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Growing of *Chlorella, Scenedesmus and Botryococus* in sewage water for biodiesel production

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ABSTRACT

Algae grown on wastewater media are a potential source of low-cost lipids for production of liquid biofuels. This study was aimed to estimate the effect of Physico-chemical characteristics of normal and sewage water (pH 7.60 and 6.60, EC 15.97and 12.36 µmol, free Co_2 1.48 and 0.74, nitrogen 0.90 and 0.50 mg/l, potassium 168.11 and 54.63 mg/l, calcium 249.52 and 112.21mg/l, magnesium 104.91 and 51.19 mg/l, sulphate 57.08 and 28.35 mg/l, chloride 98.00 and 84.63 mg/l, carbonates 362.18 and 32.64 mg/l and bicarbonates 1138.30 and 253.33mg/l in sewage and normal water respectively) on Chlorella, Scenedesmus and Botryococus. The highest biomass (4.533 mg ml^{-1}), chlorophyll (15.56 µg ml^{1}), lipid (49 %), acid value (0.52mg KOH/g), density (0.885 g/cm^3), iodine value (75 mg/g), saponification value (0.125mg KOH/g), viscosity (4.8mm^2/sec), myristic acid (9.0%), oleic acid (9.3%), linolenic acid (20.1%), palmitic acid (35.3%), stearic acid (6.1%) was observed in Scenedesmus than Botryococus and Chlorella. The properties of algal oil meet all the properties given by American society for testing and materials (ASTM) D6751, ISO 15607and EN14214- Europe. Hence, it is concluded that algae can be grown better in sewage water than normal water for their oil and used as a potential feedstock for liquid biofuel production.

Key words: *Chlorella, Scenedesmus, Botryococus,* sewage water, biodiesel

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INTRODUCTION

Biofuels produced from plants have potential to replace a significant fraction of our fossil fuel needs with a renewable alternative [1] and it is believed that large-scale production of biodiesel from edible oils may bring global imbalance to the food supply. Hence, environmentalists started to debate on negative impact of biodiesel production from edible oil [2]. However, concern has grown that use of food crops for production of ethanol, biodiesel or other renewable fuels will increase food prices while having little impact on greenhouse gas emissions [3]. Hence, as a solution for competition with food versus fuel crisis, non-edible vegetable oils are found to be suitable for biodiesel production under experimental conditions [4].

US Department of Energy suggested that algae are capable of producing oil suitable for conversion to biodiesel with an aerial productivity of 20-40 times that of oilseed crops [5]. Use of fresh water for microalgae cultivation has generated great debate. Further, use of conventional media in the form of mineral salts is uneconomical. Use of algae for sewage wastewater treatment in ponds is well established [6] and algae-based treatment of dairy and

piggery waste also has been investigated [7]. Therefore, use of sewage water for cultivation of fresh water microalgal species has proven to be a feasible option.

Waste water contains macro and micro nutrients; carbon rich waste water promotes the growth rate by balancing the CNP ratio and allows microalgae to undergo mixotrophic growth with several benefits. Algae growth in wastewater treatment ponds contributes to treatment mainly through dissolved oxygen production and nutrient assimilation. However, carbon: nitrogen and carbon: phosphorus ratio in domestic sewage (C: N 3.5:1; C: P 20:1) is low compared to typical ratios in rapidly growing algal biomass (C: N 6:1; C: P 48:1) [8], this dearth of carbon leads to limitations in algae production and incomplete assimilation of sewage water nutrients by algae, research presented herein was conducted to determine utilization of waste water for microalgal cultivation which is essential for improvement of microalgal biofuel feasibility.

MATERIALS AND METHODS

Experiments were carried out to determine the algal growth and lipid productivity in normal and sewage water. The pH and EC of water samples were evaluated before usage for the experiment. The water samples are filtered and analyzed for Free CO_2, Nitrogen, Potassium, Calcium, Magnesium, Sulphate, Chloride, Carbonates and Bicarbonates by APHA procedure [9]. The reagents used for analysis were AR grade and double distilled water was used for preparation of solutions.

