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Cattle urine increases lipid content in *Chlorella pyrenoidosa*: A low cost medium for bioenergy application

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A B S T R A C T

Media requirement for microalgae cultivation adds most to the cost of biodiesel production at commercial scale. The present work aims to study the growth of green algae *Chlorella pyrenoidosa* under fogg`s medium and modified fogg`s medium by replacing KNO_3 with urea at different concentrations 0.15, 0.20 and 0.25% (w/v). To reduce the cost of urea, cow urine (CU) was utilized to grow the *Chlorella sp.* in different volume fractions as 5, 7.5, 10, and 12.5 % (v/v). Biomass production in 7.5% cow urine was achieved 1.93 g l^{-1} that is almost double in comparison to normal Fogg's medium (0.82 g l^{-1}). Cellular biochemical components such as lipid, protein and carbohydrate are determined quantitatively. The lipid content is found to be 32.7 % in 10 % of cow urine and 22.7 % in 7.5 % cow urine that is much higher than the *Chlorella sp*. grown under Fogg`s media (7%). The protein content is enhanced to 50.17% and carbohydrate reduced by half in comparison to normal fogg's medium cells. The extracted lipid is converted into fatty acid methyl esters (FAME) and characterized by GC-MS. The FAME produced from cow urine grown cells showed suitable composition to prove its application as biofuel.

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INTRODUCTION

Biodiesel from microalgae is a new promising industry for sustainable energy source because of their high photosynthetic efficiency and ability to grow in extreme environmental conditions [1]. The ability of algae to survive under different environmental conditions, to a large extent is due to changing pattern of cellular lipids as well as by modifying lipid pathways [2]. Researchers are focused in developing new and cheap technologies to produce biodiesel from micro algae not only due to high lipid productivity but also lack of competition with food, water and land [3]. By adopting integrative approach, a simple solution can be designed for large scale production of microalgal biodiesel.

Algae has shown huge growth potential under nutrient deficient conditions, which makes it the role model for studying its molecular pathways for enhancing desired lipids. Recently conducted experimentation reveals that various physiochemical stress conditions can induce varying expression patterns for lipids [4]. Literature shows improvement in microalgae biomass and lipid production by modifying various nutrient conditions such as nitrogen [5, 6], carbon source[2] [7]; salinity and iron content of the medium also affect algae growth [8]. Extensive work is done on production of biodiesel from algae but the fuel is still costlier than normal diesel with the price of US$ 1.25/lb and US $0.43/lb, respectively, which obstructs large-scale applications of algae biofuel [9]. Media composition requires the expensive operation cost and therefore limits its availability at larger scale. There is a need to use cost effective media for growth of algae for bioenergy application.

The present study focused on the cultivation of *Chlorella pyrenoidosa* using an alternative low cost nitrogen source that is a waste and which can be advantageous for commercial production of biodiesel. Cattle urine is a rich source of nitrogen and other microelements; total N ranging from 6.8 to 21.6 g l^{-1}, of which an average of 69% is present in the form of urea [8]. Among the organic nitrogen sources, urea is found

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2 EIA, "Gasoline and diesel fuel update," March 2010, http://tonto.eia.doe.gov/oog/info/gdu/gasdiesel.asp

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the best nitrogen source for culturing *Chlorella sp*. [10, 11]. We have chosen *Chlorella pyrenoidosa* for our study, as the species has potential to grow under variety of environmental conditions with high lipid accumulation [12, 13]. A comparative analysis is done for growth and biochemical composition of the *Chlorella sp.* under cow urine (CU) medium and normal Fogg`s medium for its biofuel application.

Comparison of media cost

In our present study, cow urine is used for cultivation of *Chlorella pyrenoidosa.* The cost of 10 % CU medium is estimated approximately \$0.025 l^{-1}, which is almost half of the price of Fogg's medium that costs approximately \$0.04 l^{-1}.

