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Research Article

MICROALGAE FOR INDUSTRIAL WASTEWATER TREATMENT AND BIODIESEL PRODUCTION

Preeti Sant[1,2], Shaishav Sharma[1], Aesha Patel[1], Ekta Shah[1], Gaurav Dixit[1], Reshma L. Patel[2], Arti Pamnani[3] and Adepu Kiran Kumar[1*]

[1]Bioconversion Technology Division, Sardar Patel Renewable Energy Research Institute, Vallabh Vidyanagar, Anand – 388120, Gujarat, India
[2]Department of Civil Engineering, Birla Vishwakarma Mahavidyalaya Engineering College, Vallabh Vidyanagar, Anand – 388120, Gujarat, India
[3]Department of Civil Engineering, B & B Institute of Technology, Vallabh Vidyanagar, Anand – 388120, Gujarat, India

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ABSTRACT

The present study focuses to investigate the potential of a robust microalga strain, *Ascochloris* sp. for bioremediation of raw dairy wastewater by studying its biomass yield, biomass, aerial and lipid productivities along with lipid %. Control experiment using NPK nutrient (19:19:19) was used for the study. In both indoor as well as outdoor cultivation studies, biomass (g/L/d) and areal productivity(g/m2/d) were found to be higher in raw dairy wastewater (indoor-0.27& outdoor 0.29) as compared to NPK nutrient (19:19:19) (indoor- 0.14 & outdoor 0.15). Moreover, raw dairy wastewater recorded the maximum lipid content (~28.0%)as compared to NPK nutrient (19:19:19)(~16%). Post harvesting water was further subjected to activated carbon filtration and resulted in >90 %, >75 % and >95% reduction in COD, nitrate and total phosphate respectively. Lipid from microalgal biomass was extracted through soxhlet method using different solvents viz. hexane, chloroform, isopropyl alcohol, petroleum ether and diethyl ether. Maximum lipid was obtained through chloroform (~28%). Further the lipid obtained was subjected to acid catalyst based transesterification to convert the triglycerides to FAME (biodiesel) which resulted in 70% conversion efficiency. The properties of the algal biodiesel obtained was investigated which were compatible to the ASTM standards. Also Gas chromatographic analysis of the biodiesel was done which indicated the presence of saturated and unsaturated compounds in the sample.

INTRODUCTION

Recently, issues such as, wastewater treatment and energy crisis are the most researched topics in the field of environment. Industrialization the increasing anthropogenic activities have led to excessive disposal of effluents into water bodies posing water quality issues and damaging aquatic ecosystems. Another is extensive use of fossil fuels, which has comprehensively increased in the recent times and are recognized as unsustainable as it contributes to major global concerns like climate change and greenhouse gas (GHG) emissions. Use of microalgae in biological water treatment methods has attracted interest of various researchers due to its significant role in carbon sequestration, pollution mitigation by removing major pollutants such as N (41%), P (30%), and S (30%) [1], and also because they produce valuable biomass, which can be used for several purposes. Some industrial effluents are rich in nutrients which are suitable for microalgal growth and hence suitable for simultaneous bioremediation and microalgal biomass generation. Dairy effluent when utilized for microalgae cultivation leads to bioremediation of waste water along with algal biomass generation [2]. The integration of microalgal cultivation and bioremediation processes reduces the overall capital expenditure on post-treatment processes of wastewater for safe discharge. More over the biomass generated through bioremediation could be used for energy production[3]. Here, in this line, we have comprehensively evaluated a bioremediation process for generation of

Corresponding author:* **Adepu Kiran Kumar
Bioconversion Technology Division, Sardar Patel Renewable Energy Research Institute, Vallabh Vidyanagar, Anand – 388120, Gujarat, India

microalgae biomass and clean water from 100 % raw dairy wastewater using *Ascochloris* sp. in a column photobioreactor (CPB). A comparative analysis of lipid extraction method using pretreatment strategies and physico-chemical properties of the recycled water was also investigated. The generated biomass was then utilized to produce biodiesel; later characterization of the produced algal diesel is done.

