

Evaluation of autotrophic and mixotrophic regimen *Chlorella pyrenoidosa* cells in various wastes water for its biochemical composition and biomass production

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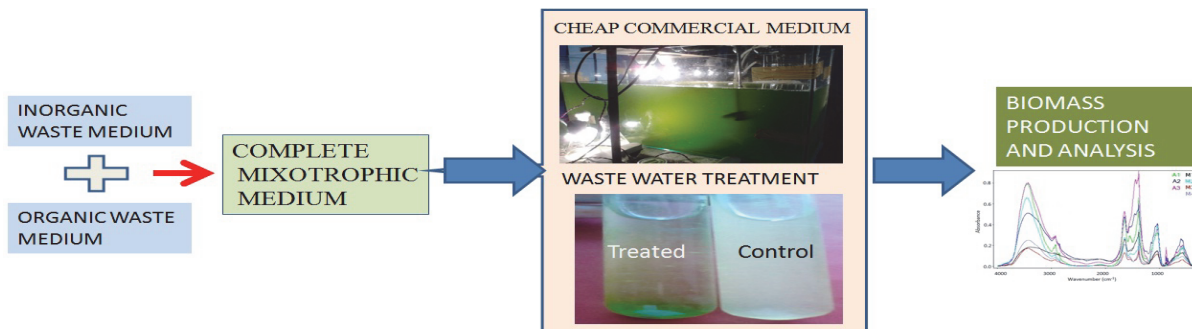
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ABSTRACT

The present study investigates the possibility of integrating an existing industrial large scale biomass production with the treatment of waste water in which a mixture of organic and inorganic rich pollutants was used as a medium. This study suggests that the replacement of a defined medium with a complete mixotrophic medium gives a significant statistical difference in terms of growth parameters i.e. biomass production and specific growth rate. The green microalga *C. pyrenoidosa* was cultivated under different mixotrophic conditions for evaluation of biomass production. Inorganic defined fog's medium supplemented, with raw dairy wastewater led to 1.37g/L biomass production in comparison to 1.2g/L obtained with pure glucose revealing 14.16% increase. The study also involves the supplementation of raw dairy wastewater as an organic carbon source in an inorganic medium comprising municipal treated water and reverse osmosis (RO) treated wastewater and attained 2.4g/L and 1.6g/L of biomass respectively, as compared to 0.3g/L and 0.16g/L obtained in the wastewaters alone revealing 700% and 900% increase respectively. Mixotrophic regimen cells as analyzed by a 2D Fourier transform infrared (FTIR) spectroscopy for its biochemical content revealed that fog's blended raw dairy waste (RDW) regimen cells had maximum Carbohydrate/Amide ratio. The study suggests that the mixotrophic regimen *C. pyrenoidosa* cells can show appropriate growth in a mixture of waste waters and the same comes out to be a cost effective and feasible alternative commercial medium for biomass production without requiring any expensive organic carbon sources in the culture medium.

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Keywords: *C. pyrenoidosa*, autotrophic, mixotrophic, wastewater, biomass, biochemical composition.

INTRODUCTION

The current research and developments in biofuel has considerably been shifted toward alternative and efficient biomass feedstock. Algae biomass as its primary source of feedstock for biofuel conversion technologies has seen as flip side of fossil fuel (*Scott et al., 2010*). Microalgae, unlike fossil fuel, the CO₂ is taken out of the atmosphere and release oxygen in the environment by the algae cultivation (*IEA, 2013; Koller et al., 2012*). Due to the present downhill condition of potential reserves and increased exploitation of fossil fuels causes energy insecurity and climate change by increasing greenhouse gas (GHGs) emissions led the scientists thinking towards the cultivation of algae using waste land (*Wang et al., 2008*). Algal fuels have attractive features like it can generate with the help of wastewaters, biodegradable and relatively harmless in nature towards the environment incase spilled in oceans and can also bloom up with minimal environmental impact on fresh water resources (*Brennan & Owende, 2010*). However, the challenge lies in commercialization of microalgae biomass and its capacity towards the fuel production. The main obstacle here is related to high production costs, low efficiency in terms of biofuel over biomass production and issues which were related towards the sustainability of this technology for a long term which were also equally important (*Koller et al., 2012*). It has been observed that the recent research in the area of biofuel production is emphasized towards the production of biomass from microalgae for a broad range of usance like nutrition for animals and humans, cosmetics products, health sector products, agricultural products in terms of fertilizers and intensively biofuels (*Slade & Bauen, 2012*). The photosynthetic microalgae utilize nutrients in the form of nitrogen and phosphorus released from synthetic fertilizers that in turn increase the overall biomass production and cultivation cost (*Amin, 2009; Slade & Bauen, 2012*). An alternative access to diminish the involvement of synthetic fertilizers in culture medium is to employ wastewater

as a medium, which are organically as well as inorganically rich pollutants (Johnson et al., 2014).

