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Enhancing starch accumulation/production in *Chlorococcum humicola* through sulphur limitation and 2,4- D treatment for butanol production

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ABSTRACT

Depleting fuel resources is a global concern worldwide due to the unstable and cost of fuel resources. Increased transportation has gradually depleted the fossil-based fuel resources leading to find a cost-effective, readily available, and renewable source. Considering these issues, various private and government organizations have focussed on producing bio-based fuels from natural sources. In this scenario, algae are a potential emerging source of feedstock or biomass for biobutanol production, which can effectively replace fossil fuels and their environmental drawbacks. The present study focussed on evaluating the potential of freshwater microalga *Chlorococcum humicola* isolated from temple pond as feedstock for biobutanol production using *Clostridium acetobutylicum*. The results indicated that *C. humicola* produced 846.33 $\mu g mg^{-1}$ of starch under full strength Chu10 medium. While under sulphur and phosphorus limitation, the accumulation of starch was 947.33 $\mu g\ mg^{-1}$ and 766.67 $\mu g mg^{-1}$, respectively. Also, *C. humicola* was exposed to different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D). At 10$\mu g ml^{-1}$ of 2,4-D, the highest starch concentration of 989$\mu g mg^{-1}$ was achieved in *C. humicola*. Finally, starch in *C. humicola* were hydrolysed and ABE fermentation was performed using *C. acetobutylicum* under anaerobic condition in a 5 L automated fermenter. After 72 h of fermentation, the fermented broth is analyed in Gas Chromatography showing the fermented product containing Acetone: Butanol: Ethanol. The present study is the first report on the production of biobutanol from *C. humicola* isolated from Temple pond. This study emphasizes the importance of local isolates of microalgae as a third-generation substrate to produce butanol to replace fossil-based fuels.

1. Introduction

Fast-growing transportation and industrialization have increased the current demand for fuel, and increased fuel price has given emergence to a major fuel crisis. Also, the growing environmental impact of burning fossil fuels has a significant effect and created the need for a better alternative [1,2]. Therefore, scientists are doing substantial research to find a replacement for fossil-based fuel. The focus is to find a sustainable, renewable, cheaper, and eco-friendly source to reduce dependency on fossil fuels [3,4]. Biofuels can be considered a potentially suitable alternative to fossil fuels, which can be more feasible for commercialization and utilization for transportation [5–7]. Production of biofuel will have a more significant impact on providing local employment and reduce CO_2 emission [8,9]. Biobutanol is one of the most standard and smoothly blending biofuels used for several industrial applications [10,11]. High energy density and the molecular similarity of biobutanol to gasoline make it convincingly superior to other biofuels like bioethanol or methanol. It has also used in the engine as fuels in pure form or blend with diesel [12,13]. Biobutanol has low vapor pressure and low volatility, making it easier for transportation and storage in metal pipelines and containers. Apart from their application as engine fuel, it is used as a solvent in food, pharmaceuticals, and paints, making them applicable in a variety of fields [10,14–16]. But still, the production of biobutanol remains a significant concern due to the cost and low production yield. Therefore, researchers are now focussing on utilizing biomass rich in carbohydrates to produce

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biobutanol [10,11]. Among the different biological substrates, microalgae are one of the most resourceful and readily available biomasses for biobutanol production.

Microalgae are a diverse group of simple eukaryotic and photosynthetic organisms dwelling in a wide range of environments [17–20]. It contains high value natural products for pharmaceuticals, food, fodder, and fuel. In addition to their high-value biomass and fast growth rate, microalgae can be cultivated in seawater as well as on non-arable land, and do not compete for resources with conventional agriculture [1,21]. Microalgae accumulate significant amounts of lipids and carbohydrates [22,23]. Besides protein, antioxidants, minerals, polyunsaturated fatty acids, and vitamins of commercial importance are also reported [24–26]. Considering the scope of renewable energy and the issue with environmental protection has led to the production of biofuel using microalgae [22,23,27,28]. Microalgae are the most efficient biomass producer accounting for 30−50% fixation of atmospheric CO_2 [29–32]. The act of photosynthesis not only provides food and energy to the other aquatic organisms but also replenishes the water with the much-needed oxygen to the microbes and the other aquatic forms to carry out the biochemical activities [33]. Reports suggest that microalgae are potential substrates for biobutanol production [12].

Generally, temple ponds are primary water sources used for general purpose and maintain water balance and a suitable microclimate. They primarily intended for rainwater harvesting; almost all the temples in Tamil Nadu and other adjacent states are been associated with ponds of various sizes. However, presently the importance of temple tanks is waning, and many of the temple ponds are been neglected, disused, or used as a garbage dump, and in some cases, children use the dry temple ponds as playgrounds. A brief survey of literature on phycological studies of this region revealed that only a single report is available on the microalgal diversity of two temple ponds in Puducherry [18]. The present study is an attempt to examine, record algal variety, and analyse the physicochemical composition of the pond water. The microalga *C. humicola* isolated from the temple pond is used for ABE fermentation studies using *C. acetobutylicum*. Nutrient starvation and 2,4- D treatment attempted to enhance the starch content of *C. humicola*. It is the first and foremost report on enhancement of starch in *C. humicola* isolated from temple ponds for biobutanol production.

2. Material and methodology

2.1. Chemicals and bacterial strain

All the chemicals used in the study were purchased from HiMedia (Mumbai, India) and Sigma Aldrich (St. Louise, USA). The bacterial strain *Clostridium acetobutylicum* 11,274 was obtained from the Microbial Type Culture Collection (MTCC). The *C. acetobutylicum* spores are maintained at 4 °C under anaerobic conditions. For the experiment, the spores are activated by growing for 12−24 h at 37°, and later it was heated at 70–80 °C for 10 min before sub culturing in Reinforced Clostridial Medium (RCM). The strain was then grown over nigh in RCM and used for the experiment.

2.2. Geographical locations of the sample collection site

Phytoplankton diversity analysis were performed out in three temple tanks located in Pondicherry, South India. The three temples tanks used in this study are Sitanandha Swamy temple (Pond1) [Lat: 11° 24′ 3.9780′′ N; Long: 79° 24′ 14.3964′′ E], Sri Krishna temple (Pond2) [Lat: 11° 52′ 18.7932′′ N; Long: 79° 42′ 48.2724′′ E], and Sri Sengazhuneer Amman Temple (Pond3) [Lat:11.8864 °N; Long: 79.7177 °E] within Pondicherry.