MATERIALS AND METHODS

Materials

Analytical grade chemicals were purchased from the local vendors which were used during the study for microalgal growth in synthetic media, lipid extraction studies and other biochemical experiments. Physico-chemical characterization of raw dairy effluent and recovered water includes chemical oxygen demand (COD), chloride, pH, total dissolved solids (TDS) phosphates, nitrates, ammonia, and sulphates [4]. Raw Dairy Wastewater (RDW) was collected from the effluent treatment plant of Anand Milk Union Limited or Amul Dairy situated in Anand, Gujarat, India. In this study, RDE represents undiluted 100% raw dairy wastewater, unless specified separately.

Microalgae cultivation conditions

Indoor

In this study, *Ascochloris sp*. was used for cultivation and bioremediation of dairy effluent. Microalga was adapted for growth in raw dairy wastewater and NPK nutrient (19:19:19) in different ratios in 500 mL flasks. Microalgal cells were transferred into fresh culture medium every fortnight. All indoor cultivation experiments were performed in a temperature, humidity, and light regulated growth room maintained at 25 ± 1^{O}C in the presence of light (approx. 3300 – 4000 W/m^2) by providing light/dark cycle of 8:16 h. The flasks were manually shaken at regular intervals of 24 h.

Outdoor

Outdoor cultivation of microalgae was performed in column photobioreactor (CPB). CPB is a cylindrical vertical column made of polyacrylic material. The dimensions of the CPB are 1220 mm length and 300 mm diameter with a working volume of 60 L. Inlet and outlet ports for supply of medium and sample collection were provided on the top and bottom of the reactor respectively. The top of the reactor was covered with a lid having an opening in the centre to insert the hollow tube. Air compressor of 0.35 hp capacity was provided to the culture at 0.75L/min rate for 15 min1 h on/off cycle for preventing bacterial growth. All the outdoor culture studies were performed in 100 % raw dairy wastewater without any alterations. Volumetrically 10 % inoculum was added to the dairy effluent after filtering the effluent through a 100 GSM nonwoven geotextile membrane to eliminate sand and other aggregated particles. Temperature and light intensity were measured regularly during the course of the study. Samples were collected periodically and evaluated for growth, biomass and lipid productivity and physico chemical analysis of dairy effluent as well as post microalgae treated water. Biomass, areal and lipid productivities were calculated using the equations given below[5].

$$\text{Areal biomass productivity } (g/m2/d) = \frac{\text{(Final Culture Density (g)} - \text{Initital Culture Density (g) X Working Voulme (L))}}{W Batch\ run\ time(d) X\ Carpet\ Area\ Occupied\ (m2)} \quad (1)$$

$$\text{Lipid Content (\% w/w)} = \frac{\text{Lipid(g)}}{\text{Weight of dry biomass (g)}} \text{X } 100 \quad (2)$$

$$\text{Area lipid productivity (g/m2/d)} = \text{Areal biomass productivity (g/m2/d) X Lipid \%} \quad (3)$$

$$\text{Volumetric lipid productivity (g/L/d)} = Volumeteric\ Biomass\ Productivity(g/L/d) \text{ X Lipid \%} \quad (4)$$

Optical Measurement, specific Growth rate and bIomass and Lipid Productivity

Microalgae cultivated in dairy wastewater were sampled every 24 hours and centrifuged at 10000 rpm for 15 min. The pellet was dried at 60^{O}C and weighed to determine dry weight of microalgal biomass (g/L)[6]. The optical density of the microalgal culture was observed at 600 and 680 nm by UV-Vis spectrophotometer (Schimadzu, USA). Lipid contents were also quantified using conventional soxhlet method as described in later sections.