Photosynthetic microalgae can be cultivated using CO₂ for carbon source and light for energy source in open/closed photobioreactors (PBR) (Chen et al., 2009). However, the autotrophic culture mode has several disadvantages which include long cultivation time period and low biomass production. Furthermore, questions about the environmental impact can be raised regarding usage of inorganic nutrients in large quantities (Sialve et al., 2009) and simultaneously contributes to the production cost of algal biomass (Knothe, 2010). Hence, a practicable substitute i.e. wastewater was introduced for the microalgal biomass production and could be used as a culture medium as well as a source of water. A recent report indicates that practices involve the use of algae for bioremediation (Mahapatra et al., 2014; McGinn et al., 2012; Saratale et al., 2010; Subashchandrabose et al., 2012; Yu et al., 2009). A recent report indicates that mixotrophic chlorophycean members (*Chlorococcum* sp.) are known to grow faster in nutrient rich condition (Madhab Mahapatra & Ramachandra, 2010). *C. pyrenoidosa* grown in organically rich glucose medium led to the increase in production of biomass and in turn biofuel production (Kong et al., 2012). Furthermore exogenous sugars, such as sucrose, glucose, galactose, fructose, lactose, and mannose were assimilated and transported by microalgae at different rate of efficiencies (Andrade & Costa, 2007; Shi et al., 1999; Sun et al., 2008). Regardless of providing higher biomass and biofuel production mixotrophically in comparison of autotrophic conditions, there is about <80% cost increase while using the organic carbon source in culture medium as compared to the cost of the defined culture medium (Bhatnagar et al., 2010). Consequently, the need of the hour is to find out the way for the reduction of carbon cost and simultaneously production of higher biomass.

The solution of the problem is development of cheap wastewater medium containing organic as well as inorganic nutrients for maximum growth yields (Liang et al., 2009). Organic carbon sources (as hexoses and pentoses) in dairy wastewater were used to facilitate the sustainability of biofuel industries (Abreu et al., 2012; Kothari et al., 2012; Prajapati et al., 2014; Srinivasan & Subramaniam, 2009; Wang et al., 2009). This capacitates the dairy industry to provide a technology to conserve water, an efficient and simultaneously aggrandize cost of waste water treatment of dairy industries (Mahapatra et al., 2013). Recently algal growth have regained on using household wastewater effluents as a microalgae inorganic growth medium (Mahapatra et al., 2013). The reason for the above is based on an appropriately balanced quantity of micronutrients, macronutrients and dissolved

salts (McGinn et al., 2012; Osundeko & Pittman, 2014; Talbot & delaNoue, 1993; Xin et al., 2010). Potentials of the microalgae cultivated in municipal wastewater effluent in batch and continuous mode have been reviewed. Many previous studies have reported the treatment of industrial, municipal and agricultural wastewater by micro algal culture systems (Franchino et al., 2013; Ji et al., 2013; Lau et al., 1995; Lau et al., 1998; Li et al., 2011; McGinn et al., 2012; Singh et al., 2012; Srinivasan & Subramaniam, 2009; Talbot & delaNoue, 1993; Tam & Wong, 1989; Williams, 2003). Such type of schemes has significant applications in the reduction of nutrient release in associated streams by assimilatory uptake of nitrogen that in turn decreases the rate of eutrophication of receiving streams and also provides oxygen for nitrification and biological organic matter oxidation (Kothari et al., 2013; Mata et al., 2010; Park et al., 2010; Wolanski et al., 2009). A novel thought, mixotrophic wastewater treatment has been considered at the laboratory scale and can be easily upgraded to large scale industries by recycling of nutrients and recovery of energy in a cost efficient manner (Subhash et al., 2014; Venkata Mohan et al., 2014). Hence, the objective of the present study was to evaluate and compare autotrophic and mixotrophic growth of *C. pyrenoidosa*, by utilizing a raw dairy waste as organic medium combined with various inorganic rich wastewater media as a cultural medium. It is a unique study of its own kind which was not carried out so far using the criteria in which dairy by-product and municipality water taken together as organic source and inorganic carbon source respectively for cultivation of *C. pyrenoidosa*.

METHODS AND MATERIALS

Microalgal Strain and Inoculum Preparation

Microalgal strain of *Chlorella pyrenoidosa* was obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, (NCL), Pune, India. This was maintained and cultivated in a fog's medium having the following composition (mg/L): MgSO₄.7H₂O (200), K₂HPO₄ (200), CaCl₂.H₂O (100), Fe-EDTA solution (5mL) prepared by adding 0.00745g/L Na₂EDTA and 0.00557 g/L FeSO₄.7H₂O; H₃BO₃ (286), MnCl₂.4H₂O (181), ZnSO₄.7H₂O (22), Na₂MoO₄.2H₂O (39), CuSO₄.5H₂O (8), KNO₃ (200), pH 7.5. Sterilized medium was inoculated with 5% v/v exponentially growing culture, having viable count of 1×10⁷ cells/mL. Autotrophic cultivation of *C. pyrenoidosa* was initially carried out in a 250mL Erlenmeyer flask containing 150mL fog's medium under 12:12 circadian cycle in the static conditions at 28°C for 12 days. The dry biomass and cellular composition of cells

were then measured. All of the experiments were carried out in triplicate.

Formulations of Medium and Cultivation Conditions

Five different cultivation conditions were carried out using three types of wastewater as represented in Table 1.

Table 1 Different cultivation conditions of *C. pyrenoidosa* and their respective carbon sources.

Growth conditions	Carbon sources
Autotrophic CO_2 (A1)	CO_2 in the form of 1g/L $NaHCO_3^*$ (Yeh <i>et al.</i> , 2010)
Autotrophic MWW (A2-I, II, III)	A1+ types of municipal waste water [†] Municipal secondary treated water (MST) Secondary treated chlorinated water (MScT) Tertiary treated water (MTT)
Autotrophic RO (A3)	A1 + Waste effluent from the reverse osmosis (R.O.) system [†]
Mixotrophic Glu (M1)	fog's medium + 15 g/L Glucose [§] (Muthuraj <i>et al.</i> , 2013)
Mixotrophic $Fog^+ + RDW$ (M2)	Fog's medium + 25% raw dairy waste water (RDW) [§]
Mixotrophic $RO + RDW$ (M3)	Waste effluent from R.O. system + 25% RDW
Mixotrophic $MWW + RDW$ (M4-I, II, III)	Municipal water + 25 % RDW Municipal secondary treated water (MST) Secondary treated chlorinated water (MScT) Tertiary treated water (MTT)

Note: [†]List of inorganic medium: Fog's medium, R.O. waste; ^{*}List of inorganic carbon source: $NaHCO_3$; [§]List of organic C source: RDW, glucose.