2.3. Collection of phytoplankton from the temple ponds

Phytoplankton samples from the three ponds was collected during the pre-monsoon season. Collected algal samples were filtered through mesh (42 and 200 μm size). Algae collected from substrates were preserved in clean polyethylene bags and bottles for transportation. Half of the sample (500 mL) were fixed in 4 % formalin diversity studies, while other half was used for isolation of microalgae. The samples were analysed under microscope and identified by morphological characteristics using standard monographs [34,35]. Images were recorded using a photomicroscopic system (Micros, Austria), and the diversity in each sample was analysed. The identified taxa were purified BG 11 medium, F/2 medium, and Chu 10 medium. The pure cultures were then deposited in the culture collection.

2.4. Physico-chemical analysis of the temple pond water

Physicochemical parameters like temperature, pH, ammonia, calcium, chloride, magnesium, nitrate, nitrite, carbonate, bicarbonate, sulphate, fluoride, total iron, and inorganic phosphate were analyzed using standard methods [36]. All the experiments were done in triplicates.

2.5. Isolation, identification, and cultivation of Chlorococcum humicola

The green alga, *C. humicola* was cultured and maintained in white photo-fluorescence light under 300 mE m^{-2} s^{-1} in 12/12 h light/dark photoperiod in Chu10 medium ($CaCl_2$ 0.25 g; K_2HPO_4 0.01 g; $MgSO_4.7H_2O$ 0.03 g; Na_2CO_3 0.02 g; Sodium silicate 0.04 g; Ferric citrate 3.5 mg and Citric acid 3.5 mg) at 24 °C. The axenic culture of *C. humicola* NRMCF0118 was submitted and maintained in DBT sponsored National Repository for Microalgae and Cyanobacteria – Freshwater (NRMC-F), Bharathidasan University, Tiruchirappalli, India.

2.6. Effect of phosphorus and sulphur stress on growth and starch accumulation in C. humicola

Influence of phosphorus (p) and sulphur (S) on growth and starch accumulation in *C. humicola* was determined in Chu 10 medium. To 5 ml of 10 days culture of *C. humicola* (1.6×10^5 cells mL^{-1}) was inoculated in full strength Chu 10 medium (Control) and two nutrients limited Chu10 media. For P limitation, KH_2PO_4 (source of phosphate) is replaced with KCl, and for S limiting medium $MgSO_4.7H_2O$ (source of sulphur) was replaced with $MgCl_2.6H_2O$ from Chu10 media.

2.7. Effect of 2, 4- D on the growth and starch accumulation in C. humicola

In Chu 10 medium, 1 mL (1.6×10^5 cells mL^{-1}) of *C. humicola* was inoculated in flasks containing different concentration of 2, 4- D (0, 10, 25, 50, 75, 100, 150 and 200 μgmL^{-1}). The growth rate of *C. humicola* were analysed at a 24 h interval for ten days using UV–vis Spectrometer at 520 nm. The cells were observed under the microscope at regular intervals for change in cell morphology and size.

2.8. Extraction and determination of Starch from C. humicola

The samples from the above 2 experiments were processed to determine the starch content control. About 2−5 mL of biomass was centrifuged at 3000 rpm for 5 min. The pigments was extracted using 4 mL ethanol (80 %) and incubated at 68 °C for

15 min and treated thrice with 3.3 mL of perchloric acid (30 %). Then stirred for 5 min at 25 °C for total starch hydrolysis. Then 0.5 mL of extract was cooled at 0 °C and starch was estimated using standard Anthrone method [37].

2.9. Pretreatment of C. humicola biomass

Dried biomass of *C. humicola* (5 % w/v) was pretreated in 1–5 % of H_2SO_4 at 100 °C for 20 min in a water bath. The slurry was centrifuged at 5000 rpm for 3 min. The supernatant was neutralized with $CaCO_3$ to adjust the pH to 6.5 –7.0. The supernatant was neutralized, filtered, and the filtrate was collected [38].

2.10. ABE Fermentation using Clostridium acetobutylicum - fermentation conditions

The RC medium were supplemented with 1 % of extracted starch and the fermentation were carried out in 250 mL bottles (working volume of 100 mL). Scale up of fermentation was performed in 5 L of benchtop fermenters (FERMENTEC, Korea) having 3 L of working capacity. To this, 10 % of bacterial inoculum was added, and the initial pH was set at 6.5 under 30 °C [39].

2.11. Analysis of biobutanol using gas chromatographic (GC)

A total of 10 mL fermented broth was collected, centrifuged, and filtered (0.2 μ membrane filter). Then 1 μL of filtrate is injected in GC (Shimadzu GC2014, Japan) with a Flame Ionization Detector (FID). The column used for biobutanol analysis is Rtx-5- Amines column (Restek, USA) (5 % diphenyl/ 95 % dimethyl polysiloxane). Temperature program was set as follows; Initial 60 °C for 1 min hold; ramp 10 °C/min to 70 with 1 min hold. The column flow was 22.2 mL/min. Instrument condition was as follows: nitrogen as carrier gas; FID set at 260 °C, the total run time for a single sample was 3 min.

