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Potential of wastewater grown algae for biodiesel production and CO_2 sequestration

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Algae have been proposed as a potential renewable fuel source. Photosynthetic CO_2 fixation to substrates that can be converted to biodiesel by microalgae is thought to be a feasible technology with energy-saving and environment-friendly approach. In the present study, potential of microalgae, from wastewater stabilization pond, as a feedstock for biodiesel production and CO_2 sequestration was evaluated. Mixed algae sample showed the highest CO_2 fixation rate, followed by *Chlorella* sp., *Scenedesmus incrassatulus*, *Scenedesmus dimorphus* and *Chroococcus cohaerens* (2.807, 1.627, 1.501, 1.270 and 0.786 g $L^{-1}d^{-1}$, respectively). Nile red stain was used for detection of lipid in microalgal sample which was further extracted and analysed by gas chromatography (GC). The main fatty acids present in the mixed algae sample were fatty acids with C14–C18 (>98%) that are generated after natural CO_2 sequestration. At ambient CO_2 concentration, total fatty acid methyl esters (FAME) mainly comprised of myristic acid (C14:0), 0.0718%; palmitic acid (C16:0), 2.558%; octadecenoic acid (C18:1), 28.98% and linoleic acid (C18:2), 12.54% which makes the microalgal biomass a suitable feedstock for biodiesel production and CO_2 mitigation.

Key words: Biodiesel, carbon dioxide fixation, fatty acid profile, microalgae, wastewater stabilization pond.

INTRODUCTION

With the diminishing non-renewable fossil fuel reserves, it is imperative to search for alternatives for crude oil-based fuels such as gasoline and diesel (Pokoo-Aikins et al., 2010). Concerns due to global warming and energy shortages have urged scientists to look forward to biodiesel as a replacement energy source. Biodiesel production is one promising route to complement our energy shortage. Biodiesel is a fuel that is derived from lipids, which are esters of fatty acids produced from plants or animals. Biodiesel has become immensely popular as a transportation fuel over the past decade. Biodiesel produced from plants are potential renewable alternative to replace a significant fraction of our fossil fuel consumption (Perlack et al., 2005). Common sources for biodiesel feedstock include soy, sunflower, safflower, canola and palm. However, there is growing concern that the use of food crops for biodiesel and other renewable fuels may be an uneconomical long term solution (Fargione et al., 2008). In order to address these concerns, focus from the popular feedstock has been

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shifted to the use of alternative, non-food related feedstock such as oil from algae (Pokoo-Aikins et al., 2010). Microalgae are more efficient users of solar energy than conventional agriculture (Li et al., 2008). Under controlled conditions, algae are capable of producing 40 times the amount of oil for biodiesel per unit area of land, as compared to terrestrial oilseed crops such as soy and canola (Sheehan et al., 1998) and they can grow in aqueous phase allowing efficient access to water, CO_2 and other nutrients which explains their potential for the production of more oil per unit area than other crops currently used (Pokoo-Aikins et al., 2010). Therefore, there is a larger potential for biodiesel production from algae biomass with less land requirement.

Greenhouse gases emissions in the atmosphere have also received great attention. The global increase in carbon dioxide concentration is largely due to fossil fuel use and land use change, while those of methane and nitrous oxide are primarily due to agriculture (Solomon et al., 2007). As estimated by Intergovernmental Panel on Climate Change criteria, carbon dioxide, the principal greenhouse gas, account for 76.7% (v/v), and its concentration have increased rapidly since the onset of industrialization. The microalgae-for-CO_2-mitigation strategy offers numerous advantages due to much higher growth rates, wide tolerance to extreme environments, potential for intensive cultures and CO_2 fixation abilities as compared to conventional forestry, agricultural and aquatic plants. These advantages promise high performance in the reduction of carbon dioxide (Kurano et al., 1996; Chisti, 2007). Microalgae need CO_2 (of about half of the dry algae weight) for growth and hence, reduce atmospheric CO_2 concentration contributed by industrial emissions such as power plants (Chisti, 2008). The carbon dioxide converted to biomass can further be processed downstream to produce biodiesel, fertilizer and other useful products. Thus, algae bear numerous characteristics that cause them to be a candidate biodiesel feedstock and deserve serious investigation (Pokoo-Aikins et al., 2010).

The earlier researchers suggested that a more practical approach for algae biodiesel production is to utilize wastewater for algae propagation (Sheehan et al., 1998). Treatment of municipal wastewater using algae is a well established technique (Oswald et al., 1953; Oswald, 2003). Algae growth in wastewater treatment ponds contributes to treatment mainly through dissolved oxygen production and nutrient assimilation and they are already being used by many wastewater facilities as bioremediation agents. In wastewater stabilization ponds, the algae produce oxygen from water as a by-product of photosynthesis. This oxygen is used by the bacteria and they bio-oxidize the organic compounds in the wastewater. An end-product, carbon dioxide, is fixed into cell carbon by the algae during photosynthesis. Thus, algae-based wastewater treatment is a powerful avenue for sustainable wastewater treatment and it serves the dual purpose of biodiesel source and CO_2 sequesteration. Combination of CO_2 fixation, biofuel production and wastewater treatment may provide a very promising alternative to current CO_2 mitigation strategies (Wang et al., 2008).

