporate [20]. The final sample containing fatty acid methyl ester was taken for analysis under Gas chromatography - mass spectroscopy (Thermo GC - Trace Ultra Ver: 5.0, Thermo MS DSQ II). The standard DB-MS non polar capillary column was used with Helium as the carrier gas with the flow rate of 1.0 ml/min. The column temperature was raised at a rate of 6 °C /min from 70 °C to 260 °C. One micro liter of sample was injected and 37.50 min was the run time. Flame ionisation detector was used at 250 °C and chromatogram was obtained.

# 3. Results and discussion

## 3.1 Characteristics of wastewater

The physical and chemical characteristics of wastewater collected from the treatment plant was tabulated in table 1. The parameters were found to be quite higher than the permissible limits of wastewater discharge resulting in a favorable condition for algal growth.

The carbonates and bicarbonates render in a higher degree of hardness, which is above the permissible limits. Though the pH is near neutral the salinity of the wastewater leads to increased alkalinity which makes the water suitable for the growth of marine microbe [6].

**Table 1.Characteristics of wastewater collected**

S.No.	Parameters	Values (mg/L except for pH)
1	Total Organic Carbon	800
2	Total Inorganic Carbon	12
3	Total Nitrogen	138
4	Total Phosphorous	30
5	Total Hardness	400
6	BOD	50
7	COD	670
8	pH	7.8
9	TSS	200

### 3.2 Growth and lipid yield of microalgae

Figure 1, depicts the biomass yield of five microbe culture with respect to time.

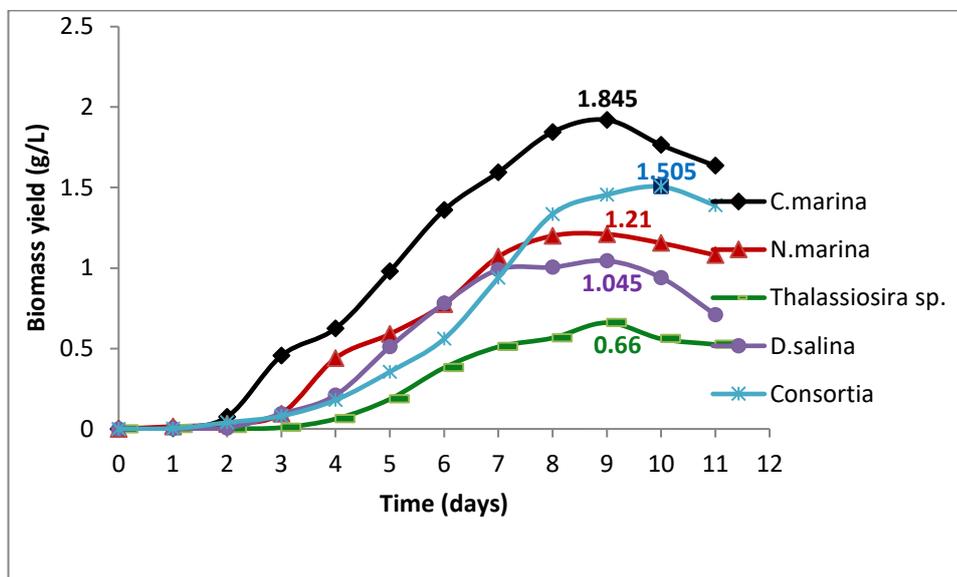


Fig.1: biomass yield

From the figure it was clear that the microalgae *C.marina* gave the maximum growth at 9<sup>th</sup> day. The maximum biomass yield of *C.marina* was 1.92 g/L followed by consortia, 1.505 g/L on 10<sup>th</sup> day. The consortium containing mixed culture of *N. marina* and *C.marina* gave highest yield on 10 days of growth. The 24 hrs delay in maximum biomass yield of consortium was because the initial lag phase required a day to initiate, which may be due to competence for food. But *N.marina* on isolation had lesser yield comparatively to

*C.marina*. In a work by Shenbaga Devi et al [28] maximum biomass yield for *Nannochloropsis marina* and *Dunaliella salina* were obtained on 9<sup>th</sup> or 10<sup>th</sup> day according to the environmental condition provided. The lipid yield was monitored and the corresponding lipid content was given in figure 2. It was clearly viewed that *C.marina* gave the maximum yield with lipid content of 42%, and the least was found in *Thalassiosira sp.* The variation in growth and lipid accumulation among different algal species relies on the macromolecular stoichiometry and storage pool, which is a function of change in environment conditions, resulting in change in C:N:P in the cell [10]. The carbohydrate and lipid storage in microalgae increases when C:N in the culture species increases with respect to nitrogen depletion resulting in decreased protein content and growth rate [7]. The evolutionary difference in macromolecular composition pertain to different phytoplankton types [31], which can be said vice versa.