Isolation of microalgae: Different fresh water samples were collected in sterilized bottles from pools, ponds and lakes of Bangalore city for isolation of microalgae and then samples were inoculated in Bold's Basal medium and incubated at 20^0C for 7 days. Aggressive culture replacement with fresh media was done to enrich the rapidly growing cells.

Purification of algae: Tube containing culture sample was centrifuged at 3000rpm for 15 minutes. After centrifugation, supernatant was discarded and cells were suspended in fresh sterile water in tube using vortex mixer to complete centrifugation-washing process. Centrifugation and washing was repeated three times to expel microorganisms in the sample.

Obtaining pure culture by streak plating technique: Washed microalgae was streaked on solidified Bold's Basal medium in aseptic condition and kept for at least fifteen days to grow. Repeated streak plating was carried out to pick up single colony from earlier streaked plates. From last streaked plates, single colonies were picked up by loop and allowed to grow in tubes and vials and the pure cultures were isolated by repeated sub-culturing.

Identification of isolated microalgae: Identification of the pure cultures was done by observing under the compound microscope [10].

Study of growth pattern of isolated microalgae: 10ml of pure culture inoculum was aseptically transferred to clean and dry sterilized conical flasks containing 500ml of normal and sewage water under laminar air flow for comparative studies. Then they were illuminated by bulbs at room temperature for 30 days and studied its growth pattern using optical density at regular interval at 540nm for *Chlorella* species and 625nm for *Scenedesmus* and *Botryococus* till values were constant and graph was plotted against OD versus days and after that a known volume of cultures is used for further studies.

Biomass estimation: Biomass was estimated by Richmond and Gobbelaar (1986) [11] method. 100 ml culture was filtered in a dried and pre-weighed Whatman Filter Paper No.1 and dried in an oven at 60°C until constant weight was obtained and then it was calculated in terms of mg/ml.

Chlorophyll estimation: Chlorophyll was estimated by homogenizing a known volume of culture and centrifuged at 8000 rpm for 10 minutes, pellet was treated with 10 ml of 95 per cent methanol, shaken well and incubated at 60°C in a water bath for 30 minutes. Supernatant was centrifuged and absorbance was measured in wave length of 652.4nm and 665.2 nm in a spectrophotometer using 95 per cent of methanol as a blank [12].

Lipid Extraction: Lipid extraction was done by using chloroform/methanol (2:1) and estimated gravimetrically by a procedure adapted from Bligh and Dyer (1959) [13] by Benemann and Tillett (1987) [14].

Determination of the physico chemical properties of the lipid: AOAC standard methods [15] were used to determine the properties like Acid value, Density, Iodine value, Saponification value and Viscosity.

Determination of fatty acid composition: Analysis of Fatty acids was carried out on Gas [16]. The GC was equipped with Flame Ionization Detector and stainless steel column, dimension 10 X 1/8; packed with 5 % DEGS-PS. Column was conditioned at 180^0C about 2 hours for attaining thermal stability before use. About 10µL of sample dissolved in hexane was loaded onto the column. Operating condition was programmed at oven temperature 150^0C (hold time 5min) with increasing rate 8^0C/min to190^0C (hold time 0 min), 2^0C/min to 200^0C (hold time 10min), injection temperature 250^0C and detector temperature 250^0C. Nitrogen was used as a carrier gas with flow rate of 20 ml/min. Concentration of individual fatty acids in the test samples were determined by comparing the peaks obtained from the GC analysis with peaks of authentic standards and n-heptane was used as an internal standard. The experimental design was completely randomized with three replicates. All data were expressed as mean values ± SE, the comparison between the mean values were tested using DMRT (P = 0.05).