In view of the above, this study was undertaken with the aim to evaluate algae biomass harvested from wastewater treatment pond of vehicle manufacturing plant in western Maharashtra region, as a source of biodiesel and to test the feasibility of an algae consortium for CO_2 sequestration. Individual performance of each microalgae present in mixed microalgal culture was also studied.

MATERIALS AND METHODS

Microalgae from wastewater

Algae biomass growing in a pond receiving wastewater from vehicle manufacturing plant in western Maharashtra region, India were collected in sterilized sampling bottles and 10 ml of the algae sample was inoculated in 50 ml sterilized standard BG-11 medium (Rippka et al., 1979) and Becker's medium (Sangolkar et al. 1989) in 100 ml Erlenmeyer flasks in five replicates. The flasks were incubated and maintained at 25±1°C under continuous illumination of cool white fluorescent lamps (75 µmol m^{-2} s^{-1}) for 14 days to obtain maximum microalgal growth.

Microalgae identification and community composition

Unfiltered pond water samples were collected in sterilized sampling bottles from a depth below 10 to 20 cm of the surface and immediately preserved with Lugol's iodine (APHA, AWWA, 2005). Samples were centrifuged using 15 ml Tarsons tubes at 280 ×*g* for 15 min. Microscopic observations were made and the total count of phytoplankton was enumerated by Lackey's drop count method using Olympus microscope BX41 (Olympus, Japan) under 100 and 400x magnification. Identification of algae taxa was carried out using taxonomic keys after Prescott (1978) and Desikachary (1959). Community structure index was used to obtain the estimation of species diversity (Figure 1). Shannon and Weaver (1949) diversity index (SWI) value was obtained using the following equation:

$$D = \sum_{i=I}^{Pi2} (\log Pi) \quad \text{(Shannon's index)}$$

Where, Pi = is the proportion of the first species. The proportions are given Pi = ni/N.

Determination of lipid content by nile red (NR) staining

Nile red (9-diethylamino-5H-benzo [α] phenoxa] phenoxazine-5-one) obtained from the Sigma-Aldrich Chemicals (Germany) was dissolved in 0.25 mg/ml acetone. Algae culture and nile red solution were mixed well in 1: 0.01 proportion for 5 min and were analysed for lipid content in the microalgae by fluorescence microscopy.