3. Results and discussion

Temple ponds are shallow freshwater bodies, which undergo periodical drying in summer, increasing the salt concentrations and undergo dilution during monsoon. These varying salt concentrations affect the microalgal community qualitatively and quantitatively. The different groups of phytoplanktons identified from three temple ponds is recorded (Table 1). Around 57 species from 29 genera was recorded from three temple ponds. Nearly half of the population (50.8 %) was Chlorophycean members closely followed by Cyanophyceae (24.6 %), Bacillariophyceae (17.5 %), and Euglenophyceae (7 %). Among the three ponds, the SitanandhaSwamy temple (Pond 2) had a higher diversity of 44 species, and the Krishna Temple (Pond1) had the next higher species diversity of 35 species. Only 15 species were been identified in Sri Sengazhuneer Amman temple (Pond 3). The low diversity found in pond 3 may be due to intense predatory pressure from zooplanktons. The class Chlorophyceae was the dominant group, followed by Cyanophyceae, Bacillariophyceae, and Euglenophyceae. Recently Sharma et al. [27] also reported that Bacillariophyceae members were dominant in a freshwater pond. Qualitatively the phytoplankton composition showed marked variation in the three temple ponds. Only four species of Chlorophyceae, such as *Chlorella vulgaris, Chlorococcum humicola, Scenedesmus denticulatus,* and *Scenedesmus quadricauda* were common in all the ponds. Probably these four algae are adapted to thrive in the varying physicochemical conditions prevailing in these ponds. *C. humicola* was selected for further study. In the remaining three groups of algae (Cyanophyceae, Bacillariophyceae and Euglenophyceae) none of the algae were repetitive in three ponds. They were found either in one or two ponds only. The diverse species composition in the three ponds (Table 1) revealed that some algal species were found only in one pond and absent in other two ponds. That indicates the uniqueness of each pond regarding the physicochemical and environmental conditions of the three temple ponds. The seven species, such as *Chlorella* sp., *Cosmarium* sp., *Monoraphidium littorale, Pediastrum boryanum, Stigeocolonium* sp., *Chroococcus minor*, and *Gyrosigma acuminatum* is limited to Sri Krishna temple pond (Pond 1). Nearly 27 % of the species (n = 12) such as *Closterium* sp., *Kirchneriella* sp., *Pediastrum duplex, Scenedesmus acuminatus, Scenedesmus bijugatus, Selenastrum* sp., *Tetraedron minimum, Chroococcus turgidus, Phormidium* sp., *Nitzschia palea, Euglena viridis, Euglena spirogyra* and *Phacus longicauda* recorded in Pond 2 only. The species (n = 5; 20 %) like *Coelastrum microsporum, Pediastrum simplex, Microcystis aeruginosa, Craticula ambigua* and *Cyclotella meneghiniana* were found in Sri Sengazhuneer Amman temple pond only. In Euglenophyceae, *Euglena viridis, Euglena spirogyra, Phacus longicauda* and another species of *Phacus* were found in Pond 2, whereas only *Phacus longicauda* recorded in Pond 3 and none in Pond 1. Thus, only 3 groups of algae recorded in Pond 1 (Sri Krishna temple).

The physicochemical characteristics of water in the three ponds have listed in Table 2. The data shows a distinct variation in almost all the test parameters. The pH (potential hydrogen) of the three ponds was between 7.1–8.1 and is within the values stipulated by WHO (6.5−8.5). Neutral to alkaline pH support better growth of microalgae due to greater availability of nutrient elements [19]. The air and surface water temperatures were in the range of 28−34 °C; Pond 3 (Sri Sengazhuneer Amman Temple) recorded maximum temperature (air 34 °C & water 32 °C). Electrical conductivity (EC) was higher than the standard value in all the ponds. Higher EC indicates the occurrence of higher ionic content in the pond waters (Agbaire et al., 2015). The total dissolved substance (TDS) was higher in Pond 1 and Pond 3 than the permissible value. However, TDS in Pond 3 was slightly lesser (420 mg L^{-1}) than the standard amount (500 mg L^{-1}, WHO).

High turbidity will reduce light penetration into the water and thus oxygen production through photosynthesis. Turbidity was higher in all three ponds than the standard value. The higher phytoplankton diversity in pond 2 is correlated with relatively low turbidity than the other two ponds. Total hardness is a measure of the mineral content in a water sample that is equivalent to the total calcium and magnesium content. The total hardness in terms of calcium carbonate was below the standard value of 600 mg L^{-1}. Calcium and magnesium contents of the three ponds were higher than the values set by WHO. However, the magnesium level was lower than calcium in all the ponds. Magnesium is essential for the synthesis of chlorophyll and involved in numerous enzyme reactions as a valid activator. Chloride ions were lower than the WHO values (250 mg L^{-1}) in Pond 2 and 3, whereas higher in Pond 1. The presence of chlorine ions represents the availability of soluble salts, which regulates the salinity of the water. Nitrate, sulphate, iron and fluoride contents were present within the prescribed standards. While inorganic phosphate was higher, sulphite and nitrite were lesser than the standard concentration. Ammonia level was found almost equal to the standard in Pond 2, but higher in Pond 1 and 3. Heavy metals are standard components present in natural waters. The pond water tested in atomic absorption spectrophotometer for determining the heavy metal or microelements. The concentration of 7 metal elements including three heavy metals (silver, cadmium, and nickel) was recorded (Fig. 1). The concentration of all the 7 metals were within the prescribed standards of Environmental Protection Agency (EPA, USA). The results of this study showed the influence of biotic/abiotic factors on the diverse composition of phytoplanktons.

Table 1
Analysis of microalgal diversity in the three temple ponds.

