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Bioremediation Of Dairy Wastewater Using Microalgae For The Production Of Biodiesel

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ABSTRACT

This study describes the feasibility for treatment of dairy waste waters in batch cultures by using selected strains of green microalgae namely *Chlorella vulgaris, Botryococcus braunii* and a mixed algal culture in indoor and outdoor. The Biomass productivity peaked on the 6th day. Best results were observed in *C. vulgaris* strain in both indoor and outdoor studies with biomass productivity of 0.51g/L, chlorophyll concentration of 0.039mg/L and lipid yield of 0.030g/L in indoor cultures whereas 0.59g/L of biomass productivity, 0.045mg/L chlorophyll concentration and 0.035g/L lipid yields were obtained in outdoor studies.. Gas Chromatography mass Spectrophotometer (GC MS) analysis of the extracted lipids showed that major components in *C.vulgaris* and mixed algae were palmitic acid, staeric acid and oleic acid where as oleic acid was the major component in *B.braunii* and palmitic acid, staeric acid were also present in minor amounts.

Keywords: ***Chlorella vulgaris, Botryococcus braunii, lipid, dairy wastewater, mixedAlgae***

1.0. Introduction

In recent years biodiesel production from algae has gained at most attention. Due to the scarcity of natural sources there is an emergency to find an alternative to replace the exhausting fossil fuels which lead to the raise of alternative biofuels (Perlack et al. 2005). Biodiesel production has been started during ages but with different feedstock like vegetable oils, animal fats etc. but the life cycle assessment from first generation biofuels frequently approached those of traditional fossil fuels mainly due to the high feed cost of vegetable oil (Antolin et al., 2002; Lang et al., 2001). Hence second generation biofuels have been raised and the goal was to improve the amount of biofuel that can be sustainably produced by using biomass from residual non-food parts of crops, such as stems, leaves and husks that are left behind once the food crop has been extracted, as well as other crops that are not used for food purposes such as switch grass, grass, Blue-green algae, Microalgae, jatropha, whole crop maize, miscanthus and cereals that bear little grain, and also industry waste such as woodchips, skins and pulp from fruit pressing, etc. Since this was a burning issue a number of researchers were going through several innovative applications using Green microalgae as a tool. easily degradable, and algae biodiesel does not contain sulphur. Hence green microalgae have become a boom to the present biodiesel production strategy.

Apart from biodiesel production green microalgae were also used as food, fertilizer, feedstock for pharmaceutical, pollution control, water treatment, dyes, agar etc. Green microalgae like plants require natural sunlight, water and carbon dioxide. Heterotrophic growth of some microalgae has been used for efficient production of biomass and lipid (Aaron Packer et al., 2010, Shi et al., 2000, 2002; Wen et al., 2002.). Green microalgae easily grew on dairy effluent and produced lipids (Woertz, et al., (2009). Green microalgae were adaptable to a maximum range of nutrient concentrations and have a capability to reproduce in a very short span utilizing the supplied nutrients, as India is very near to the equator, irrespective of any season the temperatures always remain higher than many other countries where the biodiesel research is going on.

A limited study has been carried out on the treatment of dairy wastes in shake flask cultures by using *C.vulgaris, B.braunii* and mixed algae (Tamarys et al., 2011). A notable study has not been carried out on the treatment and simultaneous lipid production in indoor and outdoor culture conditions using *C.vulgaris, B.braunii* and mixed algae comparitively. The reason for the selection of

these two particular species was that according to many researchers opinion, *C.vulgaris* can tolerate up to 50°C and can easily grow in any kind of environment particularly tolerant to sewage effluent conditions (Bhatnagar et al., 2010; Lau et al., 1995; Ruiz-Marin et al., 2010; Shi et al., 2007; Wang et al., 2010, Liling et al., 2011, O. Fenton, D. Ó hUallacháin., 2012), *B.braunii* has been selected for its high efficiency of photosynthesis and high lipid content reported amongst other species. Generally *B.braunii* cannot tolerate temperature more than 30°C but due to continuous exposure to hard temperatures for a long period of time they turned adaptive and tolerant. **(**P. Metzger, 2005). The specific objectives of the present study includes: 1) Treatment of dairy waste water in shake flasks and small open tanks as a comparison, 2) Simultaneous nutrient removal and lipid production from the algae biomass, 3) Lipid extraction and GC MS analysis.