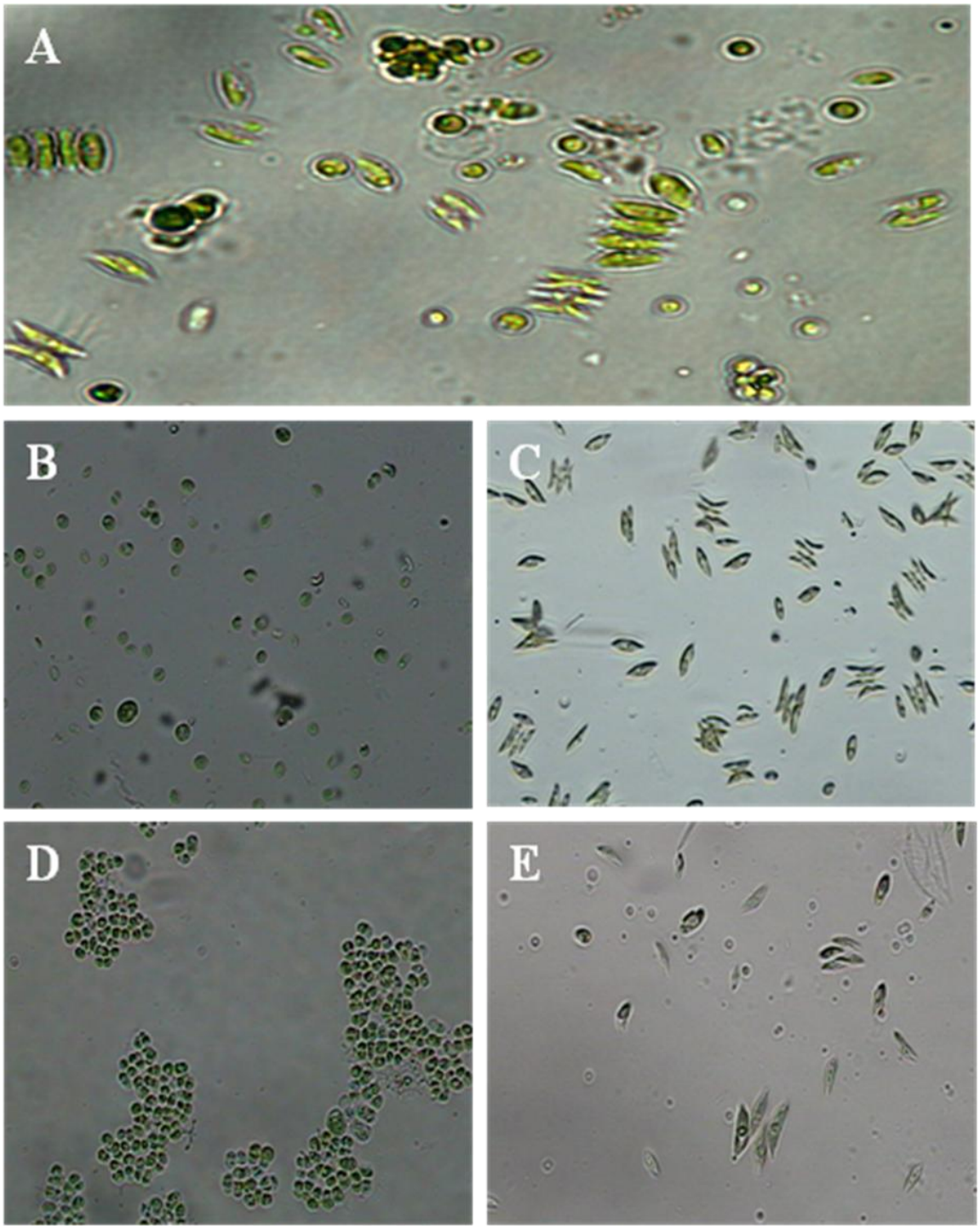

Figure 1. Microscopic images of algae diversity. **(A)** Mixed algae sample (60x); **(B)** *Chlorella* sp.; **(C)** *Scenedesmus dimorphus* (40x); **(D)** *Chroococcus cohaerens* (40x); and **(E)** *Scenedesmus incrassatulus* (40x).

Extraction of lipid from microalgal biomass

Extraction of lipid was carried out following the previously described method (Bligh and Dyer, 1959). A 15-ml glass vial containing algae biomass (0.5gm), 2 ml methanol and 1 ml chloroform were mixed and kept for 24 h at 25°C. The mixture was agitated in a vortex mixer for 2 min. 1 ml of chloroform was again added to it and the mixture was shaken vigorously for 1 min. 1.8 ml of distilled water was added and the mixture was subjected to vortex again for 2 min. The layers were separated by centrifugation for 10 min at 200 ×*g*.

Table 1. Phytoplankton species identified in the wastewater stabilization pond.

Chlorophyceae	Cyanophyceae	Bacillariophyceae	Euglenophyceae	Chrysophyceae
Chlorella sp.	*Gloeocapsa* sp.	*Triceratium* sp.	*Euglena* sp.	*Chromulina* sp.
Actinastrum sp.	*Aphanocapsa* sp.	*Synedra* sp.	*Lepocinclis* sp.	*Ochromonas* sp.
Coelastrum sp.	*Microcystis* sp.	*Navicula* sp.	*Phacus* sp.	*Urococcus* sp.
Ankistrodesmus sp.	*Chroothece* sp.	*Rhoicosphenia* sp.	*Gonyostomum* sp.	*Peridinium* sp.
Elakatothrix sp.	*Chroococcus* sp.	*Centronella* sp.		
Pediastrum sp.	*Merismopedia* sp.	*Diatoma* sp.		
Tetrastrum sp.	*Dactylococcopsis* sp.	*Surirella* sp.		
Zygnema sp.	*Aphanothece* sp.	*Nitzschia* sp.		
Chlorococcum sp.	*Oscillotoria* sp.	*Coscinodiscus* sp.		
Crucigenia sp.	*Spirulina* sp.	*Cyclotella* sp.		
Pandorina sp.	*Anabaena* sp.	*Tabellaria* sp.		
Chlamydomonas sp.	*Pleurococcus* sp.	*Caloneis* sp.		
Carteria sp.	*Gomphosphaeria* sp.	*Fragillaria* sp.		
Phacotus sp.	*Lyngbya* sp.	*Bacillaria* sp.		
Scenedesmus sp.	*Gloeocapsa* sp.			
Tetraedron sp.	*Westella* sp.			
Tetradesmus sp.				
Closterium sp.				
Staurastrum sp.				
Kirchneriella sp.				
Cosmarium sp.				
Cladophora sp.				
Spondylomorum sp.				

The lower layer was filtered through Whatman No. 1 filter paper into a preweighed clean vial. Evaporation was carried out by passing nitrogen gas and the lipid content was calculated as the difference in weights of the vial.