S.No	Name of the organism	Family	Pond 1	Pond 2	Pond 3
Chlorophyceae					
1	*Ankistrodesmus fulcatus* (Corda) Ralfs	Selenastraceae	+	+	–
2	*Chlorella* sp.	Chlorococcaceae	+	–	–
3	*Chlorella vulgaris* (Beyerinck)		+	+	+
4	*Chlorococcum humicola* (Nägeli)		+	+	+
5	*Closterium* sp.		–	+	–
6	*Closterium* sp.		+	+	–
−7	*Closterium navicula*		+	+	–
8	*Coelastrum microsporum*		–	–	+
9	*Coelastrum* sp.		+	+	–
10	*Colestrella* sp.		+	+	–
11	*Cosmarium* sp.		+	–	–
12	*Kirchneriella schmidle*		+	+	–
13	*Kirchneriella* sp.		–	+	–
14	*Monoraphidium littorale*		+	–	–
15	*Pediastrum boryanum* (Turpin) Meneghini	Hydrodictyaceae	+	–	–
16	*Pediastrum duplex* (Meyen)		–	+	+
17	*Pediastrum simplex* (Meyen)		–	–	+
18	*Pediastrum tetras* (Ehrenberg) Ralfs		+	+	–
19	*Scenedesmus armatus*(Chodat)	Scenedesmaceae	+	+	–
20	*Scenedesmus acuminatus*		–	+	–
21	*Scenedesmus bijugatus*		–	+	–
22	*Scenedesmus denticulatus*		+	+	+
23	*Scenedesmus intermedius*		+	+	–
24	*Scenedesmus obliquus* (Turpin) Kützing		+	+	–
25	*Scenedesmus quadricauda* (Turpin) Brébisson		+	+	+
26	*Selenastrum* sp.		–	+	–
27	*Stigeocolonium* sp.	Chaetophoraceae	+	–	–
28	*Tetrastrum chodat*		+	+	–
29	*Tetraedron minimum* (Braun) Hansgirg	Hydrodictyaceae	–	+	–
Cyanophyceae					
30	*Calothrix linearis* (Gardner)	Rivulariaceae	–	+	–
31	*Calothrix* sp.		+	+	
32	*Chroococcus minor*	Chroococcaceae	+	–	–
33	*Chroococcus minutus* (Kutz) Nag		+	+	–
34	*Chroococcus turgidus* (Kutz) Nag		–	+	–
35	*Chroococcus* sp.		+	+	–
36	*Merismopedia* sp.		+	+	–
37	*Microcystis aeruginosa* (Kutz)	Microcystaceae	–	–	+
38	*Oscillatoria earlei*		+	+	–
39	*Oscillatoria formosa*(Gardner)		+	+	–
40	*Oscillatoria princeps*		–	+	+
41	*Oscillatoria subbrevis* (Schmidle)		+	+	–
42	*Oscillatoria tenuis* (Bory) ex Gom		+	–	+
43	*Phormidium* sp.		–	+	–
Bacillariophyceae					
44	*Craticula ambigua* (Ehrenberg) D.G. Mann	Stauroneidaceae	–	–	+
45	*Cyclotella meneghiniana* (Kützing)	Stephanodiscaceae	–	–	+
46	*Fragilaria intermedia*		+	+	–
47	*Gomphonema* sp.		+	+	–
48	*Nitzchia sigmoidea*		+	+	–
49	*Nitzschia palea*		–	+	–
50	*Pleurosigma elongatum*		+	+	–
51	*Pleurosigma spencerii*		+	+	–
52	*Rhopalodia gibberula*		–	+	+
53	*Gyrosigma acuminatum* (Kützing) Rabenhorst		+	–	–
Euglenophyceae					
54	*Euglena viridis* (O.F.Müller) Ehrenberg		–	+	–
55	*Euglena spirogyra*		–	+	–
56	*Phacus longicauda*		–	+	–
57	*Phacus* sp.		–	+	+

+ Present; – Absent.

All the samples were isolated in Chu10 media; overall, eight microalgae were isolated from the sample collection site and identified using standard monographs and published reports [34,35]. Among them based on the fast-growing and high biomass producing potential, *C. humicola* was selected for further study (results not shown) (Fig. 2). Microalgae under nutrient stress or starvation lead to change in normal physiological activities [40]. In microalgae, starch is the primary component produced by photosynthesis [41]. Accumulation of starch is induced by altering or stressing the macro elements like nitrogen, sulphur, and phosphorus [40]. To evaluate accumulation of starch, *C. humicola* was cultured in S and P limited Chu 10 medium. Compared to the control cell (Normal Chu10), both S and P limitation reduced growth. In the absence of phosphorus, the microalgae were not able

Table 2
Analysis of physicochemical parameters of water from different ponds using Standard APHA protocol.

Physicochemical parameters	Pond 1	Pond 2	Pond 3	EPA
pH (at 25 °C)	7.2	8.1	7.1	6.5–8.5
EC (at 25 °C) μS/cm	1550	570	1320	300
TDS (at 103 °C)	1321	420	940	500–2000
Turbidity (NTU)	32	15	19	10
Total hardness	360	190	250	200–600
Calcium	96	40	65	75–200
Magnesium	30	22	18	30–50
Chloride	430	135	227	250–1000
Nitrate	7.5	3.7	8.2	50
Sulphate	42	35	28	500
Fluoride	0.33	0.3	0.39	1–1.5
Total Iron	0.21	0.15	0.23	0.3
Inorganic Phosphate	6.1	2.1	4.8	0.005–0.2
Sulphite	<0.1	<0.1	<0.1	0–0.5
Nitrite	1.7	1.1	1.3	–
Ammonia	1.8	0.54	4.2	0.5

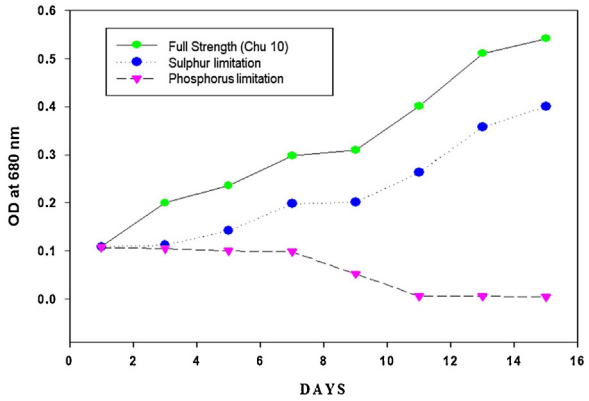

Fig. 3. Growth of *Chlorococcum humicola* NRCF0118 under Sulphur and phosphorus limitation in Chu10 medium.

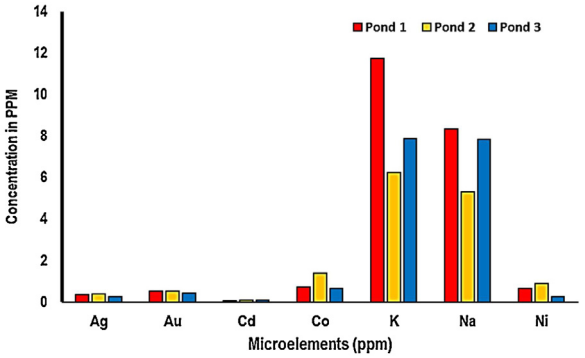

Fig. 1. Microelements concentration in the temple pond water.