MATERIALS AND METHODS

Microalgae and culture conditions

Fresh water green alga *Chlorella pyrenoidosa* was grown in 1000 ml Erlenmeyer flasks containing 500 ml Fogg's medium which contained (g l^{-1}) KNO_3, 0.2; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1; K_2HPO_4, 0.2; Na_2EDTA, 0.0745 and 1.0 ml of microelement solution consisting of H_3BO_3 2.86; $MnCl_2.4H_2O$ 1.81; $ZnSO_4.7H_2O$ 0.222; $Na_2MoO_4.2H2O$ 0.39; $Co(NO_3)_2.6H_2O$ 0.0494; $FeSO4.7H_2O$ 0.0557; $CuSO_4.5H_2O$ 0.079 pH adjusted to 7.2. The algae growth was also conducted in modified Fogg's medium by replacing nitrate with urea. The effect of urea on cell growth was investigated by varying the concentration between 0-0.25% (w/v). *Chlorella sp* was grown in cow urine medium (CU) at different concentrations ranging 5, 7.5, 10, 12.5 and 15 v/v % without adding any nutrient. The media were sterilized prior to inoculating with exponential phase fresh cells. The cultures were grown in laboratory conditions for 12 days under 24 h fluorescent illuminations (40 watt, white light) at 28°C.

Cell growth analysis

The growth of algae and biomass concentration was monitored by optical density measurement at 660 nm using UV/visible spectrophotometer (Shimadzu UV-1650). Cells were concentrated by centrifugation (Eppendorf R 5810), washed with de-ionized water and dried (60°C) to determine dry weight (expressed as g l^{-1}).

Quantification of biochemical composition

Lipid

The lipid was extracted through Bligh and Dyer method [14]. A mixture of 2ml methanol and 1 ml chloroform was made and added to 1 g algal biomass. It was kept for 24 h at room temperature to dissolve the lipids properly. The mixture was centrifuged at 3000 rpm for 10 min. Supernatant was separated 2 ml of chloroform was again added to the pellets and shaken properly. It was again centrifuged at 3000rpm for 5 mins and supernatant was separated. After adding 2 ml of 1% KCl to the supernatant separate layers will be formed. Lower layer will be pipette out and weighed.

Protein

The crude protein was determined by Lowry method by taking 0.5 ml of algal culture [15]. The absorbance of the sample was checked and the concentration was determined using standard curve.

Carbohydrate

The content of carbohydrate is estimated by the modified method of 3, 5- Dinitrosalicylic acid colorimetry using 100 mg of dry algal powder [16]. The carbohydrate content was estimated using DNS reagent and optical density of the sample was determined against the blank at 540 nm in a UV-visible spectrophotometer.

Lipid Content (%) = wt. of lipid (g) × 100/ wt. of culture (g)

Total Protein Content = wt. of protein (from BSA curve) X 100/ dry cell mass (g)

Carbohydrate Content (%) = wt. of carbohydrate (from Glucose standard curve) X 100/ dry cell mass (g)

Conversion of algal lipid to FAME

The lipid extract was esterified under acidic condition using standard method [12]. The dried oil (300 mg) was recovered with chloroform and 6 ml of NaOH (0.5 mol l^{-1}) in methanol was added. The mixture was then heated under reflux for 15 mins. After that, 18 ml of transesterification reagent (prepared from 2g ammonium chloride, 60 ml of methanol and 3 ml of sulphuric acid) was added and heated under reflux for another 15 mins and was subsequently transferred to the separating funnel. Separation of biodiesel was done using hexane and distilled water. A clear yellowish layer is recovered in the organic layer containing the FAMEs (biodiesel). After 2-3 times washing of biodiesel with water, organic layer was collected and dried in rotary evaporator. The methyl esters were then solubilised in hexane for gas chromatography- mass spectroscopy (GC-MS) analysis.