Determination of carbon content and CO_2 fixation rate

The carbon content of the dried algae biomass was determined using a model Vario EL III CHNS analyzer (Elementar Analysersystem GmbH, Germany) and calibrated using a Perkin-Elmer sulphanilic acid standard to represent 100% carbon. CO_2 fixation by each algae was calculated from the CHNS biomass carbon content values. The formula used is:

$$CO_2 \text{ fixation rate} = 0.50P \times \frac{44}{12}$$

Where, P- Biomass productivity; 44- molecular weight of CO_2; 12-molecular weight of carbon

CO_2 fixation rates were calculated from the biomass productivity by using 50% as the carbon content of dried cells (Ugwu et al., 2005).

Determination of fatty acid methyl esters (FAME) by gas chromatography (GC)

The lipid content was expressed as ratio of total lipid to biomass concentration. The lipids were measured as FAME following the direct transesterification method using a gas chromatograph (GC) equipped with a flame ionization detector (FID) (Perkin Elmer, USA). Analysis was done using a SP-2560 column (100 m x 0.25 mm I.D., 0.20 µm) supplied by Sigma (Germany) and a fatty acid standard Supelco 37 Component FAME Mix from Supelco (Bellefonte, PA, USA). Five microliters of sample was injected into the GC. The GC conditions were: injector temperature 2600°C; column: 1400°C and detector temperature: 2600°C. Helium was used as carrier gas with a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

The microalgal community of wastewater stabilization pond comprised of about 27 species of Chlorophyceae, 16 species of Cyanophyceae and 14 species of Bacillariophyceae (Table 1). Chlorophycean algae (*Scenedesmus* sp. and *Chlorella* sp.) dominated the studied stabilization pond (upto 60%) followed by Cyanophyceaen algae (*Oscillotoria* sp. and *Spirulina* sp.; upto 44%). Bacillariophyceae, Euglenophyceae and Crysophyceae were meagrely present (upto 37%) at various sampling sites. Table 2 represents the density and diversity of phytoplankton in different sites of the stabilisation pond. Shannon-Wiener Diversity Index is in the range of 2.91 to 3.66 which indicates unpolluted state of the pond water. Nutrient enrichment due to industrial wastewater and water temperature favors the algae

Table 2. Density and diversity of phytoplankton in stabilization pond.

Sampling location	Phytoplankton (No./ml)	Percentage composition of algal group					SWI
		Cyanophyceae	Euglenophyceae	Chlorophyceae	Bacillariophyceae	Crysophyceae	
Inlet	2,599	44	1	54	1	-	3.66
North-east	2,432	26	37	30	4	3	3.54
East	1,990	55	2	41	-	2	3.29
South-east	5,810	51	-	47	2	-	3.42
South	22,519	33	-	60	7	-	3.70
South-west	4,808	21	26	49	4	-	3.05
West	6,344	33	20	47	-	-	2.91
North-west	7,016	33	10	57	-	-	3.14
Centre	9,752	31	14	53	2	-	3.30
Outlet	4,873	41	3	53	3	-	3.52

SW, Shannon Wiener Diversity Index.

species diversity and abundance (Joseph and Joseph, 2002) which was very well observed in present study. Wastewater is a potential sustainable growth medium for algae feedstock and a wide range of studies have also reported microalgal growth in wastewaters including municipal (urban) sewage wastewater and agricultural manure wastewater (Pittman et al., 2011) as algae can effectively grow in nutrient-rich environments and efficiently accumulate nutrients and metals from the wastewater.

Nile red staining of microalgal sample revealed the presence of intracellular lipids (Elumalai et al., 2011). While intracellular lipid droplets of microalgae were stained in yellow and golden yellow color, chlorophyll were stained in red (Figure 2). The mixed microalgal sample showed yellow fluorescence (Figure 2A) showing high lipid content. Four algae species viz., *Scenedesmus dimorphus*, *Scenedesmus incrassatulus*, *Chroococcus* sp. and *Chlorella* sp. were found to contain intracellular lipid droplets (Figures 2B, C, D and E, respectively). As these microalgae are being globally explored for biodiesel production (Ho et al., 2010) and Nile red staining reveals the lipid accumulation (Figure 2), these algae were further isolated in pure form and analysed for their lipid content.