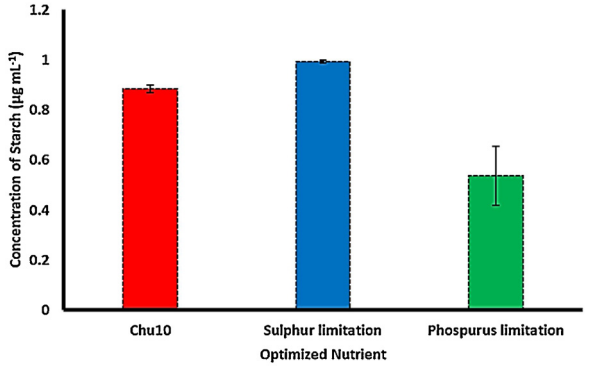

Fig. 4. Influence of sulphur and phosphorus limitation on starch accumulation in *Chlorococcum humicola* NRCF0118.

to multiply and the biomass increased after 7th day of inoculation. But under sulphur limitation the growth gradually increased (Fig. 3).

But in controversy to the growth of *C. humicola* under nutrient stress, the accumulation of starch was higher under sulphur limitation. The concentration of starch in *C. humicola* was 846.33 $\mu g mg^{-1}$, 947.33 $\mu g mg^{-1}$ and 766.67$\mu g mg^{-1}$ in Control, S and P limited media, respectively (Fig. 4). Zhang et al. (2002) suggest that *Chlamydomonas reinhardtii* undergoes cell size increase and 10-fold accumulation starch under sulphur deprivation [42]. Reports suggest that *C. vulgaris* cultured under sulphur limitation increased the starch content up to 60 % under laboratory scale and 50 % in the outdoor cultivation system [37]. Yao et al. (2012) also reported that *Tetraselmis* subcordiformis under sulphur stress increases the productivity of starch up to 0.62 g L^{-1} d^{-1} [43]. Thus, the results of the present study and already available reports suggest that *C. humicola* accumulate more starch under sulphur deprivation under phosphorus stress.

2,4-dichlorophenoxiacetic acid (2,4-D) is a synthetic herbicide employed in agriculture [44]. It is also a type of synthetic auxin, a class of plant hormones, which helps in stimulating growth, cell division, the formation of the leaf, biosynthesis of ethylene,

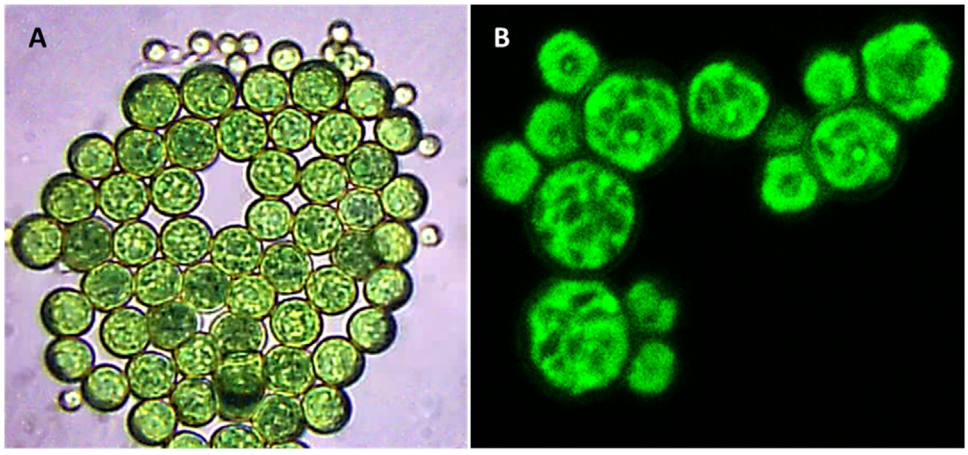

Fig. 2. Microphotograph of *Chlorococcum humicola* (A) Light Microscopic image (B) Confocal Microscopic image.

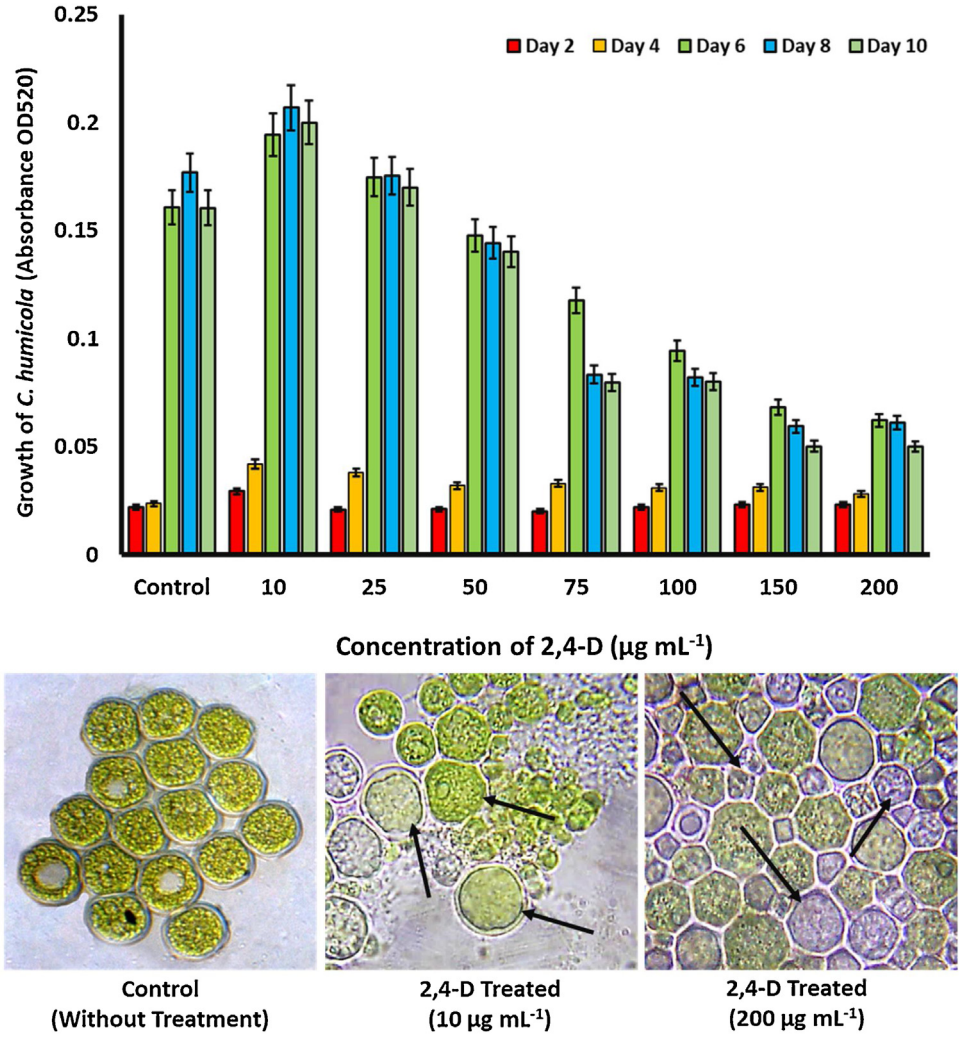

Fig. 5. Effect of different concentrations of 2,4 D on the growth rate of *Chlorococcum humicola* and microscopic analysis showing bleaching of chlorophyll at higher concentration.