Collection of Samples

Municipal wastewater samples (Municipal secondary treated water (MST), Secondary treated chlorinated water (MScT) and tertiary treated water (MTT)) were collected from the sewage treatment plant, Mohali, Panjab, India. Raw dairy influent water (RDW) was collected from the various stages of the 'VERKA' dairy wastewater treatment plant, SAS nagar, Mohali, Panjab, India. Reverse osmosis treated wastewater (RO) samples from the 'KENT' R.O.

system was collected.

All the water samples were brought to the laboratory in plastic beakers. They were inoculated with 5% algal culture and kept in light. The inoculum of strain *C. pyrenoidosa* used in this experiment was autotrophically cultivated in 250mL of Erlenmeyer flasks containing 100mL chemically defined fog's media at pH 7.5 exposed to light intensity of 533Wm^{-2} in 12:12 circadian cycles at room temperature. All the experiments last for the days till decline phase arrived and were performed in triplicates.

Optimization of Algal Growth in Waste Water

The algal strain was grown at different concentrations (25%, 50%, 75%, and 100%) of dairy influent, secondary and tertiary municipal water (effluent) and reverse osmosis treated wastewater (effluent). The experiment was set up in 250mL of the conical flask containing 150mL of varying concentration of wastewater. Homogenous algal suspension (2mL) was used to inoculate each flask.

Cell Density Determination

Cell concentration was determined by cell counting, 1mL of algal suspension taken out each time via sampling tube. It was then directly counted with a Neubauer hemocytometer and an Olympus light microscope where it represents cell density per mL. Where the microalgae cell density (10^6 cells/mL) was related to biomass production (g/L) by the equation $y = 814.85x$ ($R^2 = 0.9997$) for *C. pyrenoidosa*. Where x , y is the biomass production and observed cell density respectively.

Growth Yields Parameters

Specific growth rate (μ , d^{-1}) was calculated from the equation (1) where N_1 and N_2 were the concentration of cells. N_1 denotes the beginning (t_1) and N_2 denotes the end (t_2) of the exponential growth phase, respectively.

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1} \quad (1)$$

Biomass Production (X , g/L) was calculated from the equation (2) where C_t was total concentration of cells and V_t is the total volume of culture medium

$$X = C_t / V_t \quad (2)$$

Biomass productivity (P , g/L/d) was calculated from the equation (3) where X_{max} is the maximum concentration of biomass at the end of the batch run and t is the total duration of the run.

$$P_{\max} = X_{\max} / t \quad (3)$$

FTIR Analysis of Biochemical Content

FTIR spectroscopy of the algal cells cultivated in under different cultivation condition was carried out to determine the chemical composition of algal biomass. PerkinElmer FTIR instrument (Central Instrumentation Laboratory, Panjab University, Chandigarh, India) was used to process the FTIR spectra ranging from 400 to 4000 cm^{-1} . The characteristic peak areas of lipids, proteins and carbohydrates were calculated by integration using essential FTIR v3.10.004 software-Trial version, from Operant LLC. The amounts of biomolecules and their peak areas were correlated as the followings equations (*Pistorius et al., 2009*).

$$A_L = -2.30 + 78.96 \times T_L \quad (4)$$

$$AP = -0.27 + 12.72 \times TP \quad (5)$$

$$AC = 0.07 + 2.05 \times TC \quad (6)$$

Where T_L (mg), T_P (mg) and T_C (mg) represent the total amounts of lipids, proteins and carbohydrates, and A_L , AP and AC are characteristic peak areas of lipids, proteins and carbohydrates, respectively. Peak area integration results using eFTIR v3.10.004 from Operant LLC- Trial Version. The carbohydrate content was determined by integration of peaks between 1179 - 1140 cm^{-1} , using the calibration line $y = 0.07 \times 2.05 \times T_c$ ($r = 0.986$) in absorbance spectra. For protein, by two-point baseline correction, followed by integration of peaks between 1587 - 1495 cm^{-1} , the calibration line $y = -0.27 \times 12.72 \times T_p$ (correlation coefficient, $r = 0.994$). For Lipids, the integration boundaries were used for 2865 – 2834 cm^{-1} by two-point baseline correction the calibration equation $y = -2.30 \times 78.96 \times T_L$ (correlation coefficient, $r = 0.982$) in absorbance spectra.

Measurement of Nitrogen Concentration: Nitrate concentration was measured by the method of Taras (*Taras et al., 1971*) through spectrophotometrically (Hitachi UV–Visible spectrophotometer (Hitachi 2900), with matched silica cells of 1cm light path length). The wastewater sample was centrifuged at 4500 rpm for 10 min and the collected supernatant (1mL) was then diluted 10 times, and the KNO_3 concentration C_{KNO_3} (mM) in the dilution was determined at 220nm according to the following equation:

$$C_{\text{KNO}_3} = \text{OD}_{220} / 0.0385 \times \text{dilution} \quad (7)$$

Statistical Data Analysis

Differences in biomass production between different cultivation media got tested using the ANOVA method, and multiple pairwise comparison post hoc procedures (Dunnutt's Test) were performed when differences were significant. Kruskal-Wallis one way analysis of variance on ranks was used when the data failed tests for normality (Shapiro-Wilk) and homogeneity (Levene Median test), and transformations were unsuccessful. Statistical analyses of the above were performed using Sigma Plot 11.0.

