Environmental Science and Pollution Research (2018) 25:22052–22059
https://doi.org/10.1007/s11356-018-2339-x

RESEARCH ARTICLE

Pretreated animal and human waste as a substantial nutrient source for cultivation of microalgae for biodiesel production

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Received: 5 March 2018 / Accepted: 15 May 2018 / Published online: 25 May 2018

Abstract

The use of human and animal wastes for fertilization of aquaculture ponds has been practiced for thousands of years. In the present work, we have used the excreta (human urine, poultry waste, cow dung, and urine) as a nutrient source for the cultivation of *Chlorella singularis*, *Micractinium pusillum*, and *Chlorella sorokiniana* strains of microalgae. Different solid wastes were treated with 60 mM H_2SO_4 for the extraction of nutrients. After treatment, the supernatant of different solid wastes and liquid waste were diluted 5, 10, 15, and 20% to be used as a media for the cultivation of microalgae. *Chlorella sorokiniana* was able to grow in all concentration of excreta media. The maximum growth rate 140 ± 3.1 mg/L/day and lipid production (45.5 ± 2.3 mg/L/day) was obtained in 20% poultry. Among the different excreta media used for cultivation of microalgae, poultry media displayed the best results and thus, should be used for large scale cultivation of microalgae.

Keywords Microalgae · Excreta · Poultry waste · Human urine · Biodiesel

Introduction

Extensive research has been done for the development of biodiesel from microalgae. Nutrients are needed for the growth of microalgae. A significant challenge that needs to be addressed is optimization of algal biomass and lipid production technologies for developing a cost-effective commercial scale biodiesel (Wrede et al. 2014). Keeping in view the abovementioned facts the present study focuses on investigating animal, bird, and human excreta as a low-cost sustainable nutrient supply for algal biomass production.

Rapid growth of population has generated large amount of human and animal wastes (Talyan et al. 2008). Human and animal generate significant quantities of excreta as solid and liquid waste that can be used as fertilizer (Benetto et al. 2009; Remy and Jekel 2008; Tidåker et al. 2007a; Tidåker et al. 2007b). From the ancient time, human and animal excreta were used for soil fertility. Composted excreta are used as an organic fertilizer. Solid and liquid wastes are good sources of carbon, nitrogen, phosphorus, potassium, magnesium, and other micronutrients (Kelleher et al. 2002). The cultivation of microalgae without added chemicals using excreta is gaining rapid attention. The recycling of carbon, nitrogen, and phosphorus from wastewater and excreta for growing algae makes this process economically effective (Miranda et al. 2015). This leads to significant reductions in the overall costs for biodiesel production (Zhou et al. 2011). The present investigation aims to use animal, bird, and human excreta as a source of nutrient for microalgae growth and lipid production.

Materials and methods

Isolation of microalgae and culture conditions

The *Chlorella singularis* strain UUIND5 (Gen Bank accession number KY745895) and *Micractinium pusillum* strain UUIND4 (Gen Bank accession number KY484922) isolated earlier by our group from fresh water were used in this study.

Apart from the above mentioned two microalgal species, one waste water novel microalgae was also isolated for this study. For this purpose, waste water samples were collected from the mess outlets of the Uttaranchal University Dehradun,

Responsible editor: Philippe Garrigues

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Uttarakhand, India. Further, Bold's Basal Medium (BBM) for the cultivation of all the three microalgae was prepared according to the composition given by Guarnieri et al. (2013). Isolation of the novel strain was done on Bold's Basal Medium (BBM) agar plate according to Tale et al. (2014). Isolated strain was identified using forward primer ITS1-TCCGTAGGTGAACCTGCGG and reverse primer ITS4-TCCTCCGCTTATTGATATGC. Taxonomic position of novel microalgae isolate *Chlorella sorokiniana* (UUIND6) was determined by constructing phylogenetic tree using MEGA 6 software (Tamura et al. 2013).