Post harvesting process

Microalgal culture was harvested with the help of a centrifuge with 10000 rpm speed for 10 minutes at room temperature using universal refrigerated centrifuge (Kubota, Japan, Model 5922). The concentrated wet pellets obtained were dried in a hot air oven (Sedko laboratory equipment's, Ahmedabad) at 70 °C. Hourly moisture content of the sample was analyzed by taking 0.1g sample from different areas of the tray until the moisture content reached till ≤5 %. Moisture was analyzed using moisture analyzer (Sartorius moisture analyzer series, Model MA100). Post drying, the biomass was powdered and stored in sealed bags until lipid extraction. The algae free treated water was passed through an activated carbon filter column for colour removal. Physico-chemical properties of the recovered treated water were studied after every post processing step.

Lipid extraction

Lipid extraction was carried out by using soxhlet apparatus. Different solvents along with their boiling points are listed in Table 3. which were used to carry out the extraction. 10 gm dried microalgae was added into extraction thimble kept in the extraction chamber. The microalgae to solvent ratio was fixed at 1: 30. The solvent in the bottom flask was heated near to the boiling point to reflux. The extraction was performed for different time duration to optimize the yield. Lipid and solvent mixture was separated using rotary evaporator and lipid yield was calculated gravimetrically as described in eq. (2). In order to enhance the total lipid extract, dried microalgae biomass was subjected to pretreatment for cell disruption by a. microwave treatment at 100^{O}C and 2450 Hz for 5 minutes and b. autoclaving at 121^{O}C for 15 min at 1 bar pressure followed by soxhlet extraction with hexane as solvent. Control experiments were also conducted to compare the extraction efficiency.

Analytical methods

During microalgae growth, parameters *viz.* temperature, pH, conductivity and TDS were observed and recorded regularly using electrode probe based equipment (Eutech instruments

Cyberscan series 600 portable meter). Chemical oxygen demand (COD) was measured colorimetrically by adding COD solutions to water sample and digesting it in COD digester (Merck Spectroquant TR420) at 148^{O}C for 120 min and then absorbance recorded in spectrophotometer (Merck spectroquant picco)[4]. Ammonium concentration in the water samples (effluent and treated) was determined using nesslerization method using ammonium chloride as standard and absorbance recorded at 410 nm. Nitrate concentration was estimated photometrically using potassium nitrate as standard and absorbance observed at 220 nm. Phosphate content was also determined photometrically at 690nm using potassium phosphate as standard. Sulphate content was estimated by turbidimetric method using sodium sulphate as standard at 420 nm. Chloride concentration in water was determined using argentometric method [7]. The physico-chemical characterization of raw dairy effluent and microalgal treated water as shown in Table 2. Process steps involved in clean water generation from raw dairy effluent is shown in Fig 3.

Transesterification

Transesterification was performed through acid catalysis method. 5% sulphuric acid was added to the 23 g lipid in 6:1 ratio (Methanol: Lipid). The reaction was carried out inside a water bath and the mixture was then heated at 90°C for 4h. 10 ml of sample was taken in regular interval of 1h to optimize the transesterification reaction. The heated mixture was then allowed to cool down and was then transferred to a separating funnel. Hexane was added to the separating funnel and the mixture was left for layer separation. The lower layer consisted of glycerol while the upper layer consisted of the biodiesel and hexane. The hexane wash was repeated 3-4 times. The upper layer was collected and hexane was evaporated and recovered using a rotary evaporator. The obtained biodiesel was subjected to hot water wash to remove the impurities. The obtained biodiesel/FAME was stored at -20°C for further analysis. The conversion efficiency of the biodiesel produced was calculated using the eq. (5).

$$\text{Conversion efficiency (\% w/w)} = \frac{\text{Weight of biodiesel produced (g)}}{\text{Weight of lipid taken (g)}} \text{X } 10 \qquad (5)$$

Properties of biodiesel

Acid Number

To determine the acid number of the biodiesel, titration method was used. 0.1mL biodiesel was taken in a 250 mL conical flask and 50 mL of 95% ethanol and diethyl ether in 1:1 ratio was added and mixed thoroughly. The solvent-oil mixture was titrated against 0.1 M KOH using 0.1 mL of 1% phenolphthalein indicator.