RESULTS

Physical Analysis of Waste Water

Dairy waste: As of fundamental concern, effluent released from a treatment plant has better physiochemical properties than the influent. The influent was whitish in color and acidic pH (4.6) and has an offensive smell. The concentration of NO_3^- was 388.5 mg/L where as the permissibility of NO_3^- was 5mg/L. The effluent was turbid in color and has a slightly alkaline pH (7.3). The odor of effluent was slightly less offensive in comparison to influent. The concentration of NO_3^- (70.1 mg/L) was reduced by several folds in the effluent after treatment. However, their concentration is still sufficient to cause eutrophication.

Municipal waste: Water samples from the various stages of the sewage treatment plant, i.e. Municipal secondary treated water (MST), Secondary treated, but chlorinated water (MScT) and tertiary treated water (MTT). MST has brackish, highly offensive smell, visible precipitation of organic matter and concentrations of NO_3^- (192.1 mg/L). MScT has brackish but clear water and the presence of concentrations of NO_3^- (188.0 mg/L). MTT has clear in appearance and slightly low in concentration of NO_3^- (164.1 mg/L).

Reverse osmosis treated wastewater (RO): The disadvantage of RO systems is the high volume of wastewater generation i.e. 1625 mL/L of purified water. In this study, an attempt was done with the utilization of R.O. wastewater discharge for microalgae cultivation. It has clear appearance, no smell and high amount of concentration of NO_3^- (8.8mg/L)

Effect of Cultivation Conditions on Growth Parameters

Eligible concentration of wastewater as culture medium: Most favorable concentration of waste water 25% of dairy wastewater for influent and 100% for others specified effluents for algal growth were taken for further experiment (Fig. 1).

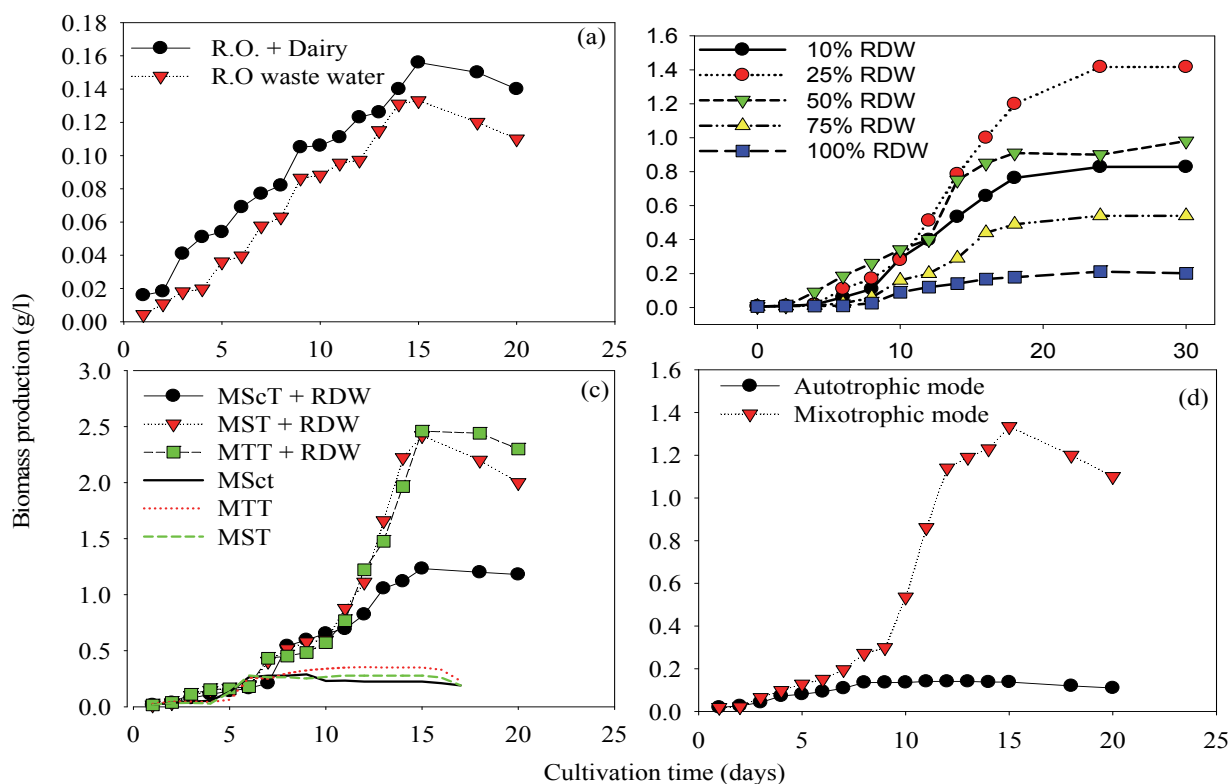


Fig. 1(a) Comparative difference of biomass production of the combined solution of M3 and RO wastewater (b) Growth of *Chlorella* in the cultivation medium of 10% diluted and 25 % diluted RDW (c) Comparative difference of biomass production in combined medium of MSct + RDW, MST + RDW, MTT + RDW, MSct, MST, MTT (d) Comparisons of biomass production in autotrophic (A1) and mixotrophic (M1) culture medium (Parameters- 12 h light; 12 h dark; 28±2°C; cultivation time 20 days; chloroampenicol (0.12mg/ml); n=3).