Preparation of animal and human excreta as a growth media

Excreta (poultry waste, cow dung, and urea) were obtained from villages nearby Dehradun. Human urea was obtained from university toilets. Poultry waste and cow dung leachates were prepared separately by adding 100 g of poultry waste/cow dung to 1 L of 60 mM H_2SO_4 in separate flasks. The mixture was stirred for 30 min and allowed to settle for 1 h. The supernatant was separated from the solids. Human urea and the separated supernatants were diluted to 5, 10, 15, and 20% in order to prepare excreta-based culture media for the cultivation of microalgae. Selected physicochemical characteristics of excreta are listed in Table 1. pH was maintained to 7. However, the aim of the present study was not to optimize the addition of macro, micronutrients to excreta but only to investigate the potential of excreta for the growth of microalgae. All cultures were incubated at 18 h light:6 h dark photoperiod (200 lmol photons m^{-2} s^{-1} illuminated externally) for 14 days. The temperature was maintained at 25 °C. The control cells were transferred into BBM and incubated under the same conditions as excreta culture.

Estimation of microalgae biomass and lipid accumulation

The microalgae growth was measured as optical density (OD) using a UV–vis spectrophotometer (UNICO model 2100 spectrophotometer). Samples were taken every 2 days for 14 days. The biomass yield was calculated according to the following equation:

$$\text{Cell dry weight (CDW) (g/L)} = 0.274\ \text{OD686 nm} + 0.002$$

The growth rate (g/L/day) was calculated according to equation

$$P = \frac{(\text{CDW}_x - \text{CDW}_1)}{t_x - t_1}$$

where

CDWx Cell dry weight at t_x time
CDW1 Cell dry weight at time t_1 (Kumar et al. 2017)

The lipid accumulation in microalgae cells was measured by nile red method (Arora et al. 2016).

Total lipid extraction

Total lipids were extracted from microalgae cells by modified method of Bligh and Dyer (1959). In brief chloroform–methanol was used in 1:2 ratio (*v*/v). The total lipids extracted were measured gravimetrically. Lipid productivity was measured by the following equation (Kumar et al. 2018a):

$$\text{Lipid yield}\% = \text{lipid content(g)}/\text{dry algae biomass(g)}$$
$$\text{Lipid productivity(mg/L/day)} = \text{Biomass productivity} \times \text{lipid yield}(\%)/100$$

Presence of triacylglycerols (TAGs) was confirmed on silica gel plate according to Kumar et al. (2018b) method.

Transesterification of algal lipid into biodiesel

Lipids were transesterified into biodiesel by methanolic sulfuric acid (6%) (Kumar et al. 2018c). The fatty acid methyl esters (FAMEs) were analyzed using gas chromatography-mass spectroscopy (GC-MS; Agilent technologies, USA) (Arora et al. 2016).

$$\text{Yield of biodiesel (wt\%)} = \frac{\text{obtained biodiesel weight (g)}}{\text{algal lipid (g)}} \times 100$$

Different properties of biodiesel were determined by empirical formulas given by Arora et al. (2016).

Determination of pigments, protein, and carbohydrate content

The growth performance of microalgae in different excreta medium was studied by chlorophyll estimation. Total chlorophyll was estimated by equations given by Lichtenthaler (1987). Total protein and carbohydrate contents were estimated by the Kumar et al. (2018a) method.

Statistical analysis

Each experiment was repeated three times ($n = 3$). These results have been reported as mean ± SD. The data was further validated using Graph Pad Prism software (version 6.0f) with probability $p < 0.05$.

Table 1 Physicochemical characterization of different excreta used for the preparation and for the cultivation of microalgae

Parameter	Human urine	Cow urine	Cow dung	Poultry waste
pH	6.1	8.1	7.8	7
Nitrogen (N, %)	8	17	0.7	1
Phosphorus (P, %)	1	2	0.2	0.6
Calcium (Ca, %)	0.20	0.34	0.50	0.83
Potassium (K, %)	2.4	3.0	0.9	0.9

Results and discussion

Isolation and identification of novel microalgae strain

The waste water microalgae isolated was identified as *Chlorella sorokiniana* UUIND6 (Gen Bank accession number KY780616). The phylogenetic analysis was done according to 18S rRNA *Chlorella* sp. sequence basis (Fig. 1).