The formula for calculating acid number is as follows:

$$\text{Acid number of biodiesel } \left(\text{mg}\frac{\text{KOH}}{\text{g}}\right) = \frac{\text{56.1 X Normality of the KOH solution X Volume of KOH used}}{\textit{Weight of the sample taken}} \qquad (6)$$

Ash Content

The ash content is a measure of the mineral content and other inorganic matter in the sample. To determine the ash content in the biodiesel, 5 g of biodiesel sample was taken in a pre-weighed crucible and placed inside a muffle furnace (550°C) for 3h. After 3h, the biodiesel was burnt completely to ash, the crucible was taken out and was placed in a desiccator. The final weight of the crucible was noted. The formula for calculating the ash content is given below.

$$\text{Ash content of biodiesel (\%)} = \frac{\text{Initital weight of the crucible} - \text{Final weight of the crucible}}{\textit{Weight of the sample taken}} * 100 \qquad (7)$$

Viscosity

The kinematic viscosity of the algal biodiesel sample was determined using Viscometer (Fungi Lab). A low viscosity adapter with circulation jack *et al* ong with a spindle was used to perform the test. 16mL of biodiesel was poured into the heat chamber of a viscometer and heated up to 30°C. Readings were noted after the completion of the experiment.

Relative Density

1 ml of biodiesel sample was taken in a pre-weighed eppendorf tube and weight of the eppendorf was noted. Density of the biodiesel sample was measured by the conventional mass: volume calculation

Calorific Value

A digital bomb calorimeter (IKA C 5000) was used to determine the calorific value (CV) of the algal biodiesel. 1mL of biodiesel was taken in the container and placed in the bomb cell, and a cotton thread hanging from a nichrome wire was dipped into the biodiesel. The bomb cell was filled with oxygen gas. The bomb cell was placed inside the bomb calorimeter instrument and the readings were noted after the completion of the experiment.

Flash & Fire point

The biodiesel was kept inside the flash and fire point apparatus (Pensky Marten Flash point apparatus), attached with a thermocouple. 55 mL the algal biodiesel sample was taken and placed in a brass sample cup. The temperature at which the spark came out first was noted as the flash point of the biodiesel, while the temperature at which a continuous flame appeared was noted as the fire point of the biodiesel.

Gas Chromatographic Analysis

The composition of algae biodiesel (FAME) produced was determined by using well established GC analysis. GC analysis was used to study the chemical composition of algae biodiesel. The major peaks were identified using Perkin Elmer, USA, Auto System XL instrument using Flame Ionization Detector (FID) detector (100 $^{\circ}$C - 450°C). The sample was optimized through a capillary column was used. A sample of 0.6 µl (0.5 mg of algae biodiesel in 1 ml of hexane) was injected under the split mode of Perkin Elmer, USA, Auto System XL equipped with a FID.

RESULTS & DISCUSSION

We comprehensively evaluated the outdoor cultivation of *Ascochloris* sp. in 100% raw dairy effluent in CPB followed by lipid extraction studies for maximizing the lipid production. Post harvesting water was further subjected to activated carbon filtration and resulted in >90 %, >75 % and >95% reduction in COD, nitrate and phosphate respectively. Also the properties of

biodiesel were tested which when compared with ASTM standards were complying the ASTM standards. The result clearly indicates that the integrated concept of microalgal based bioremediation of dairy wastewater to cultivate microalgae for pollutants removal along with biodiesel production is a feasible option.

Microalgae Cultivation

Growth of *Ascochloris* sp. in indoor cultivation was highest in raw dairy wastewater as compared to NPK nutrient (19:19:19) with biomass yield (g/L) of 1.93, whereas NPK nutrient biomass yield (g/L) (19:19:19) was 0.93on 7th day (Table 1.). The outdoor cultivation (Fig 1.) also showed similar results where cultivation was highest in raw dairy wastewater. The biomass yield (g/L) of raw dairy wastewater and NPK nutrient (19:19:19) is 2.9, 1.06 respectively. All outdoor cultivation experiments were performed in CPB under ambient light and temperature. After 7 days, no major increase in biomass and lipid yields was observed as evident from Table 1.