Table 3 presents the total lipid content of isolated strains. *Chlorella* sp. revealed total lipid content (7.8 wt%) higher than other isolated strains followed by *S. incrassatulus* (6.5 wt%) and *S. dimorphus* (4.5 wt%) while *Chroococcus coherens* showed the lowest lipid content. Lipid content (5 to 20%) in microalgae under optimal conditions is reported to increase upto 20 to 50% under unfavorable conditions (Hu et al., 2008). The genus *Chlorella* (Chlorophyta) has been reported to show lipid content at around 14% of dry weight (Chisti, 2007) which can increase upto 46% dry weight under stress conditions (Hu et al., 2008). However, under N-deficient culture conditions, lipid content in *Chlorella* sp. increased to 63.64 from 40.98% (Herrera-Valencia et al., 2011). *S. obliquus*, *Scenedesmus quadricauda* and *S. dimorphus* were reported to show 12 to 14, 1.9 and 16 to 40% lipid content, respectively, when cultivated under normal conditions. Under N-deficiency, *S. obliquus, S. quadricauda* and *S. dimorphus* showed 43, 31 and 34% lipid content, respectively, in terms of dry cell weight (Mandal and Mallick, 2009; Goswami, 2011). This shows that algae produce more lipids (w/w) under stressed conditions but the amount of algae produced is also reduced (Griffiths and Harrison, 2009; Prartono et al., 2010). Under nutrient depletion, microalgae produce more lipids which gets accumulated in the cell. 30 to 50% CO_2 levels were also found to be favorable for the accumulation of total lipids and polyunsaturated fatty acids (Makareviciene et al., 2011; Tang et al., 2011). In the present study, the microalgae were grown in optimum nutrient media at ambient CO_2 (0.03%) for 14 days. Though less lipid accumulation was observed by the test algae species as compared to previous researches, the resultant lipid content reflect the potential of studied microalgae as a source of biodiesel. When grown under nutrient limitations, the test algae are expected to accumulate more lipid (Pokoo-Aikins et al., 2010).

To determine the viability of using microalgae as a carbon dioxide sequestration option, carbon dioxide fixation rates of the test microalgae were studied. The maximum CO_2 fixation rate was shown by mixed algae sample which could be the combined result of all the algae species present in the sample. In Tang et al. (2011) studies the maximum biomass concentration and CO_2 biofixation rate were 1.84 g L^{-1} and 0.288 g $L^{-1}d^{-1}$ for *S. obliquus* SJTU-3 and 1.55 g L^{-1} and 0.260 g $L^{-1}d^{-1}$ for *Chlorella pyrenoidosa* SJTU-2, respectively at 10% CO_2 concentration. In present studies, both *S. dimorphus* and *S. incrassatulus* showed 4.4 and 5.2 times higher CO_2