Characterization of FAME by GC-MS

Fatty acid composition was determined using GC-MS. The amount of sample injected was 1µl. Fatty acid methyl esters (FAMEs) were identified by comparison of the retention times with those of the standard (Supelco TM 37 component FAME mix, Sigma-Aldrich Co.)

RESULT AND DISCUSSION

Biomass and lipid accumulation of Chlorella sp. under different growth medium

The growth curve of Chlorella pyrenoidosa was plotted under different media formulations i.e. Fogg`s media and cow urine supplemented medium (CU). The autotrophic cells showed higher cell growth and late stationary phase in cow urine as compared to Fogg`s media. The growth of Chlorella sp. under 10 % cow urine medium reached 1.811g l^{-1} while it is only 0.768 g l^{-1} under Fogg`s medium on 10th day after inoculation. The growth of Chlorella sp is found faster in cow urine medium as it reached its maxima within 10 days of inoculation. As shown in Figure 1, lag period of the cells grown under CU medium is shorter compared to the cells grown under normal Fogg's medium that also contribute to higher cell growth.

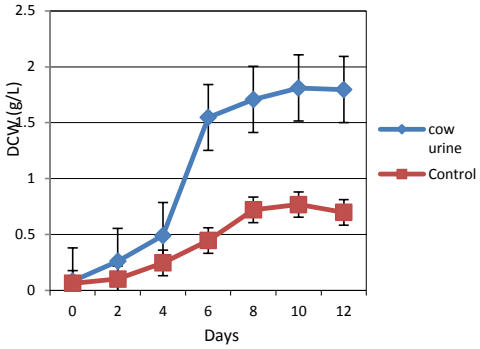

Figure 1. Growth of Chlorella sp. in Fogg`s medium and 10% cow urine medium

The growth of Chlorella sp was optimized by varying the concentration of cow urine (5, 7.5, 10 and 12.50%). The dry cell weight (DCW) of algae was obtained 0.34 g l-1 in 5%, 1.93 g l-1 in 7.5%, 1.80 g l-1 in 10% and 0.60 g l-1 in 12.50 % of cow urine supplemented medium (Figure 2). The cell growth in Fogg's medium was recorded as 0.82 g l-1 within the same time interval. Cow urine is not a toxic waste material; possess 95% of water, 2.5% nitrogen sources, and the remaining 2.5% mixture of glucose, minerals, salts, hormones and enzymes [17]. Antimicrobial and germicidal properties of cow urine (gomutra**)** are due to the presence of urea (strong effect), creatinine, swarn kshar (aurum hydroxide), carbolic acid, other phenols, calcium and manganese [18]. The nitrogen content of 7.5 and 10% CU medium are found quite suitable for growth of *Chlorella pyrenoidosa*.

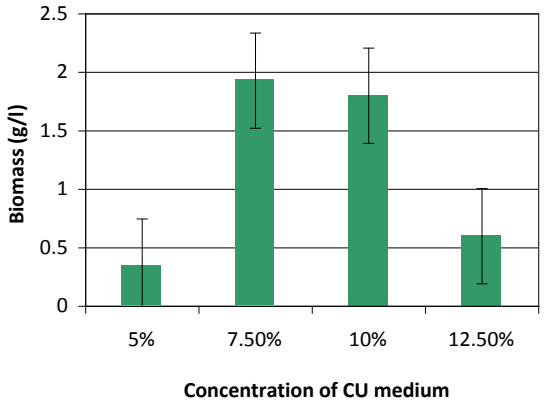

Figure 2. Biomass production of microalga in different concentrations of cow urine

The highest growth was obtained in 7.5 and 10% concentration of cow urine medium among different concentration applied. The lipid production was compared in cells kept in these two concentrations of cow urine and found tremendous increase than normal fogg`s medium cells. The lipid production was recorded 32.7% in 10% CU medium, 22.7% in 7.5% CU medium and 7% in normal fogg's medium cells (Figure 3). Biomass production is slightly higher and lipid is lower under 7.5% CU in comparison to cells grown under 10% CU medium. Increased lipid content is an attractive finding regarding bio-diesel application; hence concentration of cow urine was further optimized to get maximum lipid content in cells.