RESULTS AND DISCUSSION

Normal and sewage water samples were analyzed for pH; it was varied from 7.60 to 6.60 and electrical conductivity of water samples was also observed as 15.97and 12.36 µmol. If electrical conductivity of the water samples were less than 1 dSm-1 indicating that water samples contain less salts. The microalgae have a unique feature of growing under these pH and EC conditions. Therefore, these water samples are a good source for production of microalgal biomass and in turn biodiesel production (Table 1, Figure 1, 2).

Algae has a great ability to fix CO_2 thus it can be used to reduce greenhouse gases with higher production of microalgal biomass and consequently higher biodiesel yield [17,18].High free CO_2 content in the sewage water makes it a good medium to grow algae, here the free CO_2 is 1.48 and 0.74 in sewage and normal water respectively (Table 1, Figure 3).

Nitrogen limitation increases lipid content in some species [19, 20] here the estimated nitrogen is 0.90 and 0.50 mg/l in sewage and normal water respectively (Table 1, Figure 4).

The other parameter like Potassium (168.11 and 54.63 mg/l), Calcium (249.52 and 112.21mg/l), Magnesium (104.91 and 51.19 mg/l), Sulphate (57.08 and 28.35 mg/l), Chloride (98.00 and 84.63 mg/l), Carbonates (362.18 and 32.64 mg/l) and Bicarbonates (1138.30 and 253.33mg/l) (Table 1, Figure 4) are recorded high in sewage and normal water respectively which favors more growth of algae in sewage water compared to normal water, is in agreement with the findings of Sharma and Ashwath 2006 [21] that it also creates opportunities for commercial biomass production and sequestration of excess minerals in the plant system

Table 1: Physico chemical parameters of water used for the growth of microalgae

Parameter	Normal water	Sewage water
pH	6.60	7.60
EC µmol	12.36	15.97
Free CO_2	0.74	1.48
Nitrogen(mg/l)	0.50	0.90
Potassium(mg/l)	54.63	168.11
Calcium(mg/l)	112.21	249.52
Magnesium(mg/l)	51.19	104.91
Sulphate(mg/l)	28.35	57.08
Chloride(mg/l)	84.63	98.00
Carbonates(mg/l)	32.64	362.18
Bicarbonates(mg/l)	253.33	1138.30

Values are the means ± SE of three replicates each. Data were subjected to analysis of variance and compared for significance according to DMRT (P=0.05).