Analysis of algal biomass and lipid productivity

In the present study, it was recorded that the maximum biomass productivity was attained in 20% PCSO (poultry—as growth medium for algae strain—*C. sorokiniana)* followed by 10% CDCSO (cow dung—as growth medium for algae strain—*C. sorokiniana*) > 10% HUCS (human urine—as growth medium for algae strain—*C. singularis*) > 10% CUCSO (cow urine—as growth medium for algae strain—*C. sorokiniana*) (Fig. 2 and Table 2). Novel isolated strain of *Chlorella sorokiniana* from waste water showed the maximum growth (140 ± 3.1 mg L^{-1} day^{-1}) in 20% poultry. Control—*C. sorokiniana* had the growth rate 174 ± 4.1 mg L^{-1} day^{-1}. Markou et al. (2016) have reported the maximum growth of *C. vulgaris* in 15 and 20% of poultry litter. Agwa and Abu (2014) have also reported the growth of *Chlorella* sp. in poultry waste. The maximum lipid productivity (45.5 ± 2.3 mg L^{-1} day^{-1}) was obtained in *Chlorella sorokiniana* (20% poultry waste).

In cow dung, all the strains were grown at 10 and 15%. *Chlorella sorokiniana* showed the maximum growth at 10% (120 ± 3.1 mg L^{-1} day^{-1}).

In human urea, *Chlorella sorokiniana* and *Chlorella singularis* at 5 and 10% showed the growth. The maximum growth (110 ± 2.1 mg L^{-1} day^{-1}) of *Chlorella singularis* was reported in 10% urine. Growth of all the strains were completely inhibited in absolute, 15 and 20% dilutions of human urea. In these dilutions, the cultures turned yellowish at 4th day and could not survive further. In contrast to our study, Tuantet et al. (2014) reported that *C. sorokiniana* was able to grow on raw urine (without any dilution). In our study, *Chlorella singularis* showed the maximum growth at 10% dilution factor. Similar findings have also been reported by Zhang et al. (2014) for *Chlorella sorokiniana* on human urine. Adamsson (2000) have used 2% human urine for *Scenedesmus acuminatus* microalgae cultivation. Jaatinen et al. (2015) have reported the cultivation of *Chlorella vulgaris* on diluted human urine with biomass productivity 0.73 g VSS L^{-1}. Piltz and Melkonian (2018) successfully recycled nutrients from human urine using microalgae with removal efficiencies for N and P of 13.1 and 94.1% and growth rate of 7.2 g dry weight m^{-2} day^{-1} respectively.

In 10% dilution of cow urea, growth was recorded in all microalgal cultures. Growth of all the strains was completely inhibited at 15 and 20% cow urine dilutions. In these dilutions, the cultures turned yellowish at day 4 and could not survive further. The maximum growth (100