2.0. Materials and methods

2.1. Organism isolation and cultivation

Mixed cultures of green microalgae were collected at random from different water bodies where algal blooms were seen. The cultures were examined under Olympus CX21 light microscope at 100X magnification and found the presence of *Chlorella sp, Spirogyra, Botryococus, Oscillatoria* etc. Wild strains of *Chlorella vulgaris* and *Botryococcus braunii* were isolated and cultivated in Modified bold basal medium and Chu 13 medium respectively. They were identified by microscopic observation according to the descriptions of Chlorophyceae in the literature.

2.2. Dairy wastewater source and characterization

Dairy waste water was obtained from AP dairy industry, Hyderabad, AP, India which involves in processing raw milk into products such as consumer milk, butter, cheese, yogurt, condensed milk, dried milk (milk powder), and ice cream, using processes such as chilling, pasteurization, and homogenization. Typical by-products include buttermilk, whey, and their derivatives. The initial physicochemical characteristics like pH, Alkalinity, Chemical Oxygen Demand (COD), Chloride, Phosphate, Sulphate, Nitrate, Nitrite, Ammonia, Total Organic Nitrogen, Total solids, Total Dissolved Solids, Total suspended solids, Volatile Suspended Solids, Volatile fatty acids, Most Probable Number and Total bacterial count were analyzed (table.1) according to Standard methods for examination of water and waste water (APHA 20th edition).

2.3. Setup of indoor culture conditions

Algal cultures of *C.vulgaris*, *B.braunii* and Mixed algae were inoculated into three different Erlenmeyer flasks of 250ml capacity each containing 100ml dairy wastewater medium. An uninoculated control was run subsequently containing only dairy wastewater. The cell growth was measured in terms of optical density read as absorbance. Initial optical density was noted at 660 nm using a Shimadzu UV 2450 UV-VIS spectrophotometer to know about the cell density based on which the cell growth was implicated on a dry weight basis. The culture flasks were fixed in a shaking incubator operated at a rotating speed of 120rpm where the cultures were agitated in circular movement. Temperature and Light were maintained at 32°C and 6000lux delivered from cool fluorescent 30W tubes respectively. Maximum biomass yield was observed in exponential phase (Day 7). The cultures were harvested during late-stationary phase (Day 13) which was noted by the chlorophyll response i.e. the color change from green to yellowish-brown indicating the perishing chlorophyll and lipid accumulation.

2.4. Light/dark regime

The growth rates of shake flask microalgae cultures were studied under 6 different light and dark cycles viz., 4:20, 8:16, 12:12, 16:8, 20:4 and 24:0 (E. Jacob-Lopes, et al., 2009) to know the ambient photoperiod for the artificially illuminated indoor cultures.

2.5. Setup of outdoor culture conditions

The above mentioned three microalgal cultures were inoculated into three different Plastic open tanks of 200L capacity containing 100L dairy waste water medium (Green et al., 1995). The open tanks were equipped with a baffle system and a pump to mix the entire medium. The pump was operated at 40rpm which generated a flow rate of 0.15m/s (Craggs, 2005). Light and temperature conditions were monitored every half an hour to know the ambient condition required for the growth of green algae. Temperature reached up to 37°C during the mid-day in spring. All the other parameters were followed as mentioned in indoor cultures.

2.6. Chlorophyll estimation

Green algal cells were ground with mortar & pestle in diethyl ether. Sonic manual stirring was carried

out to shear plasma membranes and later the extract was subjected to Centrifugation with REMI R 24 Research Centrifuge where components were separated by density. Sample tubes attached to a rotor were spun at high revolutions creating high gravitational forces. Cell extract was first subjected to slow speed at 1000 rpm for a short time of 10 mins to pellet out heavy cellular debris. Pellet was thrown out and supernatant is kept. Supernatant was then subjected to higher speed 10,000 rpm and for longer time of 15-30 mins to pellet out chloroplasts. The pellet was kept and washed with distilled water dried to room temperature and weighed. Then it is placed in diethyl ether and subjected to spectroscopic analysis for estimation of total chlorophyll content. Chlorophyll content is measured as chlorophyll a at 420nm and 660nm and chlorophyll b at 440nm and 600nm as they absorb blue and red lights at these particular frequencies