Figure 1 Outdoor cultivation of *Ascochloris* sp. in dairy wastewater after 7 days

Table 1 Biomass and lipid productivities of *Ascochloris* sp. in different culture media

Cultivation	Biomass (g/L)	Biomass productivity (g/L/d)	Areal biomass Productivity (g/m^2/d)	Lipid (%)	Lipid productivity (g/L/d)
		Indoor			
RDW	1.93	0.27	ND	29.83	0.01
NPK	0.93	0.14	ND	18	0.02
		Outdoor			
RDW	2.09	0.29	17.01	28	0.07
NPK	1.06	0.15	10.17	16	0.02

Harvesting and post Harvesting

After obtaining the wet concentrated w*et al*gal biomass obtained from CPB produced 0.8 Kg of w*et al*gal biomass (Fig 2(a)) having 85% initial moisture content and was able to produce 0.12 Kg of dry biomass (Fig 2(b)). The supernatant obtained after centrifugation was utilizable water which meets the Indian Central Pollution Control Board (CPCB) water quality standards designated for Type E class of water.

A

B

Figure 2 Microalgal biomass ; (a) Concentered w*et al*gal biomass, (b) Dry powdered algal biomass

Physico-Chemical Characterization of raw and Treated water

Microalgae free water obtained as supernatant after centrifugation and activated charcoal treatment was analyzed for various parameters and compared with raw dairy effluent in order to determine its suitability for various applications. More than 90 % COD was reduced from raw dairy effluent after 7 days of microalgal treatment. Initial ammonia and nitrate concentration in the raw dairy effluent were 50.55 mg/L and 142.21 mg/L which were reduced to 10.11 mg/L and 32.70 mg/L respectively after microalgal bioremediation. These results were similar to that reported by[8] but were with mixed algal and diatom cultures. Also, sulphate and chloride content were considerably reduced from 598 mg/L to 0.58 mg/L, and 220 mg/L to 44 mg/L, respectively. Physico-chemical characteristics of the treated water were within the limits of standards set by Central Pollution Control Board (CPCB) under Type E class of water best use for irrigation purposes (Table 2). This study signifies the suitability of *Ascochloris* sp. for effectively reducing the pollutants' content in raw dairy effluents. This means that the treated water is utilizable water which meets the Indian Central Pollution Control Board (CPCB) water quality standards designated for Type E class of water.

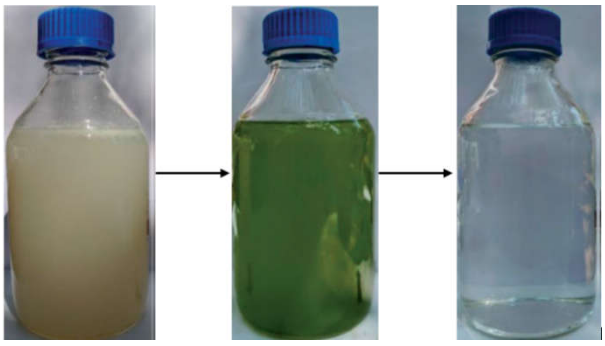

Figure 3 Process steps involved in clean water generation from raw dairy wastewater

Table 2 Physico-chemical characterization of raw dairy wastewater and microalgal treated water

Parameters	RDW	Treated water	Reduction %	CPCB Type E water
pH	3.59	8.06	-	6.0-8.5
Total Dissolved Solids (mg/L)	4016.16	1124.52	72	≤2100
Chemical Oxygen Demand (mg/L)	7289	510.23	93	≤600
Ammonia (mg/L)	50.55	10.11	82	ND
Nitrate (mg/L)	142.21	32.70	77	ND
Total Phosphate (mg/L)	0.35	0.01	97	ND
Sulphate (mg/L)	598	23.92	96	≤1000
Chloride (mg/L)	220	44	80	ND