Evaluation of growth parameters:

Out of autotrophic and heterotrophic mode of culture conditions, mixotrophic regimen cells showed higher carbohydrate accumulation and biomass production. The high cell densities 6×10^7 cells/mL or biomass 1.2g/L of mixotrophic pure glucose culture (M1) demonstrated that the carbon utilization and growth-stimulating effects of light were better as compared to the effects of inorganic carbon in autotrophic culture medium (A1) as depicted in the Fig. 1. It was observed (Table 2) that the highest values of X_{max} (2.4 g/L) and P_{max} (0.30 g/L/d) were obtained in the mixotrophic culture medium of M4III (RDW+MTT). There were relatively significant statistical differences ($P < 0.05$) observed in biomass production under different culture media following mixotrophic and autotrophic culture conditions. Overall, the highest specific growth rates of *C. pyrenoidosa* were 0.33 d^{-1} and 0.30 d^{-1} achieved when microalgae was cultivated in M4III and M2 mixotrophic culture medium respectively. The values were almost 11 and 10 times higher, respectively, compared to specific growth rate 0.03 d^{-1} obtained when cells were grown using A1 culture medium in the autotrophic mode of

cultivation. On the other hand the values were 0.53 and 0.30 times higher, respectively obtained than the specific growth rate 0.30 d^{-1} obtained when cells were grown using M1 culture medium in mixotrophic mode of cultivation.

Table 2 Growth parameters of microalgae cultivated under different autotrophic and mixotrophic culture conditions

Growth parameters	$\mu^a\text{ max (d}^{-1}\text{)}$	$X^b\text{ max (g/L)}$	$P^c\text{ max (g/L/d)}$
A1	0.03	0.14	0.12
A2-I	0.12	0.27	0.15
A2-II	0.11	0.23	0.13
A2-III	0.13	0.30	0.17
A3	0.23	0.16	0.20
M1	0.13	1.20	0.20
M2	0.3	1.37	0.28
M3	0.29	1.6	0.13
M4-I	0.29	2.21	0.30
M4-II	0.13	1.20	0.26
M4-III	0.33	2.40	0.30

Note: a=Specific growth rate; b= biomass production; c= biomass productivities

By the use of One way ANOVA, it was analyzed that biomass production of microalgae significantly increased with increasing concentration of nutrients in inorganic autotrophic culture media (Kruskal-Wallis test, $H = 23.191$ ($P=<0.001$)) and similarly biomass production significantly increased with increasing concentration of carbon sources in the mixotrophic culture media (Kruskal-Wallis test, $H = 19.243$ ($P=<0.001$)). Multiple comparisons were carried out in between different culture media and control group. Dunnett's Method was applied to isolate the autotrophic and mixotrophic medium that differs from the others significantly in terms of biomass production as represented in Table 3.

Table 3 Kruskal-Wallis One Way Analysis of Variance on Ranks. (Multiple Comparisons versus Control Group by Dunnett's Method). To isolate the best suitable media or medium that differs from the others in maximum biomass productivity as a multiple comparison procedure.

Comparisons of inorganic culture media	Difference of Ranks	Q	P<0.05
M4I vs A1	488.500	3.394	Yes
M4II vs A1	413.000	2.870	Yes
M4III vs A1	495.500	3.443	Yes
M3 vs A1	57.000	0.396	No
M4I vs M1	112	0.778	Yes
M4II vs M1	18	0.125	No
M4III vs M1	105	0.730	Yes
M3 vs M1	427.5	2.970	Yes

Assessment of Biochemical Composition of *C. Pyrenoidosa* under Autotrophic and Mixotrophic Culture Conditions

Many researchers have identified FTIR as a tool to investigate the changes in cellular components, including carbohydrates, lipids and proteins as well as a response to a nutrient stress, such as low-N (Giordano *et al.*, 2001; Heraud *et al.*, 2008; Stehfest *et al.*, 2005) and low-P (Dean *et al.*, 2012; Dean *et al.*, 2010; Sigee *et al.*, 2007). FTIR spectra of *C. pyrenoidosa* cells showed distinct absorption bands over the wavenumber range 4000–800cm⁻¹ (Fig. 2). Notice that biochemical compositions were not determined by the traditional time-consuming methods such as Bligh and Dyer, Bradford and the phenol–Sulphuric, but were analyzed simultaneously by a FT-IR spectrometer (Pistorius *et al.*, 2009), because the acyl chains of lipids, the amide groups of peptides, and C–OH, C–O–C groups of carbohydrates occurred at 2800–3000 cm⁻¹, 1500–1700 cm⁻¹ and 1000–1200 cm⁻¹, respectively (Fig. 2 a). The study showed that biochemical content of *C. pyrenoidosa* varied with different nutritional conditions as depicted in 2D FTIR spectrum (Fig.2 b).