This is the first study on use of cow urine for the growth and lipid production of *Chlorella pyrenoidosa.* The cost of cultivation medium is very important component in capital cost for biofuel production; using cow urine as growth of algae will definitely decrease the total production cost.

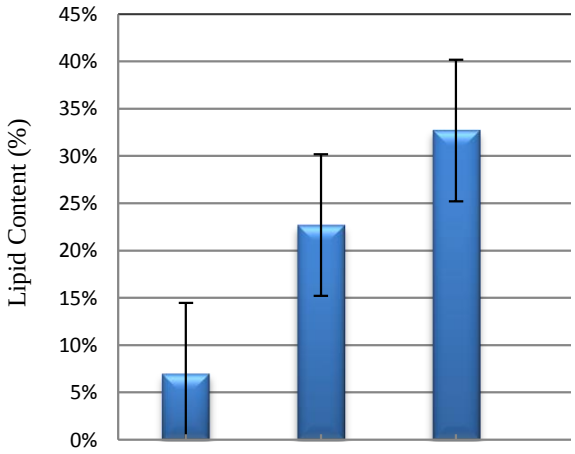

Figure 3. Effect of cow urine (CU) concentration on lipid content of *Chlorella pyrenoidosa*

To verify the component present in the CU medium responsible for increased growth and lipid accumulation in *Chlorella sp.*, the cells were grown under pure urea at different concentrations (Figure 4). In this study, the growth of cell is directly proportional to the concentration of urea applied. The highest biomass production (0.9 g l^{-1}) was obtained at the concentration of 0.25 g l^{-1}. The biomass production was only 0.145 g l^{-1} under urea starvation, and was increased to 0.57 g l^{-1} and 0.713 g l^{-1} at 0.15 and 0.20% (w/v) of urea, respectively. This finding may be attributed to the growth of the *Chlorella pyrenoidosa* under urea and also the cell growth may be supported by ammonia if produced as a hydrolysed product of urea and also found in cattle urine.

Lipid content was also affected by urea supplemented medium at different concentrations. Figure 4 shows lipid production of 0.022, 0.12, 0.09 and 0.04 g l^{-1} in 0, 0.15, 0.20, 0.25 % (w/v) of urea respectively. The maximum lipid content (21%) was estimated at 0.15 % urea (w/v) and decreases with increase in urea concentration from 0.20 to 0.25 % (data are not shown). Literature shows, urea is a low cost nitrogen source to support growth and lipid accumulation in various *Chlorella* species [11, 19, 20].

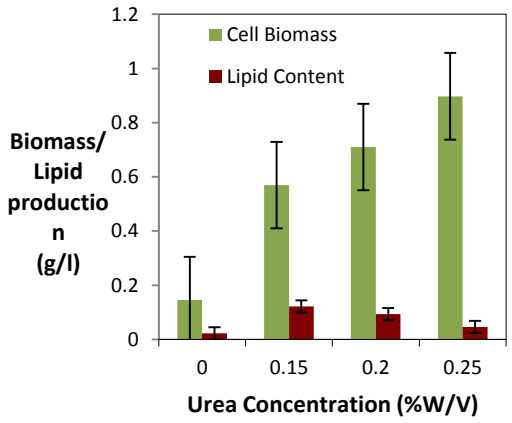

Figure 4. Effect of urea concentration on biomass and lipid production in *C. pyrenoidosa*

Determination of biochemical components

The cellular components may vary with the growth media and culture conditions. The cell grown in cow urine supplemented medium showed high protein in comparison to cells grown in normal fogg's medium (Figure 5). Protein content was estimated 50.176% in CU medium whereas it was approximately 37% in case of fogg`s medium. The lipid accumulation was enhanced to 22.7% which is much greater than the lipid extracted from cells grown in control medium (7%), while the carbohydrate was reduced from 40 to 23% in presence of CU medium. Results showed that in presence of cow urine the excess carbon from photosynthesis is channelled into storage molecule (TAGs), and carbohydrate content may be reduced [21]. An increase in protein may be induced due to high nitrogen supplementation in CU medium [22].