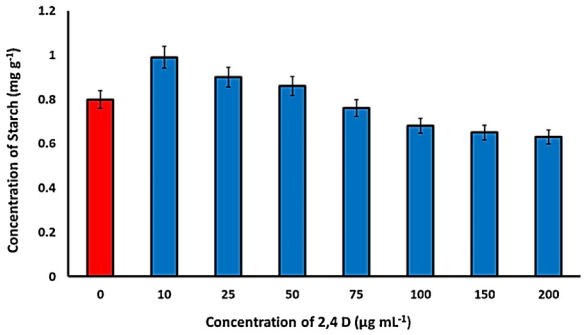

Fig. 6. Effect of 2,4-D on starch content of *Chlorococcum humicola* NRCF0118.

development of root, and fruiting in plants [45]. Based on the efficacy of 2, 4-D on the photosynthetic organism, *C. humicola* was subjected to different concentrations of 2,4-D to increase growth and starch productivity (Fig. 5). Compared to the Control, *C. humicola* cultured in 10 µg mL^{-1} showed higher growth rate in day 8. But a gradual decrease in growth was observed with an increasing concentration of 2, 4-D. At the highest level of 200 µg mL^{-1} of 2,4-D lowest growth rate was observed. Also, the level of starch content in *C. humicola* was measured, showing that starch productivity was directly proportional to the growth rate. At 10 µg mL^{-1} concentration of 2,4-D, *C.humicola* showed around 0.989 mg g^{-1} of starch at the end of day 10, while lowest starch content were recorded in cells grown at 200 µg mg^{-1} 2,4- D concentration (Fig. 6).

2,4-D at different concentrations (0, 0.5, 1.0, and 2.0 mg L^{-1}) increase the growth of Dunaliella salina and Haematococcus pluvialis [46]. Dao et al. (2018) recently reported that *Scenedesmus* sp. LX1 exposed to low dosage of 2,4-D increased growth rate and lipid productivity [47]. But this report agrees with the present work, stating the decrease in biomass with an increase in 2, 4-D concentration. Also, *Chlorococcum* sp showed better growth at a much lesser level of 2,4-D than the previously mentioned report. Therefore 2, 4-D treatment will decrease the risk of toxicity in the environment. Reports also suggest that after 96 h of exposure, the toxic concentration for 2,4-D was 1353.80 and 71.20 mg L^{-1} against algae and cyanobacteria. It also increased the pigments, macromolecule concentration, increased enzyme activity in *Microcystis aeruginosa* and *Ankistrodesmus falcatus*. It suggested that microalgae can survive a higher level of 2,4-D than cyanobacteria [47]. Thus, the present study goes in agreement with the previous report, which suggests that 2,4-D can potentially increase the biomass production of microalgae and it could be a good strategy for future biobutanol production strategies. The starch extracted

B. Retention time in GC analysis of butanol

Solvents	Retention Time (Min)
Acetone	1.45
Ethanol	1.85
Butanol	3.657

Fig. 7. (A) Production of butanol by anaerobic ABE fermentation of *C. humicola* hydrolysate in batch fermentation using *C. acetobutylicum* and (B) Retention time of Acetone, Butanol and Ethanol using GC analysis.

via the optimized condition mentioned above was exposed to batch fermentation using *Clostridium acetobutylicum*. After 5 days of fermentation as per standard protocol, the butanol was produced as per the ABE fermentation ratio (Fig. 7A & B) [48]. The ratio of biobutanol production during the fermentation shown in the result section. The present ABE fermentation protocol goes in agreement with the results reported in previous studies [48]. Thus, the present study gives a detailed note on the strategies to enhance starch content in *C. humicola* NRMCF0118 using different nutrient stress and induction of synthetic molecules for producing biobutanol.

4. Conclusion

Microalgae are important group of renewable, easily available and cost effective third generation feedstock to produce biofuels. The present study gives us the importance of *C. humicola* as source of starch for biobutanol production. Further growing *C. humicola* under nutrient stress (Sulphur and Phosphorus) and exposure to 2,4-D enhance starch accumulation. Even though under sulphur stress, the growth rate of *C. humicola* was decreased but the accumulation of starch was higher than full strength media (Chu10). Synthetic growth promoting molecule especially 2,4-D increased the growth rate at lower concentration (10 $\mu g\ mL^{-1}$) showed an increase in biomass. Still, at higher concentrations (200 $\mu g\ mL^{-1}$), it became toxic to the microalgae and resulted in bleaching of chlorophyll. Combining the nutrient stress strategy with limited concentration of 2, 4-D will directly influence the growth and starch accumulation in *C. humicola*. Therefore, the present study is more focussed in increasing the concentration of starch in *C. humicola* by applying varies chemical stress to produce cheaper and higher mass of starch for biobutanol production. This is the first report on increasing the biomass using 2,4-D and starch accumulation by nutrient stress in microalgae isolated from temple ponds for biobutanol production.

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Ganesan Narchonai: Conceptualization, Methodology, Writing - original draft. **Chitirai Arutselvan:** Conceptualization, Methodology, Writing - original draft. **Felix LewisOscar:** Investigation, Writing - original draft. **Nooruddin Thajuddin:** Supervision, Project administration, Writing - review & editing.