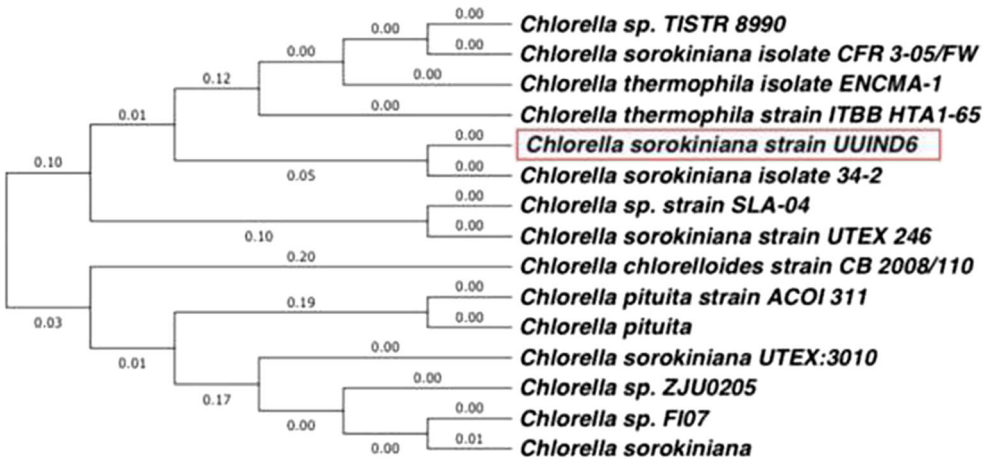
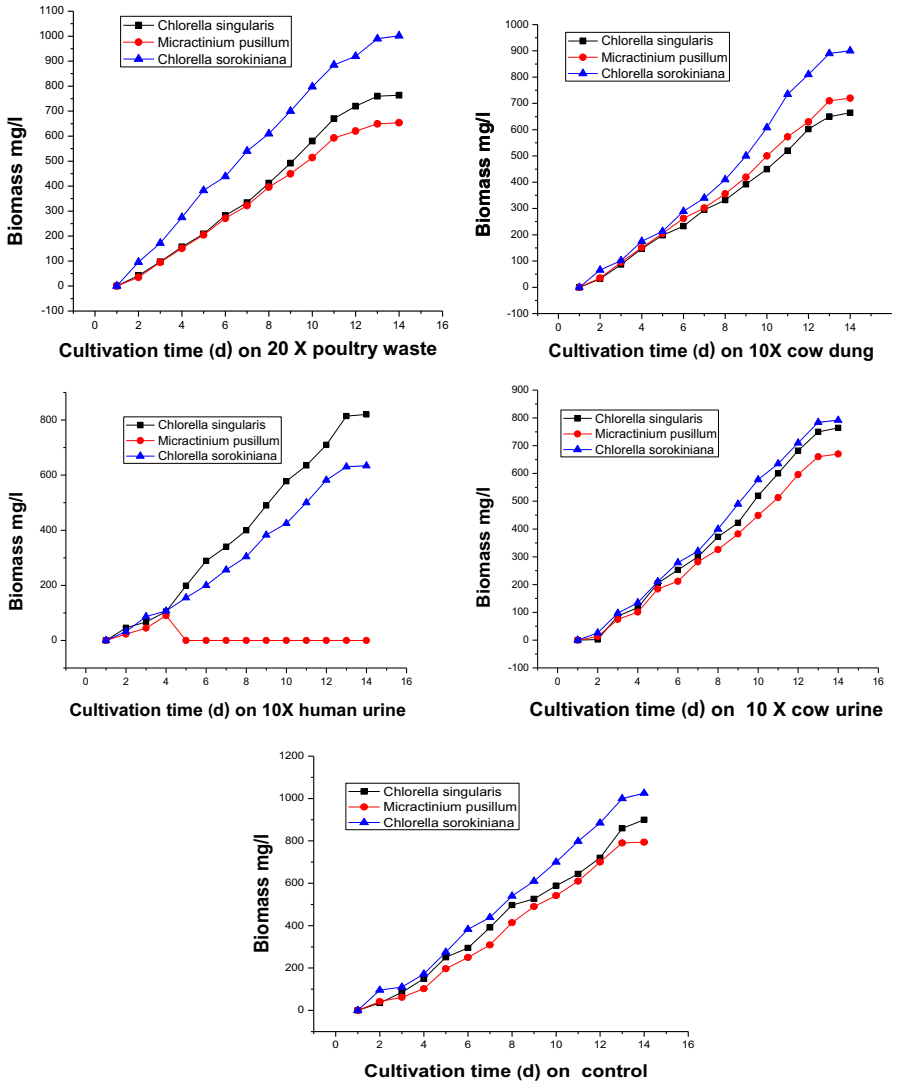
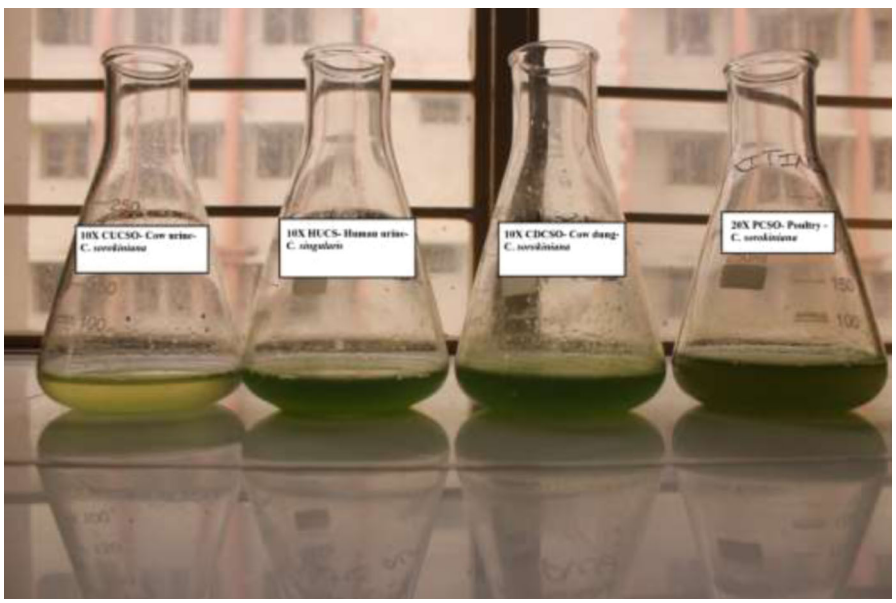