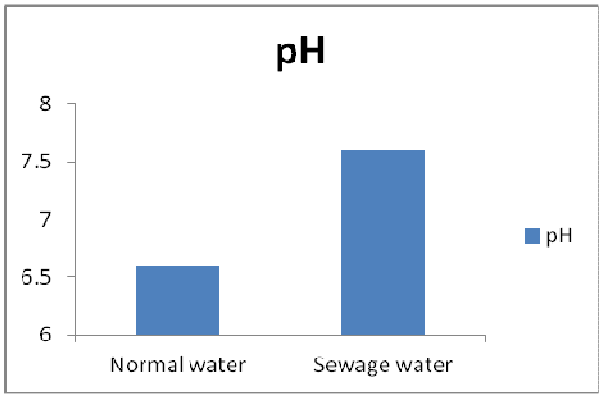


Figure 1

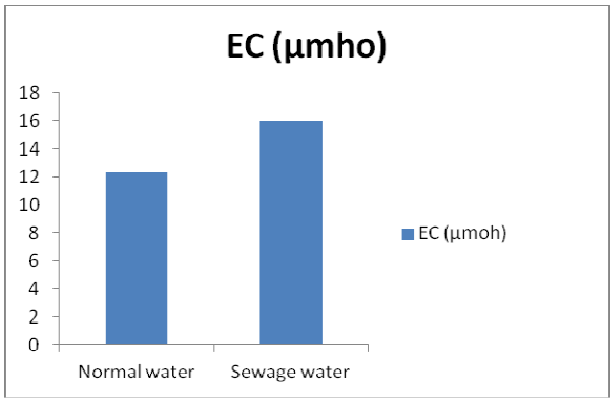


Figure 2

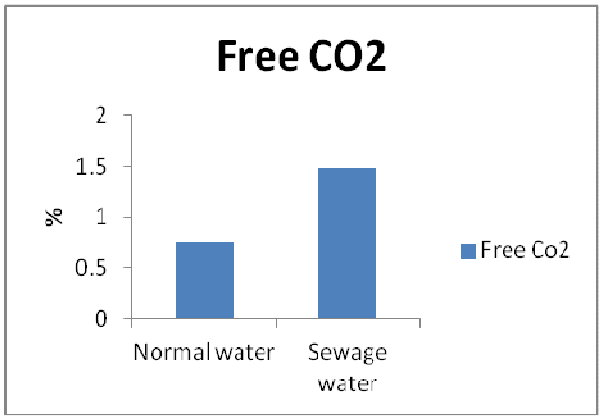


Figure 3

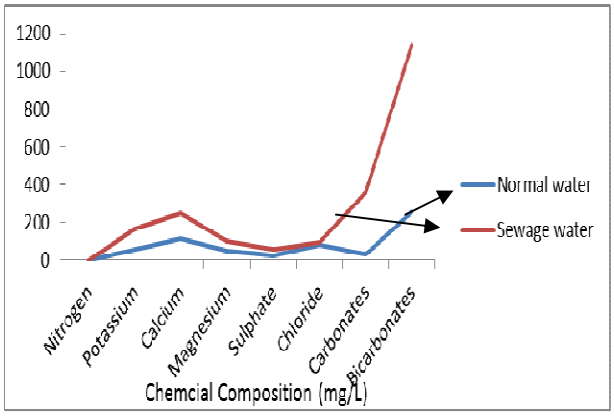


Figure 4

Fig: 1, 2, 3 and 4 Physico chemical parameters of water used for the growth of microalgae

The growth of isolates was measured in terms of biomass, chlorophyll and lipid production, highest biomass production was noticed in *Scenedesmus* 4.533 mg ml^{-1} followed by *Botryococus* and *Chlorella*. The total lipid content of biomass from the 10% dilution ranged from 8-14% and from 25% dilution ranged from 10-29% by weight. In comparison, lipid content of *Scenedesmus* and *Chlorella* cultures has been reported to range from 12-45% [22] which correlates with our results. Chlorophyll content varied significantly among the cultures *Scenedesmus* showed 15.56 µg ml^{-1} (Fig-6), this implies that increase in the biomass production also increases chlorophyll content of strains [23] and Lipid produced from the isolates *Scenedesmus* was more compared to *Botryococus* and less as compared to Chlorella, the experiments with sewage water reached steady-state biomass concentrations after 30 days, the lipid contents in *Scenedesmus*, *Botryococus and Chlorella* was 49, 43 and 39%, which concurred with the result of Mandal and Mallick 2009 [24] and average lipid content of the micro algae *Scenedesmus acutus* was 50 per cent of its dry matter as estimated by Pokethitiyook et al. 2009 [25]. Despite relatively low lipid contents observed in algal growth in normal water, high biomass production rates resulted in high lipid productivities (Table 2 and Fig 5, 6 &7).

Oil extracted from algal species was analyzed for acid value, density, iodine value, saponification value and viscosity. It was deduced from the results that highest acid value 0.52mg KOH/g, density 0.885 g/cm^3, iodine value 75 mg/g, saponification value 0.125mg KOH/g, viscosity 4.8mm^2/sec was observed in *Scenedesmus* compare to *Botryococus* and *Chlorella* (Table 2 and Fig 8, 9, 10, 11, 12).The obtained results for iodine value meet the biodiesel quality specifications (<120g I_2/100g) which makes these algal oils competitive with some vegetable oils traditionally used for biodiesel production as per EN 14111(2003) [26]. Properties of oil were comparable with study conducted by Li et al. 2007 [27] and feasibility of biodiesel production from algal consortium grown in treated wastewater was checked by Chinnasamy et al., 2010 [28] , where these results are also in conformity with

observations made by Gouveia and Oliveira 2009 [29] that microalgal lipids derived from *Scenedesmus spp.* were mainly composed of unsaturated fatty acids.