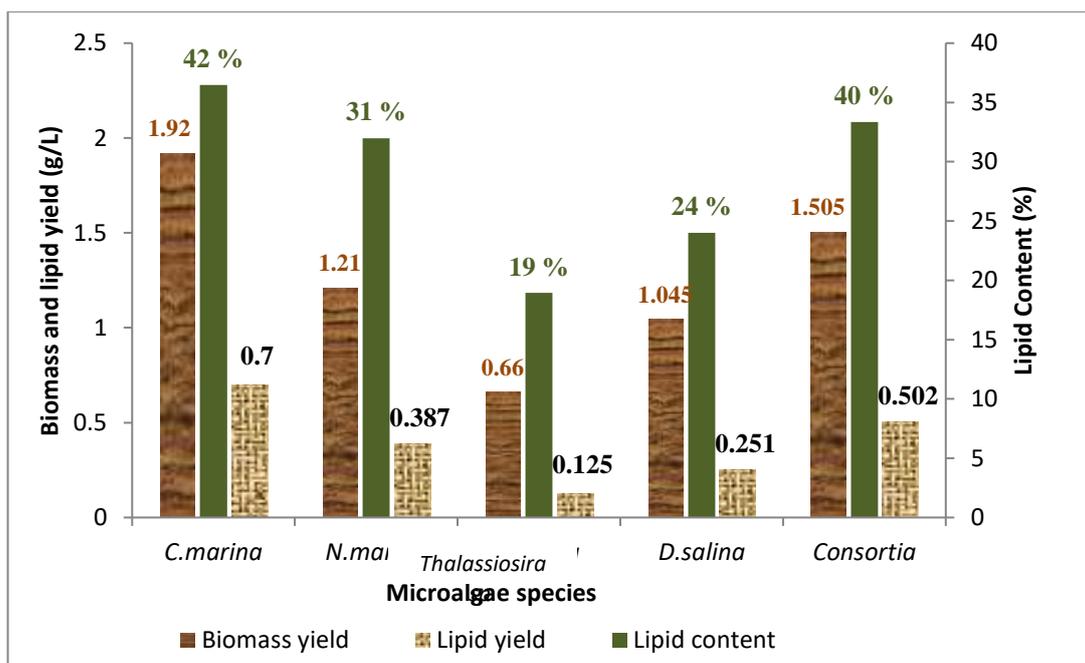


Fig.2: maximum Biomass, lipid yield and lipid content by the microalgae.

### 3.3 Substrate utilisation

The COD removal efficiency by *C.marina* was monitored every 24 hours and charted. From the graph it was clear that the removal efficiency was higher at the initial stage, which indicates the rapid substrate utilisation. *Chlorella* species under ideal condition incapable of multiplying rapidly on utilising carbon, light and water [27]. Inclined increase was seen up to 6 days, after which the removal efficiency nears saturation. The rate of substrate utilisation is proportional to microbial growth. In a study by Zoe V. Finkel [31], 22.5% lipid yield/cellular dry weight was obtained during the stationary phase, whereas 17.3% yield was obtained on active log phase. This shows that the lipid accumulation occurs on the saturation of nutrient inhibiting cell proliferation leading to lipid accumulation. The maximum removal efficiency was noted as 78%, this showed that *C.marina* has been a better species in yielding sufficient lipid alongside the higher COD removal resulting to be a promising microbe in waste management.

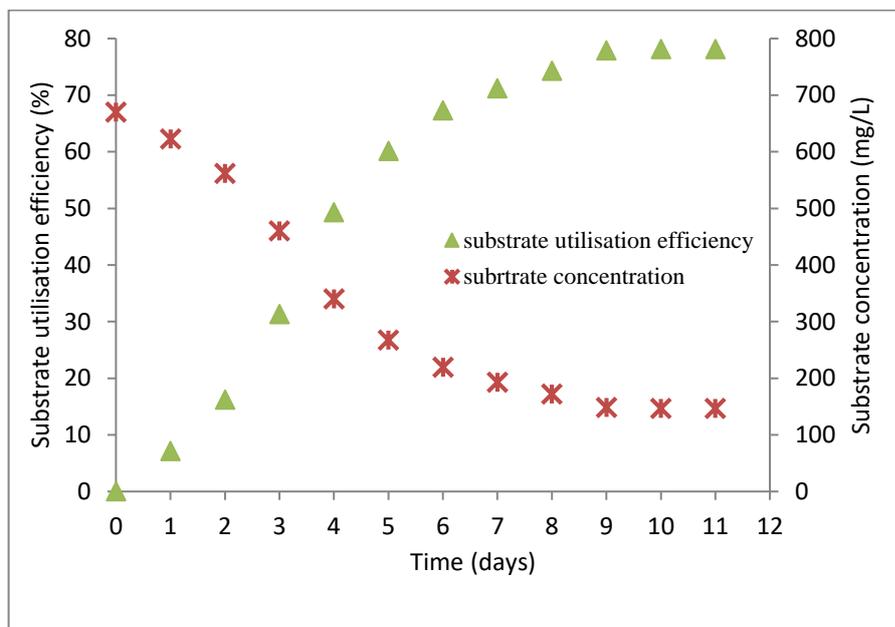


Fig.3: Substrate utilisation in terms of COD removal efficiency.