Fig. 1 Phylogenetic tree showing the relationships among partial 18S rRNA sequences of isolate *Chlorella sorokiniana* (UUIND6)


Chlorella singularis
Micractinium pusillum
Chlorella sorokiniana
Biomass mg/l
Cultivation time (d) on 20 X poultry waste
Cultivation time (d) on 10X cow dung
Cultivation time (d) on 10X human urine
Cultivation time (d) on 10 X cow urine
Cultivation time (d) on control

A


10X CUCSO- Cow urine-
C. sorokiniana
10X HUCS- Human urine-
C. singularis
10X CDCSO- Cow dung-
C. sorokiniana
20X PCSO- Poultry -
C. sorokiniana

B

◀ **Fig. 2** **a** Effect of animal, bird, and human excreta media on the growth of different micro algae cultures. **b** Cultures cultivated for 14 days at 20% PCSO (poultry—as growth medium for algae strain—*C. sorokiniana*), 10% CDCSO (cow dung—as growth medium for algae strain—*C. sorokiniana*), 10% HUCS (human urine—as growth medium for algae strain—*C. singularis*), and 10% CUCSO (cow urine—as growth medium for algae strain—*C. sorokiniana*). The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

± 1.2 mg L^{-1} day^{-1}) of *Chlorella sorokiniana* was reported in 10% urine dilution. Zhu et al. (2016) acquired the maximum biomass and lipid productivity of *Chlorella* sp. when cultivated on livestock waste compost. Tan et al. (2016) achieved 110.63–375.71 mg L^{-1} day^{-1} *Chlorella pyrenoidosa* biomass when cultured with the effluent of anaerobically digested activated sludge.

Significant differences were observed in growth rate between the excreta media and the control media. This could be due the lack of some of the nutrient constituents in excreta media (as no extra nutrients were added to the excreta media) that affected the growth of algal cells (Markou et al. 2016). In the present study, growth of all microalgae was not reported in all the concentrations of excreta media. This may be due to contamination with pathogens (Chojnacka 2007). These contaminants may be eliminated by sterilizing the excreta media (Block 2001). This will further lead to the increase in cost for microalgal biomass production (Noüe and Pauw 1988). According to the present findings, among the different excreta media used, poultry media was the best medium for cultivating microalgae. This media can thus be used for large scale cultivation of microalgae by adding extra nutrients. From this study, we have selected maximum biomass producing microalgae for further study viz, PCSO—poultry—*C. sorokiniana*, CDCSO—cow dung—*C. sorokiniana*, HUCS—human urine—*C. singularis*, CUCSO—cow urine—*C. sorokiniana*, and CCSO—control—*C. sorokiniana.*

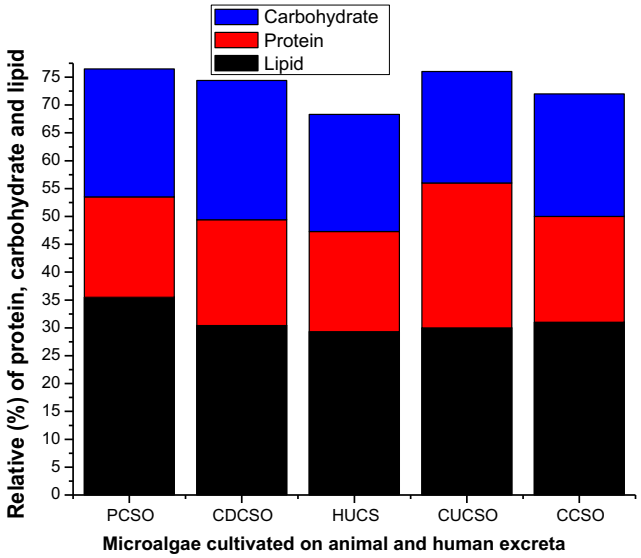


Fig. 3 Relative percentage of protein, carbohydrate, and lipid content of higher biomass microalgae cultivated on PCSO (poultry—as growth medium for algae strain—*C. sorokiniana*), CDCSO (cow dung—as growth medium for algae strain—*C. sorokiniana*), HUCS (human urine—as growth medium for algae strain—*C. singularis*), and CUCSO (cow urine—as growth medium for algae strain—*C. sorokiniana*). The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