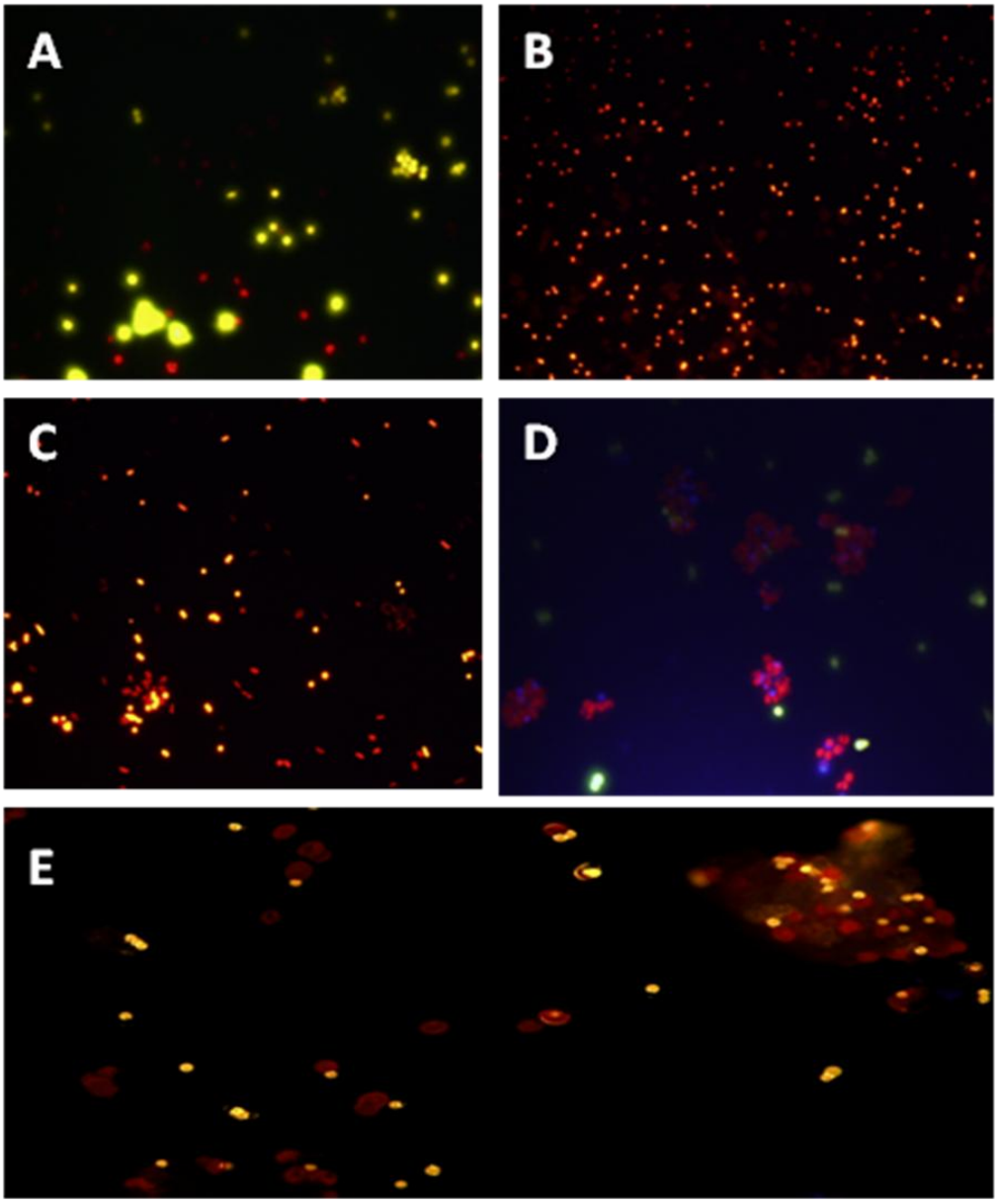

Figure 2. Microscopic images of algae samples stained with Nile red. **(A)** Mixed algae sample; **(B)** *Scenedesmus dimorphus*; **(C)** *Scenedesmus incrassatulus*; **(D)** *Chroococcus cohaerens* and **(E)** *Chlorella* sp. fluorescence with yellow and golden yellow color oil droplets.

Table 3. Biomass concentration, lipid content and CO_2 fixation efficiency of biodiverse samples of algae after 14 days of cultivation in batch culture.

Algae species	Biomass concentration (g L^{-1})	Lipid content (g g^{-1})	CO_2 fixation rate (g L^{-1} d^{-1})
Mixed algae sample	1.67 ± 0.003	0.24± 0.015	2.807 ± 0.01
S. dimorphus	0.71± 0. 08	0.04± 0.024	1.270± 0.05
S. incrassatulus	0.88± 0.03	0.06± 0.011	1.501± 0.04
C. cohaerens	0.43± 0.01	0.01± 0.017	0.786± 0.01
Chlorella sp.	0.92± 0.02	0.07± 0.02	1.627 ± 0.03

Each data indicates the mean ± SD which were measured from five replicates.