Declaration of Competing Interest

There is no conflict of interest for this manuscript

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References

[1] A. Pugazhendhi, S. Shobana, P. Bakonyi, N. Nemestóthy, A. Xia, G. Kumar, A review on chemical mechanism of microalgae flocculation via polymers, Biotechnol. Rep. 21 (2019)e00302.
[2] T. Mathimani, A. Pugazhendhi, Utilization of algae for biofuel, bio-products and bio-remediation, Biocatal. Agric. Biotechnol. 17 (2019) 326–330.
[3] S. Anto, S.S. Mukherjee, R. Muthappa, T. Mathimani, G. Deviram, S.S. Kumar, T. N. Verma, A. Pugazhendhi, Algae as green energy reserve: technological outlook on biofuel production, Chemosphere 242 (2020)125079.
[4] J.M. Jacob, R. Ravindran, M. Narayanan, S.M. Samuel, A. Pugazhendhi, G. Kumar, Microalgae: A prospective low cost green alternative for nanoparticle synthesis, Curr. Opin. Environ. Sci. Health (2020).
[5] G. Kumar, T. Mathimani, E.R. Rene, A. Pugazhendhi, Application of nanotechnology in dark fermentation for enhanced biohydrogen production using inorganic nanoparticles, Int. J. Hydrogen Energy (2019).
[6] A.P. Saravanan, T. Mathimani, G. Deviram, K. Rajendran, A. Pugazhendhi, Biofuel policy in India: a review of policy barriers in sustainable marketing of biofuel, J. Clean. Prod. 193 (2018) 734–747.
[7] A.P. Saravanan, A. Pugazhendhi, T. Mathimani, A comprehensive assessment of biofuel policies in the BRICS nations: implementation, blending target and gaps, Fuel 272 (2020)117635.
[8] J. Sharma, S.S. Kumar, V. Kumar, S.K. Malyan, T. Mathimani, N.R. Bishnoi, A. Pugazhendhi, Upgrading of microalgal consortia with CO2 from fermentation of wheat straw for the phycoremediation of domestic wastewater, Bioresour. Technol. 305 (2020)123063.
[9] S. Shanmugam, T. Mathimani, S. Anto, M. Sudhakar, S.S. Kumar, A. Pugazhendhi, Cell density, Lipidomic profile, and fatty acid characterization as selection criteria in bioprospecting of microalgae and cyanobacterium for biodiesel production, Bioresour. Technol. 304 (2020)123061.
[10] A. Pugazhendhi, T. Mathimani, S. Varjani, E.R. Rene, G. Kumar, S.-H. Kim, V.K. Ponnusamy, J.-J. Yoon, Biobutanol as a promising liquid fuel for the future-recent updates and perspectives, Fuel 253 (2019) 637–646.
[11] J. Dharmaraja, S. Shobana, S. Arvindnarayan, M. Vadivel, A. Atabani, A. Pugazhendhi, G. Kumar, Biobutanol from lignocellulosic biomass: bioprocess strategies, Lignocellulosic Biomass to Liquid Biofuels, Elsevier, 2020, pp. 169–193.
[12] U. Rajak, P. Nashine, T.N. Verma, A. Pugazhendhi, Performance and emission analysis of a diesel engine using hydrogen enriched n-butanol, diethyl ester and Spirulina microalgae biodiesel, Fuel 271 (2020)117645.
[13] P. Shrivastava, T.N. Verma, O.D. Samuel, A. Pugazhendhi, An experimental investigation on engine characteristics, cost and energy analysis of CI engine fuelled with Roselle, Karanja biodiesel and its blends, Fuel 275 (2020)117891.
[14] U. Rajak, P. Nashine, T.N. Verma, A. Pugazhendhi, Alternating the environmental benefits of Aegle-diesel blends used in compression ignition, Fuel 256 (2019)115835.
[15] U. Rajak, P. Nashine, T.N. Verma, A. Pugazhendhi, Performance, combustion and emission analysis of microalgae Spirulina in a common rail direct injection diesel engine, Fuel 255 (2019)115855.
[16] P. Shrivastava, T.N. Verma, A. Pugazhendhi, An experimental evaluation of engine performance and emisssion characteristics of CI engine operated with Roselle and Karanja biodiesel, Fuel 254 (2019)115652.
[17] F.L. Oscar, D. Bakkiyaraj, C. Nithya, N. Thajuddin, Deciphering the diversity of microalgal bloom in wastewater-an attempt to construct potential consortia for bioremediation, J. Cur. Per. In Appl. Microbiol 2278 (2014) 92.
[18] G. Narchonai, C. Arutselvan, F. LewisOscar, N. Thajuddin, Deciphering the microalgal diversity and water quality assessment of two urban temple ponds in Pondicherry, India, Biocatal. Agric. Biotechnol. 22 (2019)101427.