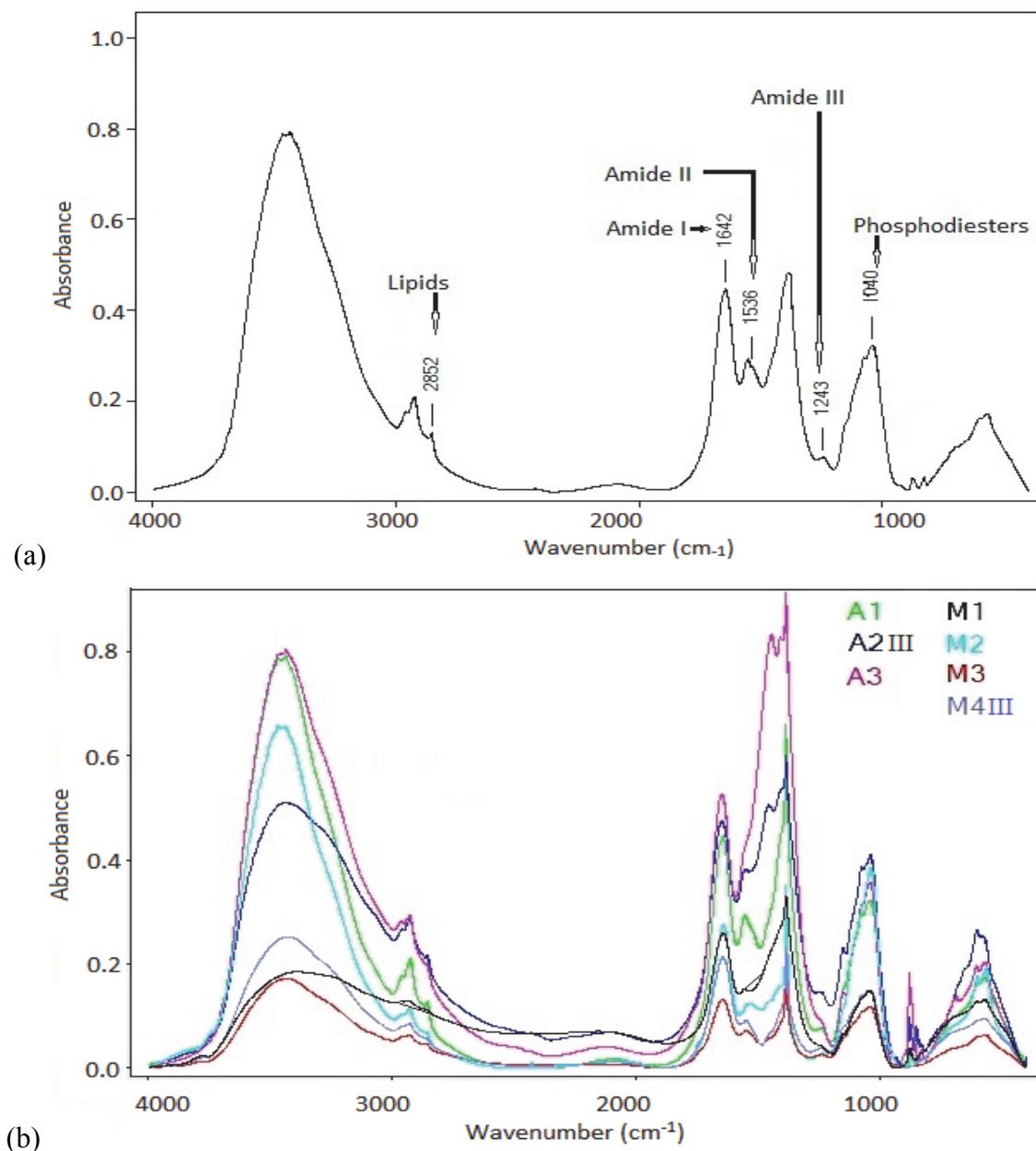


Fig. 2 (a) DRIFT spectra of *C. pyrenoidosa* in the range of 4000-400 cm⁻¹ with possible specific bands assignments (b) One-dimensional noise reduced FTIR spectrum depending on the cultivation conditions changes in different culture media.

FTIR studies showed that under mixotrophic conditions (M1-M4) carbohydrate content of *C. pyrenoidosa* was increased up to 54.0% as compared to that of 20.3%, under autotrophic conditions (A1-A3) as represented Fig 3 a. Although the lower content of nutrients in the inorganic autotrophic medium as compared to that in mixotrophic wastewater medium results in a stress over microalgal cells, which further results in the formation of high levels of lipid content 12% in autotrophic regimen *C. pyrenoidosa* cells. Similar results were found by Chandra (*Chandra et al., 2014*) where lipid content increased by 26% under nutrient deprivation. Following above, autotrophic regimen microalgal

cells showed 65% protein of total biochemical content was significantly higher than mixotrophic regimen cells (39.3%) as depicted in Fig. 3.

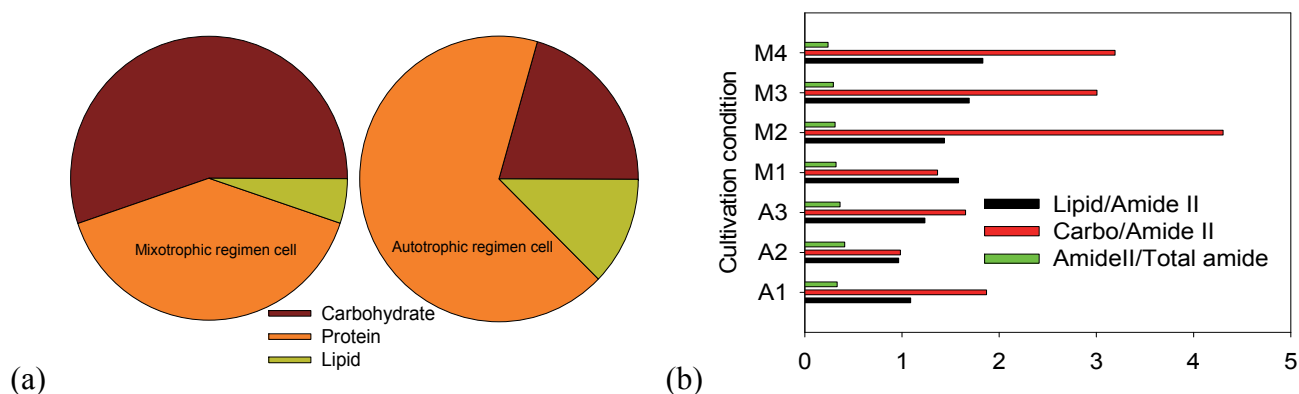


Fig. 3 (a) Comparison of biochemical composition of *C. pyrenoidosa* cells under mixotrophic (M1) and autotrophic (A1) cultivation conditions in terms of protein, carbohydrate and lipid content (b) Comparisons of biochemical content of *C. pyrenoidosa* under various culture mediums in terms of integrated infrared band area ratios