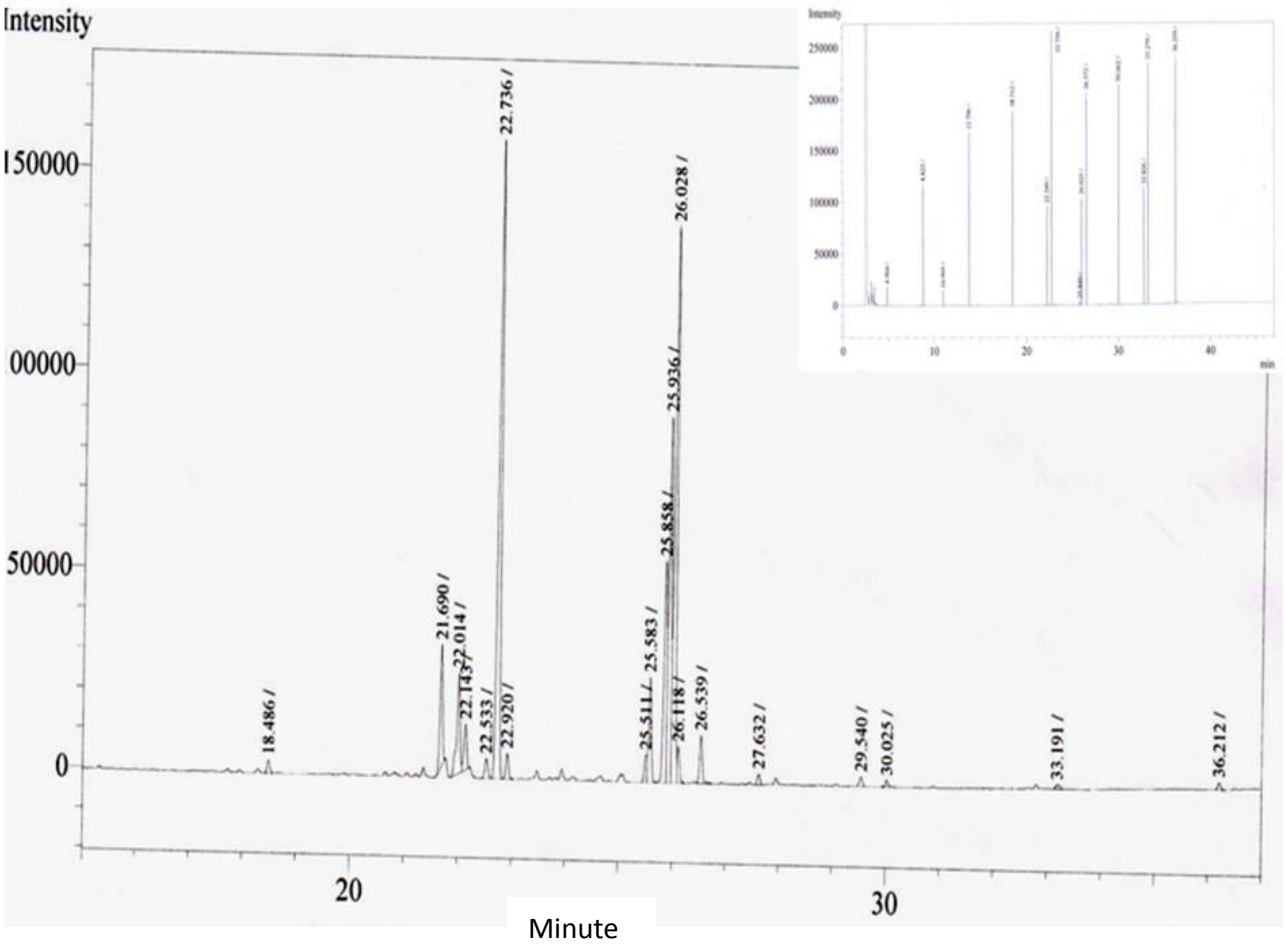

Figure 3. Gas chromatogram of biodiesel of mixed algae sample with standard FAME in inset.

fixation rate at ambient CO_2 concentration even though their biomass concentrations were 2.6 and 2.1 times lower than that of *S. obliquus* (Table 3). The CO_2 fixation rate of both the *Scenedesmus* species may increase significantly with increasing CO_2 concentration. Similarly, *Chlorella* sp. showed biomass concentration and CO_2 fixation rate of 0.928 g L^{-1} and 1.672 g L^{-1} d^{-1}, respectively, at ambient CO_2 concentration (Table 2). As compared to *C. pyrenoidosa* isolated by Tang et al. (2011), the CO_2 fixation rate of *Chlorella* sp. was 6.26 times higher at 1.67 times lower biomass concentration.

Sydney et al. (2010) showed the maximum biomass concentration and CO_2 biofixation rate for *C. vulgaris* as 1.94 g L^{-1} and 0.252 g L^{-1} d^{-1}, respectively, at 5% CO_2 concentration. In the present studies, though the isolated *Chlorella* sp. showed lower biomass concentration, its CO_2 fixation rate was 6.5 times higher than that of *C. vulgaris* (Sydney et al., 2010), at ambient CO_2 concentration. *C. coherens* showed less CO_2 fixation rate and biomass concentration, that is, 0.786 g L^{-1} d^{-1} and 0.438 g L^{-1}, as compared to other three isolated algae and consequently its contribution towards overall CO_2 fixation was very low. Makareviciene et al. (2011) found increased biomass growth of *Chlorella* sp. and *Scenedesmus* sp. with increasing CO_2 concentration which justifies that the studied microalgae would show increased biomass concentration, under higher CO_2 concentration, leading to increased CO_2 sequestration and lipid accumulation. Figure 4 presents overall approach for integrated biofuel precursor production and CO_2 sequestration from wastewater grown algae biomass.

To evaluate the feasibility of producing biodiesel from wastewater grown microalgae, the extracted lipid was converted to methyl esters and examined for the fatty acid profile using gas chromatography (GC-FID). The main biodiesel precursors at ambient CO_2 concentration were myristic acid (C14:0), 0.0718%; palmitic acid (C16:0), 2.558%; linoleic acid (C18:2), 12.54% of total FAME. This FAME profile of mixed algae sample also indicated the presence of octadecenoic acid (C18:1), 28.984% and archidic acid (C20:0), 0.335% (Table 4 and