[19] J. Sharma, S.S. Kumar, N.R. Bishnoi, A. Pugazhendhi, Screening and enrichment of high lipid producing microalgal consortia, J. Photochem. Photobiol. B, Biol. 192 (2019) 8–12.
[20] B.R. Kumar, G. Deviram, T. Mathimani, P.A. Duc, A. Pugazhendhi, Microalgae as rich source of polyunsaturated fatty acids, Biocatal. Agric. Biotechnol. 17 (2019) 583–588.
[21] R. Praveenkumar, K. Shameera, G. Mahalakshmi, M.A. Akbarsha, N. Thajuddin, Influence of nutrient deprivations on lipid accumulation in a dominant indigenous microalga Chlorella sp., BUM11008: evaluation for biodiesel production, Biomass Bioenergy 37 (2012) 60–66.
[22] N.T.L. Chi, P.A. Duc, T. Mathimani, A. Pugazhendhi, Evaluating the potential of green alga Chlorella sp. For high biomass and lipid production in biodiesel viewpoint, Biocatal. Agric. Biotechnol. 17 (2019) 184–188.
[23] J. Sharma, S.S. Kumar, N.R. Bishnoi, A. Pugazhendhi, Enhancement of lipid production from algal biomass through various growth parameters, J. Mol. Liq. 269 (2018) 712–720.
[24] A. Pugazhendhi, D. Prabakar, J.M. Jacob, I. Karuppusamy, R.G. Saratale, Synthesis and characterization of silver nanoparticles using Gelidium amansii and its antimicrobial property against various pathogenic bacteria, Microb. Pathog. 114 (2018) 41–45.
[25] A.A.P. Ahamed, M.U. Rasheed, K.P.M. Noorani, N. Reehana, S. Santhoshkumar, Y. M.M. Imran, N.S. Alharbi, C. Arunachalam, S.A. Alharbi, M.A. Akbarsha, In vitro antibacterial activity of MGDG-palmitoyl from Oscillatoria acuminata NTAPC05 against extended-spectrum β-lactamase producers, J. Antibiot. 70 (2017) 754–762.
[26] F. LewisOscar, C. Nithya, S.A. Alharbi, N.S. Alharbi, N. Thajuddin, In vitro and in silico attenuation of quorum sensing mediated pathogenicity in Pseudomonas aeruginosa using Spirulina platensis, Microb. Pathog. 116 (2018) 246–256.
[27] J. Sharma, V. Kumar, S.S. Kumar, S.K. Malyan, T. Mathimani, N.R. Bishnoi, A. Pugazhendhi, Microalgal consortia for municipal wastewater treatment–Lipid augmentation and fatty acid profiling for biodiesel production, J. Photochem. Photobiol. B, Biol. 202 (2020)111638.
[28] S. Mona, S.S. Kumar, V. Kumar, K. Parveen, N. Saini, B. Deepak, A. Pugazhendhi, Green technology for sustainable biohydrogen production (waste to energy): a review, Sci. Total Environ. (2020)138481.
[29] T. Mathimani, A. Baldinelli, K. Rajendran, D. Prabakar, M. Matheswaran, R.P. Van Leeuwen, A. Pugazhendhi, Review on cultivation and thermochemical conversion of microalgae to fuels and chemicals: process evaluation and knowledge gaps, J. Clean. Prod. 208 (2019) 1053–1064.
[30] S.-H. Kim, A. Mudhoo, A. Pugazhendhi, R.G. Saratale, D. Surroop, P. Jeetah, J.-H. Park, G.D. Saratale, G. Kumar, A perspective on galactose-based fermentative hydrogen production from macroalgal biomass: trends and opportunities, Bioresour. Technol. (2019).
[31] E. Baldev, D. Mubarakali, K. Saravanakumar, C. Arutselvan, N.S. Alharbi, S.A. Alharbi, V. Sivasubramanian, N. Thajuddin, Unveiling algal cultivation using raceway ponds for biodiesel production and its quality assessment, Renew. Energy 123 (2018) 486–498.
[32] E. Baldev, D. MubarakAli, M. Dhivya, M. Kanimozhi, T. Shakena-Fathima, N.S. Alharbi, C. Arunachalam, S.A. Alharbi, N. Thajuddin, Facile and novel strategy for methods of extraction of biofuel grade lipids from microalgae-an experimental report, Int. J. Biotechnol. Wellness Ind. 3 (2015) 121–127.
[33] N. Thajuddin, G. Subramanian, Cyanobacterial biodiversity and potential applications in biotechnology, Curr. Sci. (2005) 47–57.
[34] T.V. Desikachary, Cyanophyta, Indian Council of Agricultural Research New Delhi, 1959.
[35] G.W. Prescott, Algae of the Western Great Lakes Area: with an Illustrated Key to the Genra of Desmids and Freshwater Diatoms, WC Brown, 1962.
[36] A.P.H. Association, A.W.W. Association, W.P.C. Federation, W.E. Federation, Standard Methods for the Examination of Water and Wastewater, American Public Health Association, 1920.
[37] I. Brányiková, B. Maršálková, J. Doucha, T. Brányik, K. Bišová, V. Zachleder, M. Vítová, Microalgae—novel highly efficient starch producers, Biotechnol. Bioeng. 108 (2011) 766–776.
[38] C.K. Phwan, K.W. Chew, A.H. Sebayang, H.C. Ong, T.C. Ling, M.A. Malek, Y.-C. Ho, P.L. Show, Effects of acids pre-treatment on the microbial fermentation process for bioethanol production from microalgae, Biotechnol. Biofuels 12 (2019) 191.
[39] N.K.N. Al-Shorgani, H. Shukor, P. Abdeshahian, M.S. Kalil, W.M.W. Yusoff, A.A. Hamid, Enhanced butanol production by optimization of medium parameters using Clostridium acetobutylicum YM1, Saudi J. Biol. Sci. 25 (2018) 1308–1321.
[40] G. Markou, D. Vandamme, K. Muylaert, Microalgal and cyanobacterial cultivation: the supply of nutrients, Water Res. 65 (2014) 186–202.
[41] Y. Li, D. Han, M. Sommerfeld, Q. Hu, Photosynthetic carbon partitioning and lipid production in the oleaginous microalga Pseudochlorococcum sp. (Chlorophyceae) under nitrogen-limited conditions, Bioresour. Technol. 102 (2011) 123–129.
[42] L. Zhang, T. Happe, A. Melis, Biochemical and morphological characterization of sulfur-deprived and H 2-producing Chlamydomonas reinhardtii (green alga), Planta 214 (2002) 552–561.
[43] C. Yao, J. Ai, X. Cao, S. Xue, W. Zhang, Enhancing starch production of a marine green microalga Tetraselmis subcordiformis through nutrient limitation, Bioresour. Technol. 118 (2012) 438–444.
[44] F.A. Chinalia, M.H. Regali-Seleghin, E.M. Correa, 2, 4-D toxicity: cause, effect and control, Terrest. Aqua. Environ. Toxicol. 1 (2007) 24–33.
[45] C. Finet, Y. Jaillais, Auxology: when auxin meets plant evo-devo, Dev. Biol. 369 (2012) 19–31.
[46] M.F. de Jesus Raposo, R.M.S.C. de Morais, Influence of the growth regulators kinetin and 2, 4-D on the growth of two chlorophyte microalgae, Haematococcus pluvialis and Dunaliella salina, J. Basic Appl. Sci. 9 (2013) 302–308.
[47] G.-H. Dao, G.-X. Wu, X.-X. Wang, L.-L. Zhuang, T.-Y. Zhang, H.-Y. Hu, Enhanced growth and fatty acid accumulation of microalgae Scenedesmus sp. LX1 by two types of auxin, Bioresour. Technol. 247 (2018) 561–567.
[48] Y. Wang, W. Guo, C.-L. Cheng, S.-H. Ho, J.-S. Chang, N. Ren, Enhancing bio-butanol production from biomass of Chlorella vulgaris JSC-6 with sequential alkali pretreatment and acid hydrolysis, Bioresour. Technol. 200 (2016) 557–564.