Chl mg/L = Chl a mg/L + Chl b mg/L

2.7. Harvest

The fully grown microalgae were bulk harvested after reaching to late stationary phase. The biomass was harvested using a double layered thin mesh filter into a large vessel. This procedure was repeated several times till maximum water content was separated. After separating, the rigid biomass is spread uniformly as a thin layer on a stainless steel plate and dried in a hot air oven at 80°C for 10-12hrs till the total moisture was evaporated. Then the dried biomass was ground in a mortar and pestle into a fine powder and stored in cool dry place.

2.8. Lipid Extraction

The dry biomass was mixed in benzyl alcohol in a ratio of 1:2 in a round bottomed flask and exposed to ultrasounds of 20MHz at 25°C in an Ultrasonicator for one hour. Later the reaction mixture was transferred onto a Buchner funnel covered with a Whitman no.1 filter paper and pressed with a pestle for effective filtration. Then the filtrate containing extracted lipid and the extracting solvent was subjected to distillation process in an oil bath containing paraffin oil maintained at 250°C as benzyl alcohol boils off at 205°C the remaining extract was microalgal lipid which was transferred into a fresh container and weighed.

2.9. Analytical Determinations

The obtained lipids were extracted with hexane in acidic medium and injected into GC MS instrument to investigate the components present in the lipid extract. An Agilent Technologies GC MS equipped with 6890N Network GC system baring 30m×250µm×0.25µm capillary column and 5973 Inert Mass selective quadruple detector is used to estimate the lipid content.

3.0. Results and discussion

Dairy waste water has proven to be good medium to feed microalgae for lipid generation. The two isolated species and mixed strains actively degraded the toxic chemical constituents like nutrients, COD, TDS, Ammonia, Nitrogen, Nitrate etc in the dairy effluent and produced notable yields of biomass and fatty acids.

3.1. Removal of nutrients and pollutants from dairy wastewater by Microalgae

In indoor cultures, COD was reduced to 90% by *C. vulgaris*, 90% by *B.braunii* and 85% by mixed algae; Ammonia was reduced to 93% by *C. vulgaris*, 92% by *B.braunii* and 90% by mixed algae. Nitrate was reduced to 91% by *C. vulgaris*, 90% by *B.braunii* and 85% by mixed algae and other nutrients were reduced to about 80% on an average by the three strains (Table.1). In outdoor cultures, COD was reduced to 95% by *C. vulgaris*, 90% by *B.braunii* and 85% by mixed algae; Ammonia was reduced to 95% by *C. vulgaris*, 93% by *B.braunii* and 92% by mixed algae. Nitrate was reduced to 96% by *C. vulgaris*, 94% by *B.braunii* and 91% by mixed algae and other nutrients were reduced to about 85% on an average by the three strains (Table.2). C.vulgaris was found to be efficient in nutrient-pollutants removal in both indoor and outdoor cultures. The treatment of the dairy waste water was efficient in outdoor open ponds.

S.no	Parameters	*Before treatment (raw dairy waste water)*	After treatment (*C.vulgaris)*	After treatment (*B.braunii)*	After treatment (Mixed algae)
1.	*PH*	*8.15*	*7.7*	*7.8*	*7.9*
2 .	*Total dissolved solids (mg/L)*	*1936*	*163*	*189*	*206*
3.	*COD (mg/L)*	*1760*	*176*	*208*	*288*
4.	*Volatile fatty acids (mg/L)*	*160*	*11.5*	*24*	*46*
5.	*Alkalinity as $CaCO_3$ (mg/L)*	*980*	*110*	*120*	*120*
6.	*Chlorides (mg/L)*	*745.5*	*85.2*	*88.75*	*92.3*
7.	*Nitrates (mg/L)*	*118*	*10*	*11.6*	*18*
8.	*Sulphates (mg/L)*	*460*	*31.6*	*36*	*37.5*
9.	*Phosphates (mg/L)*	*14*	*0.9*	*1.2*	*1.6*
10.	*Total solids (mg/L)*	*2342*	*200*	*234*	*258*
11.	*Total suspended solids (mg/L)*	*406*	*37*	*45*	*52*
12.	*Total Kjeldhal Ammonia (mg/L)*	*37.24*	*2.38*	*2.88*	*3.19*
13.	*Kjeldhal Nitrogen (mg/L)*	*8.96*	*0.56*	*0.84*	*1.12*
14.	*Volatile Suspended Solid (mg/L)*	*290*	*95*	*105*	*120*