In the present work, FTIR was used to evaluate the effects of cultivation conditions on *C. pyrenoidosa* grown in batch culture in terms of integrated infrared band area ratios as in Table 4.

Table 4 Integrated infrared Band area values of *C. Pyrenoidosa*

Band Area (cm ⁻¹)	Designation
I 3000–2800	Lipids
II 1705–1575	Amide I
III 1575–1480	Amide II
IV 1350–1240	Amide III
V 1200–900	Carbohydrates

The present results have stated that the carbohydrate yield is better in the biomass grown on the influent of dairy wastewater (M2), when compared with the results obtained with the biomass grown in the laboratory fog's media (A1). This has been found that biomass production of microalgae and protein increases with increasing concentration of nitrogen in culture medium (Piorreck et al., 1984). Ueno (Ueno et al., 1998) reported that microalgae has the ability to accumulate high carbohydrate content and can be used as a potential source of ethanol production. It has been reported by many researchers that nutrient limitation usually nitrogen in the growth medium is responsible for lipid accumulation in microalgal cell (Cheng et al., 2012; Karemore et al., 2013; Klok et al., 2013; Li et al.,

2013; Liu et al., 2008; Pedro et al., 2013; Xin et al., 2010; Zhang et al., 2014).

Under mixotrophic and autotrophic culture conditions, biochemical composition biosynthesis was changed with culture condition as depicted in Fig. 3. Dry biomass were evaluated and it was found that carbohydrate and lipid synthesis occurred, as the content of these components changed from 55.3% and 5.9% mixotrophically to 22% and 13.2% autotrophically in dry biomass for carbohydrate and lipid, respectively. As protein is a major chemical component of microalgae is an important feature of rapidly growing cells with low carbohydrate content, and found it low when cells have reached stationary phase because of more carbon is introduced into carbohydrates and/or lipids (Gatenby et al., 2003; Glassford et al., 2013; Pancha et al., 2014; Piorreck et al., 1984). The study reported that protein ranged from about 38.7% to nearly 64.7 % of cellular dry weight depending on cultivation conditions

DISCUSSION

Inferring, from the study it could be stated that the alternative renewable energy technology, specifically, bioenergy technologies will be exemplary in providing the fossil fuel energy. In a developing country like India, bioenergy technologies are much more suitable economically, particularly biofuel production from algal biomass using wastewater. Nonetheless, the conventional wastewater treatment has been unconvincing due to high energy input and difficult processing. Treatment of effluent from industries by the use of algal biomass make the grade of two major sectors, one is to diminish the pollution load from environmental sector and on the flip side, reduction of energy load from the energy sector by biofuels production (Wang et al., 2008). This research suggests the possible future aspects of using a mixture of various wastewaters as mixotrophic culture medium and it comes out to be a cost effective and feasible alternative commercial medium for biomass production (Fig. 4).

As of fundamental concern, different nutritional conditions were significantly influenced the biochemical content and biomass productivity at the end of cultivation. In this study, cultivation conditions got tested were selected on the basis of their potential suitability of various wastewater as a culture medium for maximum biomass production. It would be a fair assumption that microalgae can adapt easily and multiply well in different ecological environment. Li (Li et al., 2014) reported that with the addition of 4 g/L glucose, maximum biomass dry weight (DW, 3.55 g/L) in the mixotrophic culture were 5.4 and 5.2-fold of those under the photoautotrophic, which is quite high and rare, however it states that organic carbon utilization and growth-stimulating effects of light were better as

compared to the effects of inorganic carbon in autotrophic condition culture. Correlated to these, we achieved the high biomass output 2.4 g/L with specific growth rate (0.30 d^{-1}) in organically rich mixotrophic culture medium in wastewater.