### 3.4 Fatty acid analysis

The fatty acid profile of the samples extracted (Fig.4) was obtained from Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectroscopy. In the spectrum, frequency is represented in  $\text{cm}^{-1}$ , measuring the number of wave cycles per cm. Absorption of a CO stretch of esters corresponding to two asymmetric vibrations of O-CO (aliphatic) within  $1275$  to  $1185$   $\text{cm}^{-1}$  and O-R stretch at  $1160$  to  $1055$   $\text{cm}^{-1}$  have been identified as very important in FT-IR analysis of oils (Mueller et al., 2013). The peaks between  $2850$  to  $3000$   $\text{cm}^{-1}$  specifies the strong stretching of C-H in aldehydes ( $\text{CH}_3$ ,  $\text{CH}_2$ , and  $\text{CH}$ ), here the peaks were visualised at  $2922$   $\text{cm}^{-1}$  and  $2853$   $\text{cm}^{-1}$  which were ascribed to the C-H stretching in  $\text{CH}_3$  and  $\text{CH}_2$  [9,21]. At  $1744$   $\text{cm}^{-1}$  and  $1710$   $\text{cm}^{-1}$  vibration due to C=O (ester) stretching [15],  $1463$   $\text{cm}^{-1}$   $\text{CH}_2$  bending, and at  $1377$   $\text{cm}^{-1}$   $\text{CH}_3$  bending can be attributed to the glycerol group [29]  $721$   $\text{cm}^{-1}$   $\text{CH}_2$  rocking vibration. This spectrum ascribed the signals of fatty acid, and also matched with the spectra obtained by Rabelo, S.N. et al, 2015, for soybean oil. The result proved the lipid obtained from *C.marina* to be promising in biodiesel production by means of transesterification.