15.	*Most Probable Number (MPN/100 ml)*	*4*	*1*	*1*	*2*
16.	*Total bacterial count (CFU/ml)*	*>110*	*<20*	*<29*	*<32*
17.	*Volatile fatty acids (mg/L)*	*60*	*9.5*	*11.8*	*20.6*

Table.1 Dairy waste water treatment using the three algal strains in shake flasks

S.no	Parameters	*Before treatment (raw dairy waste water)*	After treatment (*C.vulgaris)*	After treatment (*B.braunii)*	After treatment (Mixed algae)
1.	*PH*	*8.15*	*7.5*	*7.7*	*7.7*
2.	*Total dissolved solids (mg/L)*	*1936*	*90*	*112*	*124*
3.	*COD (mg/L)*	*1760*	*80*	*176*	*256*
4.	*Volatile fatty acids (mg/L)*	*160*	*4.5*	*9.7*	*11.1*
5.	*Alkalinity as $CaCO_3$ (mg/L)*	*980*	*90*	*105*	*120*
6.	*Chlorides (mg/L)*	*745.5*	*70.9*	*89.65*	*85.2*
7.	*Nitrates (mg/L)*	*118*	*4.5*	*6.3*	*9.8*
8.	*Sulphates (mg/L)*	*460*	*29*	*36*	*38*
9.	*Phosphates (mg/L)*	*14*	*Nil*	*0.2*	*0.5*
10.	*Total solids (mg/L)*	*2342*	*112*	*156*	*172*
11.	*Total suspended solids (mg/L)*	*406*	*22*	*44*	*48*
12.	*Total Kjeldhal Ammonia (mg/L)*	*37.24*	*1.79*	*2.29*	*2.68*
13.	*Kjeldhal Nitrogen (mg/L)*	*8.96*	*0.28*	*0.56*	*0.84*
14.	*Volatile Suspended Solid (mg/L)*	*290*	*65*	*95*	*105*

15.	***Most Probable Number***	***4***	***1***	***1***	***2***
	(MPN/100 ml)				
16.	***Total bacterial count (CFU/ml)***	***>110***	***<10***	***<17***	***<21***
17.	***Volatile fatty acids (mg/L)***	***60***	***4.5***	***6.7***	***11.1***

Table.2 Dairy waste water treatment by the three algal strains in open tanks under continuous medium circulation.

3.2. Ambient light/dark regime

Among the six different light/dark regimes the three species showed best growth response in 8h:16h light/dark cycle with vigorous mixing at 120rpm rotation speed which enabled better nutrient supply in equal proportions enabling fast growth in such a short photoperiod. *C.vulgaris* reached to maximum cell density on day 6 during the exponential phase. *B.braunii* and mixed algae have shown maximum cell density on day 7 later on they passed through a stationary phase and maximum lipid yields were noted during the late-stationary phase (Day 13) during which the harvesting was carried out.

3.3. Cell growth analysis

Microalgae have shown higher growth yields in the outdoor compared to indoor cultures. Among the three species *C.vulgaris* was more efficient in biomass growth in indoor as well as outdoor. Growth rates of *B.braunii* were good but not as better as *C.vulgaris* due to temperature barrier. Mixed algal species yielded little lesser compared to the other two. In indoor cultures *C. vulgaris* yielded 0.51g/L biomass on a dry weight basis (Fig.1), *B. braunii* yielded 0.41g/L (Fig.2) and mixed algae yielded 0.23g/L (Fig.3). In outdoor cultures the light intensities and temperature conditions were continuously monitored as the cultures were maintained in open atmospheric conditions.