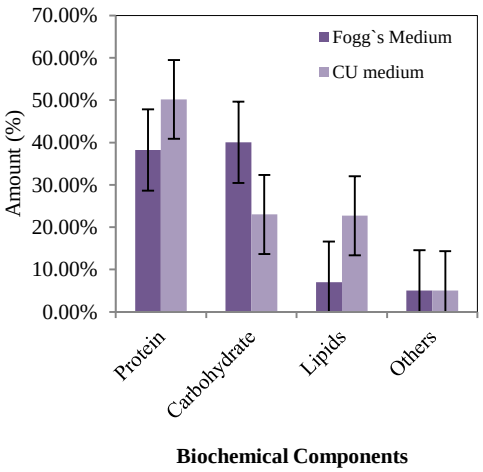

Figure 5. Biochemical components of *C. pyrenoidosa* in Fogg's medium and cow urine (CU medium).

In the present work, cells grown in CU medium does not show decrease in protein while there was decrease in carbohydrate and increase in lipid, so the growth has not been disturbed by increase in lipid. Similar result obtained in *C. vulgaris* when grown in presence of acetate and glycerol [23].

FAME analysis

Fatty acid profile of the algae grown in Fogg's medium and CU medium was determined and the results are presented in Table 1 (a & b). The fatty acid composition was calculated based on specific fatty acid percentage over the total fatty acid of lipids of each sample. The fatty-acid produced from cells grown under fogg's medium showing linolenic acid (18:3; 37.55%) as the highest content followed by palmitic (16:0;14.3%), oleic (18:1; 12.3%) and linoleic acid (18:2; 8.7%) (Table 1a). The FAME profile of cells grown under CU medium is quite different showing highest content of palmitic (16:0; 21.56%) and oleic (18:1; 21.33%) followed by linoleic (18:2; 13.69%) and linolenic acid (18:3; 10.39%). Two fatty acids palmitoleic (16:2; 5.58%) and stearic (18:0; 3.26%) are also present in CU cells those are not present in fogg's cells (Table 1, b). In presence of CU medium, the total content of saturated fatty acid is found to be 25 % , monounsaturated fatty acids is 24 % and polyunsaturated fatty acids is 31% while in cells The proportion of saturated, monounsaturated and polyunsaturated fatty acids are better in CU grown cells, which meets the requirements of the European Standard

Peak Num-ber	Retention time	Compound	Area (%)
4	11.35	3,7,11,15-tetramethyl-2-hexadecen-1-ol ($C_{20}H_{40}O$)	0.42
12	17.33	Hexadecanoic acid methyl ester ($C_{17}H_{34}O_2$)	21.56
13	17.57	7- hexadecenoic acid methyl ester ($C_{17}H_{32}O_2$)	2.94
15	18.87	9,12- hexadecadienoic acid methyl ester ($C_{17}H_{30}O_2$)	5.58
17	19.82	9,12,15-octadecatrienoic acid methyl ester ($C_{19}H_{32}O_2$)	10.39
19	21.91	Octadecanoic acid methyl ester ($C_{19}H_{38}O_2$)	3.26
20	22.26	9-octadecenoic acid methyl ester($C_{19}H_{36}O_2$)	21.33
22	23.13	9,12-octadecadienoic acid methyl ester ($C_{19}H_{34}O_2$)	13.69
26	24.86	5,8,11,14,17-Eicosapentaenoic acid methyl ester ($C_{21}H_{32}O_2$)	2.12