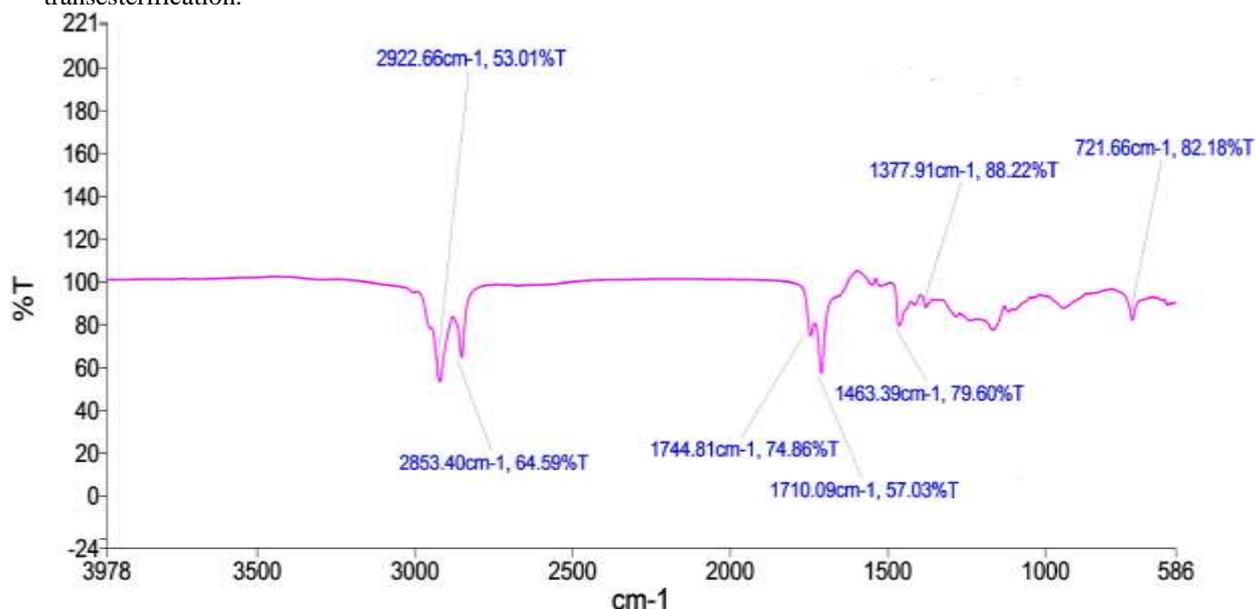


Fig.4: FTIR spectrum of lipid obtained from *C.marina*

### 3.5 Transesterification

Transesterification involves the formation of fatty acid methyl ester (FAME) which is commercially called biodiesel. The biodiesel yield obtained on insitue transesterification was 0.59 g/L. It was proven that, the organic solvents like petroleum ether, n-hexane and chloroform drives to efficient extraction of lipid from the cell, than other solvents [30]. This may be due to the fact that these solvents are non-polar which have the ability to dissolve long chain triglycerides and also miscible with alcohol which provides homogenous catalysis. Thus the insitue transesterification worked well, and the biodiesel content was measured to be 30.7%.

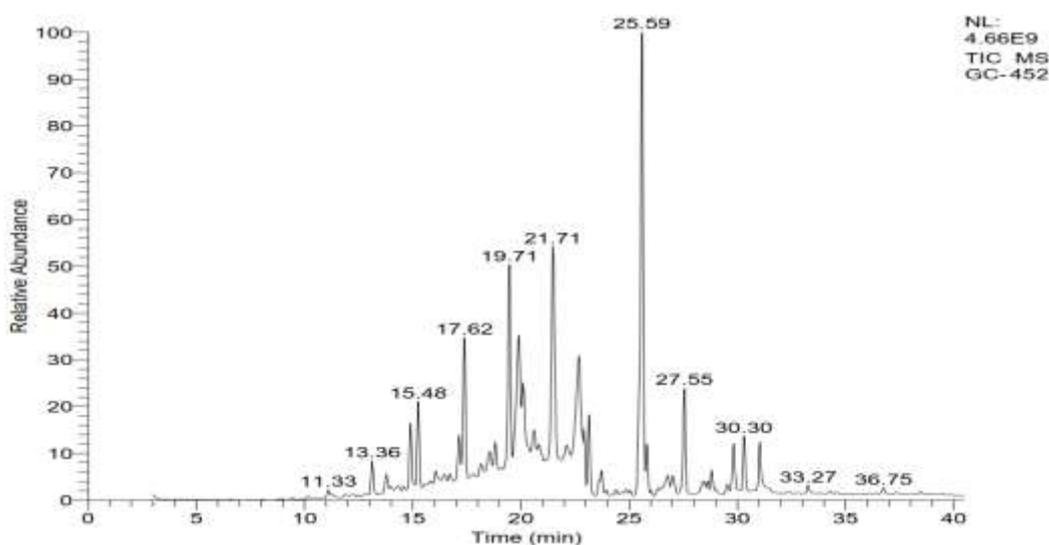


Fig:5. GC-MS chromatogram of fatty acid methyl esters from insitue transesterification of *C.marina*

S.No.	Retention time (min)	Fatty acid esters	Lipid number	Degree of saturation	% of FAME
1	15.48	Methyl myristate	C14:0	Saturated	5
2	17.625	Methyl palmitate	C16:0	Saturated	9
3	19.71	Methyl stearate	C18:0	Saturated	14
4	21.71	Methyl linoleate	C18:2	Polyunsaturated	17
5	25.59	Methyl behenate	C22:0	Saturated	28
6	27.55	Methyl erucate	C22:1	Monounsaturated	8
7	30.30	Methyl docosahexaenate	C22:6	polyunsaturated	3

The sample analysed using GC-MS provided the fatty acid methyl ester profile given in fig 5. The report showed the content of esters obtained from the biological cell *Chlorella marina* and tabulated in table 2. It was observed that most of the esters were found to be saturated. Methyl behenate was found to be 28%, which is saturated fatty acid ester proves the biodiesel obtained to be a promising fuel source, as the saturated fatty acid holds good cetane number and oxidative stability which are the important fuel properties for biodiesel [26]. In a work by Rasoul-Amini et al [24], on *Chlorella* sp. the fatty acid methyl esters obtained were found to

be saturated. This proves that the biodiesel obtained on direct transesterification from *Chlorella marina* can be a effective fuel source.

#### 4. Conclusion

From the study it was clear that the secondary effluent from tannery wastewater can be a promising feed stock for culturing marine micro algae *C.marina*. The lipid obtained from *C.marina* was found to be a suitable alternative in biodiesel production. The fatty acid methyl esters from *Chlorella marina* was found to be with efficient fuel properties which can be a best alternative biofuel obtained on utilising waste. Also it was additional quality that microalgae *Chlorella* was accepted as functional food which on consumption benefit human health as well [19].

#### Declaration

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