The temperatures reached maximum up to 40°C during the mid day and the light intensities were noted up to 1200X100 lux. Inside the water medium the temperature reached up to 33°C on a sunny day. The biomass yield of *C.vulgaris was* 0.59g/L, *B.braunii* yielded 0.57g/L and mixed algae could yield 0.32g/L (Fig.4) on a dry weight basis in outdoor cultures.

Among the studies carried out on treatment of different wastewaters, the highest biomass yields reported in the literature where about 81.4mg/L/day (Wang et al, 2010 in press) and 2.6g/m^2/day (Johnson and Wen, 2010) for C. vulgaris with dairy manures. The biomass yield of mixed species containing *C.vulgaris* was about 270.3mg/L/day with primary treated municipal wastewater along with CO_2 supply (Woertz, et al. 2009).

The maximum biomass yield of *B. braunii* was 300mg/L/day in piggery waste water with high NO_2-N content (Jin- Young An et al, 2003). The biomass yields in the present study were significant compared to the above reported yields due to the evidence that microalgae were able to grow and treat undigested raw wastewaters at elevated temperatures above 37^{O}C and light intensities up to 15Klux (247.5 µmol. Photons^{-1}.s^{-1}).

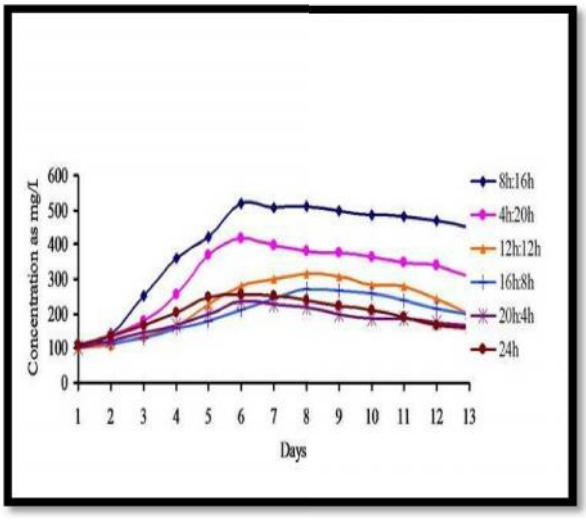

Fig1: Biomass growth patterns of *C.vulgaris* at different light/dark cycles grown in shake flasks.

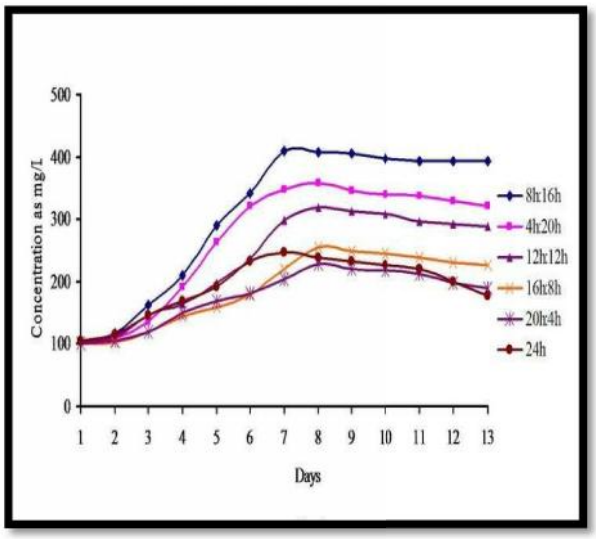

Fig 2 : Biomass growth patterns of *B.braunii* at different light/dark cycles grown in shake flasks

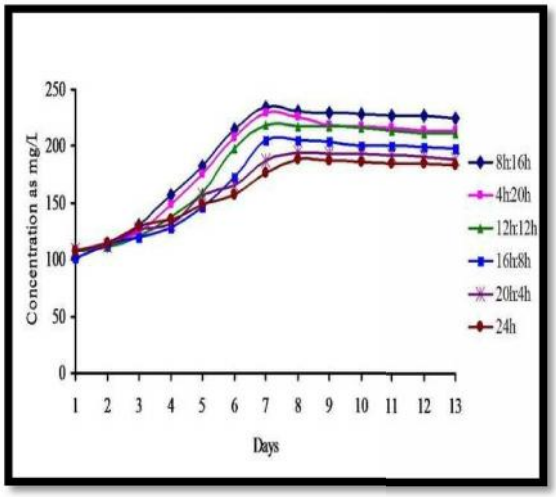

Fig 3 : Biomass growth patterns of *mixed algae* at different light/dark cycles grown in shake flasks.