Effect on pigments, protein, and carbohydrates

The effects of variation in biochemical composition of microalgae cultivated in various excreta media was

Table 2 Growth of different strains of microalgae at different concentrations of excreta media

Excreta concentration	*Chlorella sorokiniana*	*Chlorella singularis*	*Micractinium pusillum*
Poultry waste 5%	Growth	Growth	Growth
Poultry waste 10%	Growth	Growth	Growth
Poultry waste 15%	Growth	Growth	Growth
Poultry waste 20%	Growth	Growth	Growth
Cow dung 5%	Growth	Growth	No growth
Cow dung 10%	Growth	Growth	No growth
Cow dung 15%	Growth	Growth	Growth
Cow dung 20%	Growth	Growth	Growth
Human urine 5%	Growth	Growth	No growth
Human urine 10%	Growth	Growth	No growth
Human urine 15%	Growth	No growth	No growth
Human urine 20%	Growth	No growth	No growth
Cow urine 5%	Growth	Growth	Growth
Cow urine 10%	Growth	Growth	Growth
Cow urine 15%	Growth	No growth	No growth
Cow urine 20%	Growth	No growth	No growth

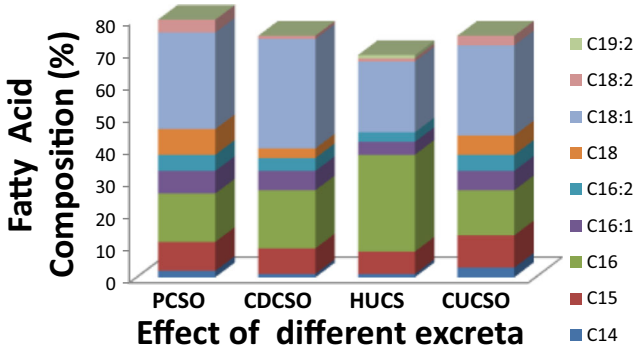


Fig. 4 Effect of animal and human excreta on fatty acid composition. PCSO (poultry —as growth medium for algae strain—*C. sorokiniana*), CDCSO (cow dung—as growth medium for algae strain—*C. sorokiniana*), HUCS (human urine—as growth medium for algae strain—*C. singularis*), and CUCSO (cow urine—as growth medium for algae strain—*C. sorokiniana*). The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

determined by analyzing pigments (Chl a, Chl b, carotenoids), carbohydrate, and protein content (Fig. 3 and Table 2). The experimental results showed that PCSO has low content of chlorophyll a, b, and (a + b); carotenoid; and protein content as compared to control. The pigment synthesis is associated with growth of microalgal cells which is further dependent on nitrogen and phosphorous (Lai et al. 2011). Increase in Carotenoids/Chl a + Chl b ratio indicates PS II depression (Pancha et al. 2014). The results of the present study indicated that lipid content (35.50% in PCSO and 31% in CCSO) and carbohydrates content (23% in PCSO and 22% in CCSO) were high as compared to the control. This could be due to decrease in protein synthesis and the excess carbon was converted into lipids and carbohydrates (Fan et al. 2014). Reduction in the photosynthesis causes an increase in NADH and acetyl CoA levels. This activates acetyl CoA carboxylase which converts acetyl CoA to malonyl CoA that leads to lipid accumulation (Praveenkumar et al. 2011). Markou et al. (2016) have also reported that protein and pigment content were lower in the poultry-based media than in the control, while carbohydrate and lipid content were higher in the poultry-based media. Increase in lipid during starvation of nitrogen was reported by Arora et al. (2016). Other excreta-based media showed less pigments and lipid contents as compared to control.