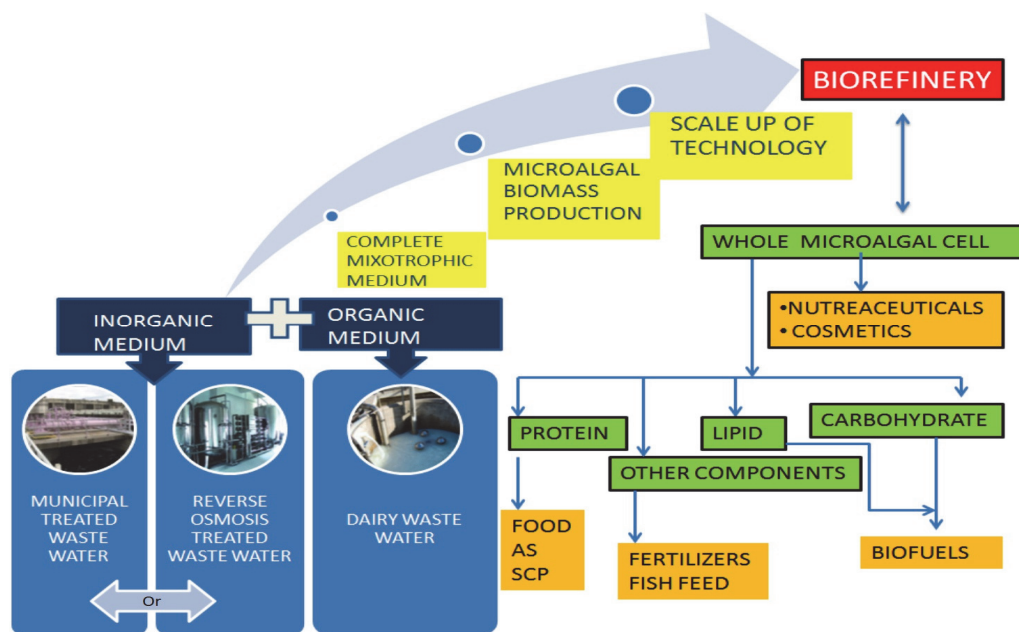


Fig. 4. The economical feasible way of biorefinery development for simultaneously waste water treatment and medium development.

These effects were in consensus to a previous written report, which proposed that higher biomass productivity and specific growth rate obtained under controlled environment in mixotrophic culture medium than autotrophic culture medium (Feng et al., 2005; Kong et al., 2012). It was found that under mixotrophic conditions, biomass production increased by 3-10 times in case of *Chlamydomonas globosa*, *Chlorella minutissima* and *Scenedesmus bijuga* as compared to that of autotrophic growth conditions (Bhatnagar et al., 2010). Mixotrophic cultivation conditions utilize both light and organic carbon source to provide higher energy efficiency than other cultivation ways. These conditions have shown to be the most effective for the yield of microalgal biomass. In the light of above the best organic C-substrate for mixotrophic growth conditions is glucose (Shi et al., 1999). Microalgae can assimilate available organic compounds as well as atmospheric CO₂ as a carbon source in mixotrophic model (Venkata Mohan et al., 2014; Wang et al., 2013). Our results showed that the specific growth rate of *C. pyrenoidosa* was strongly affected by the presence of carbon sources, the mixotrophic growth rate of *C. pyrenoidosa* was about equal to the almost 10 times of the autotrophic growth rates (Fig. 1 d). An especially important finding is that mixotrophic algae showed the increased specific growth rates with the addition of nutrients in the phase of wastewater. It was observed that the mixotrophic growth rate of *C. pyrenoidosa* surpassed autotrophic growth rates. The results agreed with

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the findings of other strains, such as *C. vulgaris* (Kong et al., 2012; Liang et al., 2009) *Chlamydomonas humicola* (Bhatnagar et al., 2010) marine *Chlorella sp.* (Li et al., 2014; Rai et al., 2013; Sarma et al., (in press) and *Nannochloropsis sp.* (Koller et al., 2012). Nutrients like calcium and phosphorus are important macronutrients which is necessary for cellular metabolism required for proper development and growth of microalgae. It was reported that approximately 0.96% total phosphorous on dry weight basis is present in the whey powders (Ozmihci & Kargi 2007; Richmond et al., 2003; Richmond & Grobbelaar, 1986). It is worth mentioning that processing techniques for casein removal from milk have a huge impact on the nutrient content of wastewater. Due to presence of eco-rich organic carbon in influent of dairywater, the biomass productivity was 280 mg/L/d in the 25% diluted influent dairywater, which is higher than an earlier report (Chinnasamy et al., 2010) of 57 mg/L/d (in open pond) and 70 mg/L/d (mixture of algal species cultured in poly bags). Correlated to these, the X_{max} and P_{max} of the combined mixotrophic medium of MTTW water and RDW water resulted in 8- and 0.78-fold increase in comparison to the values obtained in the autotrophic medium of MTTW water alone. Results comprising biomass yield (X_{max}), specific growth rate (μ_{max}) and biomass productivity (P_{max}) of *C. pyrenoidosa* cultivated under mixotrophic and autotrophic conditions are summarized previously in Table 2. Hence the result showed that there was some growth promoting factors in dairy waste so as to find higher biomass production than medium containing easily utilizable glucose as sole carbon source. Abreu (Abreu et al., 2012) reported the similar results in which the highest values of X_{max} (3.58 g/L) and P_{max} (0.43 g/L/d) using dairywater as cultivation medium. The results obtained above using the mixotrophic culture where hydrolyzed cheese whey powder solution is used which is comparable to our results. However, it remains to be determined the reduction of pollutant load of wastewater in this study. Previously, it has been studied that the alga *C. vulgaris*, consume nutrients and meanwhile diminishes COD by 61%, phosphorus by 28% and ammonium by 72%, while rendering a microbial biofilm after incubation of 5 days (Adey et al., 1996; Craggs & Sukias, 2004) has successfully reported the wastewater treatment by the microalgae base system. Similarly *Botryococcus* shows a good example of bioremediation by reducing the nitrogen and phosphorus contents of the dairy wastewater by using the alga (Shen et al., 2013).

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CONCLUSION:

A complete mixotrophic medium was developed using dairy waste water and municipal waste water. The cells displayed differences in growth parameters and biochemical composition based on differences in the growth conditions and medium composition with, biomass and carbohydrate yields increased significantly under mixotrophic cultivation condition. Biochemical composition of the algal biomass makes it interesting as a candidate for bioethanol or biorefinery. Algal cultivation in the wastewater could reduce the effluent's pollution load significantly, indicating potential for its use as an effective effluent treatment program with value addition of the waste stream.

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