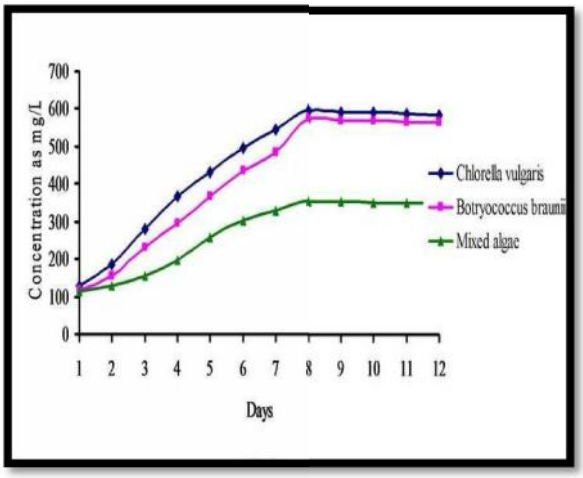

Fig 4 : Biomass growth patterns of the three strains in open tanks.

3.4. Chlorophyll content

Chlorophyll contents for the outdoor cultures were relatively high due to innumerous sunlight. In indoor cultures the chlorophyll contents were 0.039mg/L for *C.vulgaris*, 0.038 mg/L for *B.barunii* and 0.031mg/L for mixed algae (Fig. 5). In outdoor cultures the chlorophyll contents were 0.045mg/L for *C.vulgaris*, 0.043mg/L for *B.braunii* and 0.034mg/L for mixed algal culture (Fig. 6).

3.5. Lipid yields

Lipid yields were higher in outdoor cultures especially in *C.vulgaris*. In indoor cultures the lipid yields were 0.030g/L for *C.vulgaris*, 0.029g/L for *B.braunii* and 0.011g/L for mixed algae. The lipid yields in outdoor cultures were 0.035g/L for *C.vulgaris*, 0.032g/L for *B.braunii* and 0.019g/L for mixed cultures. *C.vulgaris* was efficient in lipid yield compared to *B.braunii* though the latter is known for its high lipid content, yet it could not cope up with the high temperatures as the former. Lipid contents of the mixed cultures were comparatively low. The highest lipid yields reported in the literature were about 31mg/L/day (Wang et al, 2010 in press) and 230mg/m^2/day (Johnson and Wen, 2010) for C. vulgaris with dairy manures. Mixed species containing *C.vulgaris* yielded about 24.4mg/L/day (Woertz, et al. 2009) and the high lipid yield for *B. braunii* was 69mg/L/day (Jin- Young An et al,

2003). In the present study there was no additional supply of nitrates, CO_2 and dilutions were not made to the dairy wastewaters. Also extreme light and temperatures were the major hurdles yet there were good yields.

3.6. GC MS Analysis

The lipids derived from both indoor cultures and outdoor cultures were subjected to GC MS analysis and found that palmitic acid, steric acid, oleic acid, lauric acid etc were the major components commonly present in *C.vulgaris and* mixed algal species where as in *B.barunii*, methylated esters of fatty acids i.e. oleic acid phenyl methyl ester, palmitic acid phenyl methyl ester, steric acid phenyl methyl ester, and traces of some other hydrocarbons such as methyl oleate were found to be present.

4.0 Conclusions

The present study evaluated the feasibility for dairy wastewater as a useful medium to support algal growth with simultaneous nutrient and pollutants removal with notable lipid yield. Green microalgae were able to grow in elevated temperatures up to 40˚C. *C. vulgaris* was more efficient in nutrient removal, biomass and lipid yields in both indoor and outdoor cultures among the other species selected for this study. Hence a further detailed study is needed to know how to improve the biomass and lipid yields in different microalgae strains and to study the effect of various nutrients and CO_2 concentrations on the biomass growth and lipid yields.

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