Physicochemical property analysis of fatty acid and biodiesel

TLC confirmed presence of TAGs in the total extracted lipids in PCSO—poultry—*C. sorokiniana*, CDCSO—cow dung—*C. sorokiniana*, HUCS—human urine—*C. singularis*, CUCSO—cow urine—*C. sorokiniana*, and CCSO—control—*C. sorokiniana*. The overall comparison of different excreta media used in this study affects the fatty acid profile to a large extent (Fig. 4). FAME (fatty acid methyl ester) analysis showed that palmitic acid (C16:0), 7, 10-hexadecadienoic acid methyl ester (C16:2), stearic acid (C18:0), and oleic acid (C18:1) were the major fatty acids (Table 3). Small amounts of myristic acid (C14:0) was present in PCSO. In HUCS, myristic acid (C14:0), linoleic acid (C18:2), and C19:2 were present in small amounts. In this study, oleic acid (C18:1) was present in high amount in PCSO (52%) and CDCSO (56%). Knothe (2005) reported that high amounts of oleic acid are good for oxidation stability and high cetane number of biodiesel.

Prediction of biodiesel properties

In the present study, it was recorded that PCSO 30%, CDCSO 29%, HUCS 21%, CUCSO 25%, and CCSO 30% of lipids were converted into biodiesel. FAMEs obtained from PCSO 30%, CDCSO, and CUCSO have good combustion properties similar to conventional diesel fuel. Important parameters of biodiesel are summarized in Table 4. CUCSO biodiesel showed the highest value of cetane 60 and specific gravity 0.700 kg^{-1}. Cold flow plugging property of the biodiesel derived from microalgae cultivated on different waste medium was higher as compared to commercial biodiesel.

Conclusions

All the strains of microalgae were not able to survive in all concentrations of diluted excreta media. The maximum

Table 3 Effect of different excreta on pigments

Excreta	Chl a* (μg/mL)	Chl b** (μg/mL)	Car*** (μg/mL)	Chl a + Chl b	Chl a/Chl b
Control	8.41 ± 0.04	2.40 ± 0.01	2.58 ± 0.03	10.81 ± 0.05	3.50 ± 0.03
PCSO	6.61 ± 0.02	1.07 ± 0.01	1.47 ± 0.02	7.68 ± 0.03	6.18 ± 0.01
CDCSO	4.31 ± 0.02	0.82 ± 0.03	0.94 ± 0.02	5.13 ± 0.02	5.25 ± 0.01
HUCS	1.91 ± 0.03	0.76 ± 0.03	1.03 ± 0.02	2.67 ± 0.04	2.51 ± 0.02
CUCSO	1.62 ± 0.03	0.59 ± 0.02	0.93 ± 0.04	2.21 ± 0.02	2.74 ± 0.02

* *Chl a*, Chlorophyll a; ** *Chl b*, Chlorophyll b; *** *Car*, Carotenoids

The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

Table 4 PCSO, CDCSO, HUCS, CUCSO, and CCSO biodiesel fuel properties

Properties	Units	Excreta					ASTM D6751	Commercial biodiesel
		CUCSO	PCSO	CDCSO	HUCS	CCSO		
Saponification value	(mg KOH)	115	110.16	101.82	90.26	112.10	–	
Iodine value	(g I_2/100 g)	38	39	37	30	40	–	130
Specific gravity	(kg^{-1})	0.671	0.623	0.700	0.639	0.650	–	–
Acid value, mg	KOH g^{-1}	2.4	2.2	2.2	3.2	3.0	0.8	0.50
Flash point	°C	43	42	40	38	42	93.0 min	35
Fire point	°C	51	51	48	42	54	–	–
Cetane value		57.03	53.32	60.63	50.19	51.01	47 min	47
High heating value		40.10	41.12	42.06	39.3 2	42.12		
Long chain saturation factor	(% wt)	14.7	12.3	10.6	15.6	14.3	–	–
Cold flow plugging property	°C	2.4	1.75	2.65	3.70	1.85	–	−5

PCSO (poultry—as growth medium for algae strain—*C. sorokiniana*), CDCSO (cow dung—as growth medium for algae strain—*C. sorokiniana*), HUCS (human urine—as growth medium for algae strain—*C. singularis*), CUCSO (cow urine—as growth medium for algae strain—*C. sorokiniana*). No standard limit designated by biodiesel standards

biomass productivity was attained in 20% PCSO followed by 10% CDCSO > 10% HUCS > 10% CUCSO. Thus, it can be concluded that among the different excreta media used for cultivation of microalgae, poultry media was the best media, which should be used for large scale cultivation of microalgae. Further study is needed to simulate or optimize the addition of extra nutrients and elimination of contaminants present in excreta which inhibit the growth of microalgae.

Acknowledgements We are thankful to MS. Neha Arora (IIT Roorki, UK, India) for GC-MS analysis.

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