Table 2: Growth and lipid parameters of microalgae

Parameter	*Chlorella*		*Scenedesmus*		*Botryococus*	
	Control	Sewage water	Control	Sewage water	Control	Sewage water
Biomass (mg ml^{-1})	1.790	1.833	4.421	4.533	2.454	3.566
Chlorophyll (a+b) µg ml^{-1}	8.52	8.71	14.45	15.56	9.91	11.07
Lipid %	20	39	37	49	39	43
Acid value (mg KOH/g)	0.41	0.49	0.43	0.52	0.48	0.51
Density (g/cm^3)	0.870	0.881	0.794	0.885	0.795	0.883
Iodine value (mg/g)	61	63	73	75	68	69
Saponification value (mg KOH/g)	0.190	0.210	0.126	0.215	0.156	0.211
Viscosity (mm^2/sec)	3.8	4.0	3.9	4.8	3.8	4.3
Myristic acid %	7.9	8.3	8.8	9.0	7.8	8.9
Oleic acid%	8.1	8.4	8.6	9.3	8.4	9.0
Linolenic acid%	15.9	16.6	19.3	20.1	15.90	17.1
Palmitic acid%	30.9	31.7	29.8	35.3	29.8	33.3
Stearic acid%	3.9	4.9	5.8	6.1	4.7	5.0

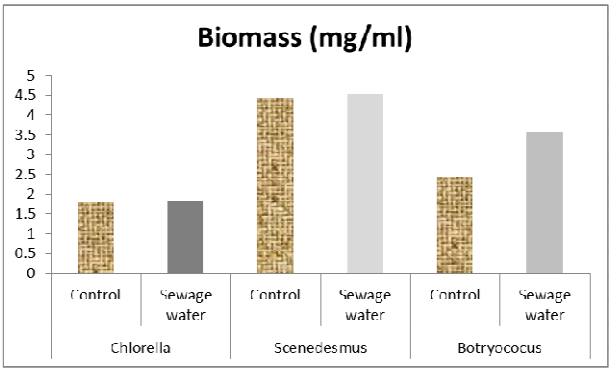


Fig – 5

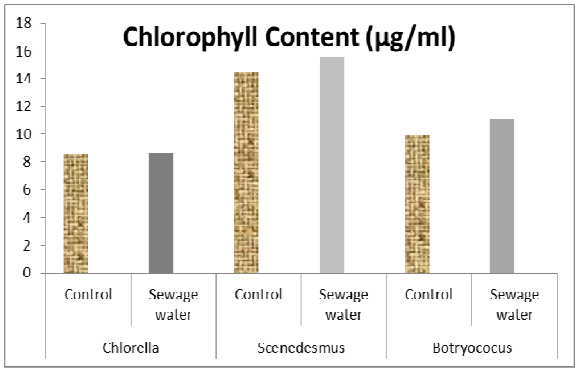
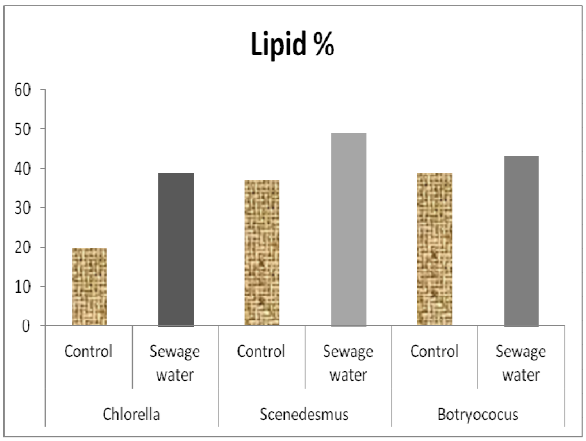