TABLE 1. Fatty acid profile

(a) *C. pyrenoidosa* grown under Fogg's medium

0	Retention time	Compound	Area (%)
4	11.90	3,7,11,15-tetramethyl-2-hexadecen-1-ol ($C_{20}H_{40}O$)	1.74
9	14.48	1-octadecyl isocyanate($C_{19}H_{37}NO$)	1.74
12	17.66	Hexadecanoic acid methyl ester ($C_{17}H_{34}O_2$)	14.31
13	17.94	7- hexadecenoic acid methyl ester ($C_{17}H_{32}O_2$)	2.48
14	18.29	2,5-dimethyl-4-hexene-3-01($C_8H_{16}O$)	3.09
15	18.87	1,8,10-tetradecatriene($C_{14}H_{24}$)	2.99
16	20.20	9,12,15-octadecatrienoic acid methyl ester ($C_{19}H_{32}O_2$)	37.55
17	20.75	4,7,10,13,16,19-docosahexanoic acid methyl ester ($C_{23}H_{34}O_2$)	3.73
20	22.57	9-octadecenoic acid methyl ester($C_{19}H_{36}O_2$)	12.35
21	23.44	9,12-octadecadienoic acid methyl ester ($C_{19}H_{34}O_2$)	8.73
24	25.23	5,8,11,14,17-Eicosapentaenoic acid methyl ester ($C_{21}H_{32}O_2$)	1.91

(b) C. pyrenoidosa grown under CU medium

EN 14214 for biodiesel production [25]. In our study cow urine; a rich source of nitrogen is used to cultivate algae and the effect is found to be very positive in respect to both biomass and lipid accumulation.

CONCLUSION

The proportion of saturated, monounsaturated and polyunsaturated fatty acids are better in CU grown cells, which meets the requirements of the European Standard EN 14214 for biodiesel production. In our study cow urine; a rich source of nitrogen is used to cultivate algae and the effect is found to be very positive in respect to both biomass and lipid accumulation.

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Persian Abstract

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چکیده

محیط کشت مورد نیاز برای کشت میکروجلبک ها بیشترین هزینه را برای تولید بیو دیزل در مقیاس صنعتی در بر دارد.در این پژوهش هدف مطالعه رشد جلبک های سبز *Chlorella pyrenoidosa* در محیط *کشت fogg و محیط کشت fogg*اصلاح شده با جایگزینی KNO_3 *با اوره در غلظت های مختلف ۰,۱۵،۰,۲،۰,۲۵ (w/v) می باشد. برای کاهش هزینه های اوره از اوره گاوی برای رشد* ***Chlorella sp*** در نسبت های حجمی مختلف ۵،۷,۵،۱۰،۱۲,۵(v/v) استفاده شد.توده زیستی تولیدی برای ۷,۵٪ اوره گاوی *1.93g/l* به دست آمد که تقریبا در مقایسه با محیط کشت *fogg* معمولی دو برابر شده است.(*0.82g l^{-1}*).ترکیبات بیو شیمیایی سلولی مانند لیپید،پروتیین و کربوهیدرات به طور کمی تعیین شدند.میزان لیپید در ۱۰٪ اوره گاوی ۳۲,۷٪ و در ۷,۵٪ اوره گاوی ۲۲,۷٪ تعیین شد که بسیار بیشتر از ***Chlorella sp*** *رشد داده شده در محیط کشت fogg می باشد(۷٪).میزان پروتیین تا ۵۰,۱۷٪ افزایش پیدا کرد و کربوهیدرات در مقایسه با محیط کشت نرمال fogg به نصف کاهش پیدا کرد.لیپید استخراج شده به متیل استرهای اسید چرب تبدیل شد(FAME)و با GC-MS شناسایی شد.FMAE تولیدی از سلول های رشد داده شده با اوره گاوی ترکیب مناسبی را برای استفاده به عنوان سوخت زیستی نشان دادند.*