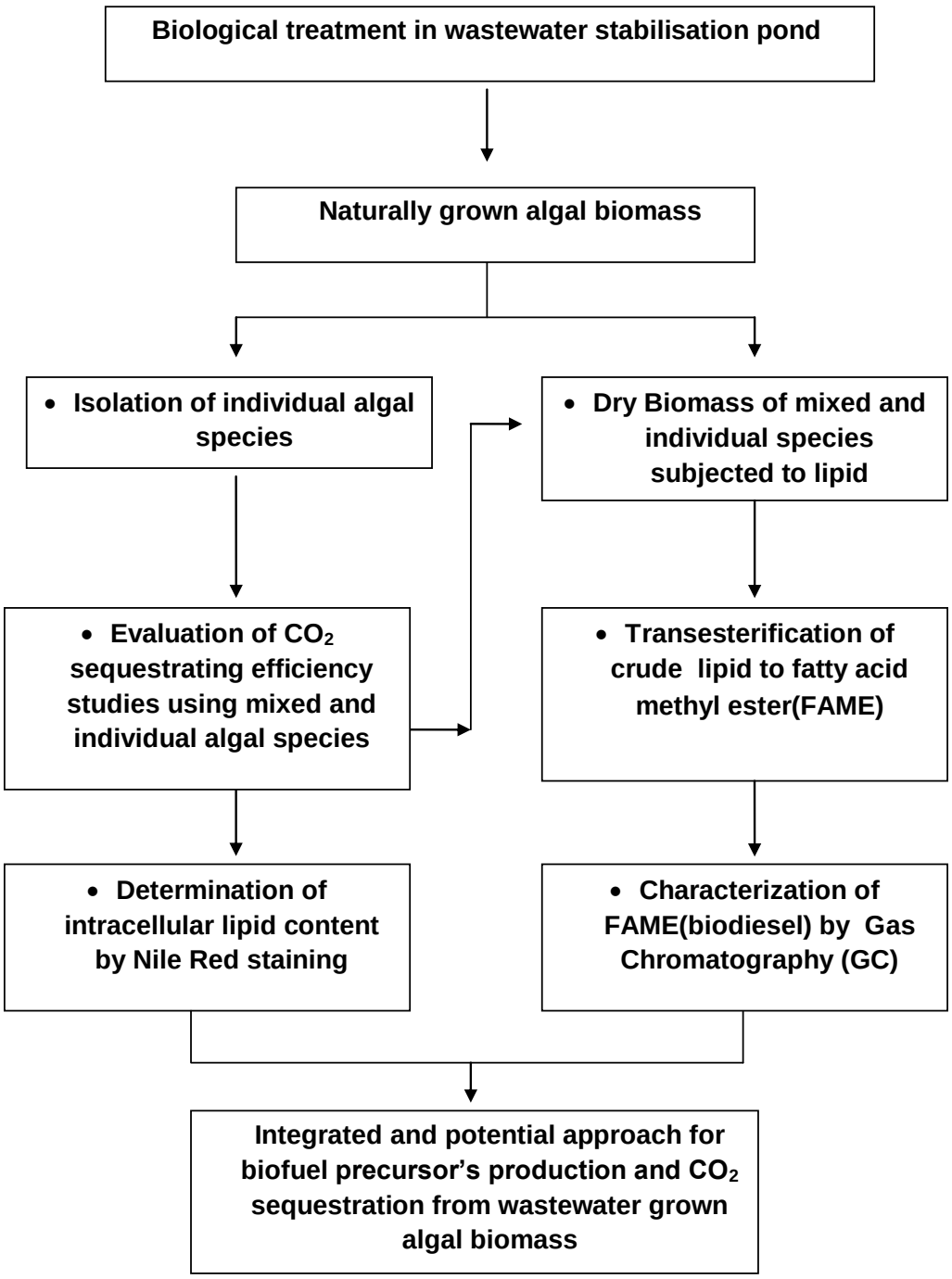

Figure 4. Stepwise analysis for integrated biofuel precursor production and CO_2 sequestration from algae biomass grown in wastewater.

Table 4. Fatty acids composition of algae biomass from stabilization pond.

Fatty esters (%)	Myristic (C14:0)	Palmitoleic (C16:1)	Palmitic (C16:0)	Linoleic (C18:2)	Linolenic (C18:3)	Octadecenoic (C18:1)	Arachidic (C20:0)	Erucic (C22:1)
Mixed algae sample	0.0718	2.556	34.016	12.541	20.44	28.984	0.335	ND

ND, Not detected.

Figure 3). The fatty acid composition in green algae has been reported to range from C-14:0 to C-20:0 (Xu et al., 2006; Petkov and Garcia, 2007; O'Grady and Morgan, 2011) and majority of lipid-producing algae species have a similar lipid profile which is, in general, equivalent to vegetable oil from land plants suitable for biodiesel production (Xu et al., 2006). These results reveal that the production of biodiesel from microalgal lipids will indeed prove profitable and when grown under appropriate nutrient and CO_2 conditions would lead to an ideal high-biodiesel yielding process and efficient CO_2 sequestration.

The results of this study have verified that the industrial wastewater could support good growth of diverse microalgal species and potential applicability of using microalgae biofixation to sequester carbon dioxide and biodiesel production. The wastewater algal biomass was mainly dominated by Chlorophyceae which showed the potential for biodiesel production besides CO_2 sequestration, thus, contributing to both CO_2 mitigation and alternate energy source. Wastewater grown algae is a cost-effective source of biodiesel production which caters for the thrust area of alternate energy and CO_2 mitigation and a cost-effectiveness analysis was used to aggregate private and external costs and derive the social cost of each fuel as reported by Kovacevich and Justus (2010).

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