Fig – 6



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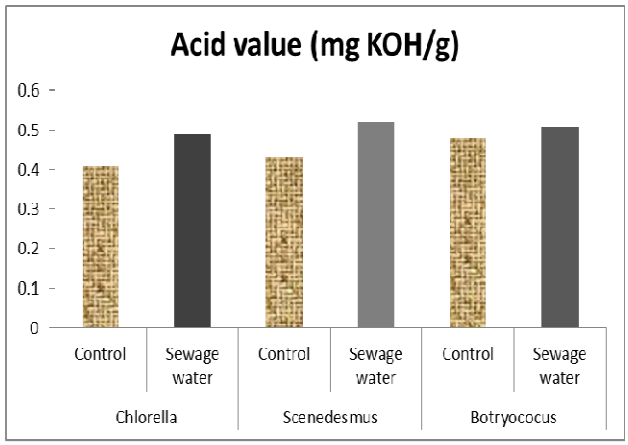

Fig – 8

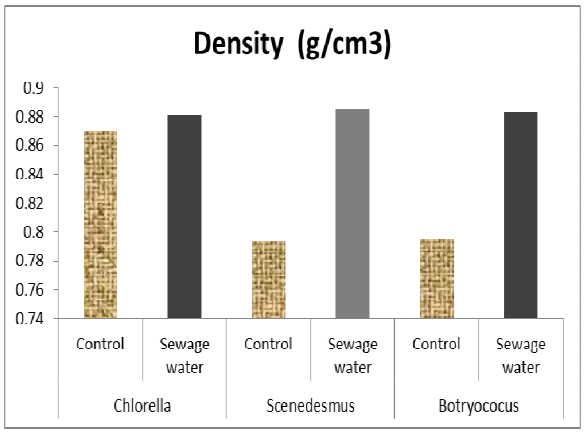

Fig – 9

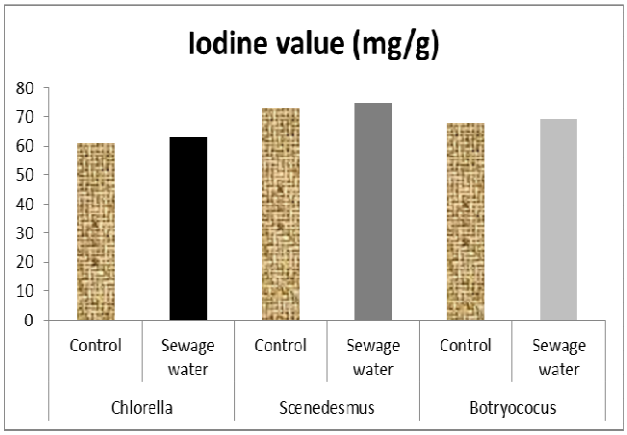

Fig – 10

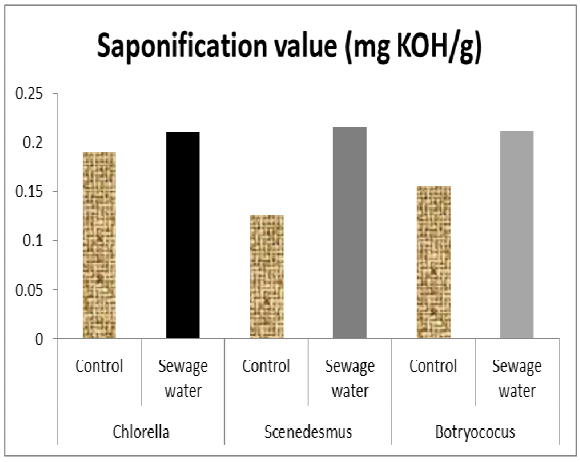

Fig – 11

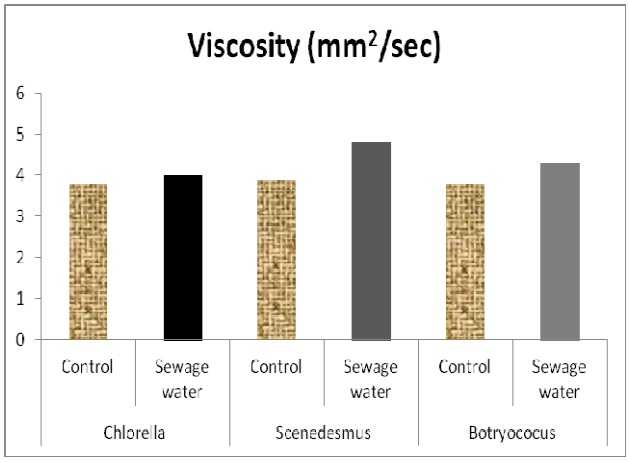

Fig – 12

Fig: 5, 6 and 7-growth parameters and 8, 9, 10, 11 and 12 -lipid parameters of microalgae

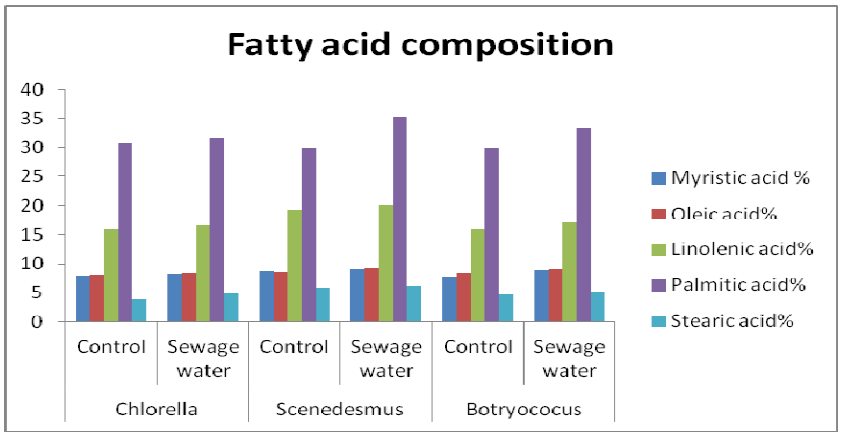

Fig 13 – Fatty acid composition of algae

GC–MS analyses of the fatty acids through chromatograms illustrate and are depicted in Table 2 and Figures 13. The results are in agreement with earlier studies of [30, 31,32, 33], Myristic acid 9.0%, Oleic acid9.3%, Linolenic acid 20.1%, Stearic acid 6.1% was observed to be higher in *Scenedesmus* compare to *Botryococus and Chlorella*

which is grown in sewage water than normal water. These results indicate that the algae exhibited adaptability to a wide range of pH, EC, free CO_2, nitrogen, potassium, calcium, magnesium, sulphate, chloride, carbonates and bicarbonates in sewage water. Where Palmitic acid was also highest in *Scenedesmus* i.e., 35.3% compared to *Botryococus* (33.3%) and *Chlorella* (31.7%), the results of important fatty acid composition in *Scenedesmus* are in agreement with earlier study where there is higher palmitic content [34] The percentage of saturates were higher followed by mono-enes, di-enes and in all the three algal species.

CONCLUSION

Integration of municipal wastewater treatment with algal biofuel generation is an economically viable and attractive option for meeting the decentralized energy demand. Efficiency of lipid extraction from algae was found to differ according to species, cell disruption and associated extraction methods. Highest lipid was extracted from *Scenedesmus* followed by *Botryococus* and *Chlorella.* This study also contributed data on lipid productivity of wastewater grown algae, a rarely addressed topic, while this is many times higher than that of terrestrial oil plants, higher productivity is a goal of continuing research. In addition, the suitability of the lipids for fuel production by transesterification and other means needs to be determined. Overall, the waste-to-biofuel approach of this study avoids many of the cost and food competition issues of other biofuel feedstocks.

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