ND: Not defined

Cell Disruption & Lipid extraction

The results from the cell disruption indicate that microwave technique to the most suitable pre-treatment methods which were able to break the most number of cells. Control experiment was performed using hexane as solvent without applying any pretreatment method. Among pretreatment methods, microwave method was found to extract maximum lipid (25%) with hexane solvent. Further, microwave pretreated microalgae was subjected to lipid exctraction using different solvents as mentioned in table 3. The highest lipid was extracted using chloroform as solvent (28%). This experiment establishes microwave and chloroform as best pretreatment method and solvent for microalgae lipid extraction, respectively (Table 3).

Table 3 Microalgal lipid extraction in different conditions

Cell disruption technique	Solvent	Boling point (°C)	Lipid (%)
Control	Hexane	68.0	13.0
Autoclave (121°C for 15 min)	Hexane	68.0	18.0
Microwave (5 min with 30s pulse)	Hexane	68.0	25.0
	Chloroform	61.2	28.0
	Diethyl ether	34.6	19.0
	Petroleum ether	51.0	23.0
	Isopropyl Alcohol	82.5	20.0

Transesterification

A total of 23 g of lipid was used for the transesterification reaction which yielded 16.1 g (18 mL) of algal biodiesel (Fig 4.). The conversion efficiency was calculated to be 70%.
5

Figure 4 Algal biodiesel

Properties of biodiesel

All the properties of the algal biodiesel were within the ASTM standard limits (Table 4.). To estimate the acid number of the produced algal biodiesel titration method was used. The acid number of the algal biodiesel (10 mg KOH/g), but it does not harm the engine parts. Both viscosity and density of the algal biodiesel at 30°C (5.05 mm^2/s and 0.8686 g/m^3, respectively) which indicates good atomization and complete combustion of the biodiesel inside the engine and a healthier engine life. Even though the CV of the produced algal biodiesel in this experiment was lower (39 MJ/Kg) than that of petro based diesel, it was higher than the CVs of coal or standard biodiesels like palm and Jatropha[9]. The flash and fire points of the biodiesel were much higher (150 and 156°C, respectively), and thus better, than those of petro-diesel because higher flash and fire points reduce the chance of unexpected fire hazard. The ash content of the algal biodiesel produced here was 0.001%. So the algal biodiesel can be used in an unmodified CI engine.

Table 4 Properties of algal biodiesel with corresponding ASTM standards

S. No.	Properties	Microalgal Biodiesel	ASTM Standards
1	Acid Number (mg KOH/g of oil)	10	< 0.5
2	Density (g/cm^3)	0.87	0.8-0.9
3	Kinematic viscosity (mm^2/s)	5.05	1.9-6
4	Ash content (%)	0.001	< 0.01
5	Calorific value(MJ/Kg)	39	-
6	Flash point (°C)	150	> 93
7	Fire point (°C)	156	-

Gas Chromatographic Analysis

The analysis majorly showed the presence of five saturated which are palmitic (16:0), arachidic (20:4), myristic (14:0), lauric (12:0), and stearic (18:0:) and four unsaturated which are linolenic acid (18:3), linoleic (18:2), oleic (18:1), and palmitoleic fatty (16:1) acids in the algae biodiesel chromatograms.

CONCLUSION

This study is an attempt to reduce the pollution load of wastewater and generate the algal biomass at the cost of nutrient rich dairy industry wastewater. This is an integrated process for bioremediation of dairy effluent with generation of microalgal biomass and recovery of > 80 % water suitable for irrigation and cleaning purposes. Besides this the harvested algal biomass has been successfully utilized to produce biodiesel which can be blended with convention diesel. This study provides a solution to the increasing challenges of industrial effluent handling, decreasing ground water levels and energy security issues.

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