Desalination and Water Treatment
www.deswater.com

doi:10.5004/dwt.2018.23040

131 (2018) 187–193
November

Ultrasound intensified saccharification of *Chlorella vulgaris* isolated from municipal wastewater

T. Sivakumar[a], P. Senthil Kumar[b,c,*]

[a]*Department of Chemical Engineering, Coimbatore Institute of Technology, Civil aerodrome post, Coimbatore-641014, India, Tel. +919791546900, email: sivakumarthangavelu1@gmail.com (T. Sivakumar)*

[b]*Department of Chemical Engineering, SSN College of Engineering, Kalavakkam, Chennai – 603110, India, Tel. +919884823425, email: senthilchem8582@gmail.com (P. Senthil Kumar)*

[c]*SSN-Centre for Radiation, Environmental Science and Technology (SSN-CREST), SSN College of Engineering, Chennai 603110, India*

Received 5 June 2018; Accepted 27 August 2018

ABSTRACT

This study assesses the potential of *Chlorella vulgaris* to exploit the nutrients in the municipal wastewater and yield abundant biomass for biofuel production, sequestering CO_2 in a photo-bioreactor. During the cultivation, about 78.4% of COD, 91% nitrogen and 92.9% phosphate were removed resulting a good biomass concentration of 3.6 g/L of which the carbohydrate content accumulated was about 60%. Saccharification was carried with mild acid or mild alkali with and without application of ultrasound. On assessment, the ultrasound assisted mild acid saccharification (at 8% v/v) resulted the highest sugar yield of 153 mg/g dry biomass. The hydrolysed biomass was fermented using *Saccharomyces cerevisiae*, to yield maximum concentration of 68.8 mg of bioethanol/g of algae in 72 h. These findings suggest that municipal wastewater with sufficient nutrients supplement can be directly used for mass cultivation of micro algae, which is an essential source of carbohydrate for bioethanol production.

Keywords: Chlorella vulgaris; Municipal wastewater; Sequestration; Carbohydrate; Ultrasound

1. Introduction

The fast growth of the world population and rapid development of a number of developing economies have both led to piercing increase in global energy consumption [1]. Energy consumption is unavoidable with the majority currently derived from fossil fuels and it is projected to increase by 56% by 2040 [2]. Among the 88% of the carbon based non-renewable fuels provided for the world energy demand, 55.2% of it are used by transport sector [3]. Thus many countries are turning their attention to the development of new alternative fuels which could help replace petroleum-diesel. And among the renewable energy alternatives, biofuels are considered the most promising liquid fuel source [4,5].

Micro algae have lately been considered as a predominant feedstock of third generation biofuel production mainly due to its capability to grow faster by utilizing wastewater and consuming carbon dioxide or flue gas in non-arable land. Though biodiesel being a major product from micro algae containing high lipid content [6], interest in bioethanol has recently increased due to carbohydrate rich micro algal biomass [7]. Moreover, the carbohydrates of micro algae are mainly in the form of starch and cellulose which is much easier to convert to monosaccharides or sugar when compared to lignocellulosic materials (the second generation bioethanol feedstock) [8,9].

Micro algae also play a pivotal role in the sequestrations of CO_2 in order to reduce the impact of CO_2 on global warming. Micro algae utilizes around 1.83 kg of CO_2 for every kilogram of algal dry cell weight which is annually around 54.9–67.7 tonnes of CO_2 that can be sequestered correspond-

*Corresponding author.

ing to annual dry weight biomass production rate of 30–37 tonnes per hectare [10]. Latest developments have been made to the design and operation of photo bio-reactors to attain high-efficient CO_2 capture and microalgae yield [11,12]. Thus, micro algae would be conceivable solution for the treatment of wastewater in an environmentally safe manner and economic, a part of their prospective to be used as feedstock for biofuels and sequestration of CO_2.

Micro algal cultivation generally requires organic and inorganic nutrients and carbon dioxide in the presence of sunlight through photosynthesis. Since wastewater consists of both this nutritional supplement, micro algae can be cultivated in it and can help in bioremedy of wastewater. Use of micro algae for nutrient removal was first demonstrated by Oswald et al. [13], and had been shown to efficiently utilize the nutrient source of wastewater [14,15]. In earlier studies, a selection of waste waters including agricultural, municipal and industrial sewage were used to study nutrient (nitrogen and phosphorus) removal by micro algae cultivation [16]. Diverse types of microalgae such as *Chlorella vulgaris*, *Botryo coccus braunii*, *Scenedesmus obliquus*, *Chlamydomonas reinhardtii* and *Spirulina maxima* have been used for autotrophic, heterotrophic and mixotrophic cultivation in sewage [17], and said to have high carbohydrate content (>40% of the dry weight) [7]. Among these species, *C. vulgaris* known as one of the wildest growing micro algae was found to readily uptake the nutrients from the wastewater and with carbohydrates being 37–55% of its dry weight [18].

The starch in chloroplasts and cellulose/polysaccharides on cell wall are the main source of carbohydrate in green algae [19], which are not freely fermentable for the production of ethanol. Hence the polysaccharides of micro algae should be hydrolysed to fermentable sugars prior to ethanol fermentation [20]. Overall, the chemical (acid and alkaline) and enzymatic hydrolysis are the two common hydrolysis methods used for this purpose. The acid hydrolysis is the most commonly used method since it is faster, easier and inexpensive [21] than the enzymatic method which is slower and much expensive being a benign process [22].

Ultrasound is generally used to disrupt the micro algae cells at relatively low temperature by generating intense sonic pressure waves in the liquid medium. Moreover, this method does not require chemicals or beads during the process, which helps to reduce the cost. Under the specific condition, the sonic pressure waves generate a series of micro bubbles cavitation, which results in the formation of shock wave with sufficient kinetic energy to rupture the rigid cell, envelops of micro algae and thus facilitate the distribution of lipids and carbohydrates into the exocellular medium. Furthermore, performing the acid hydrolysis with ultrasound assistance can accelerate the process with much efficiency at shorter time. Ultrasonic-assisted simultaneous saccharification and fermentation of pre-treated oil palm fronds for bioethanol production has been carried out effectively [23].

In this study, the pre-treated municipal wastewater was used to cultivate *Chlorella vulgaris* in an aerated photo bioreactor, treating the wastewater and sequestering CO_2. The final composition of the wastewater and the carbohydrate content of the micro algae in percentage of its dry weight were determined. The effects of various hydrolysis methods (Mild acid or mild alkali with and without sonication) and conditions on ultrasound-assisted saccharification of the micro algal biomass were investigated. The sugar yield obtained by various hydrolysis processes was compared.

2. Materials and methods

2.1. Micro algae strain and culture

Chlorella vulgaris was isolated from a nearby water body in Chennai as per standard procedure [24]. Prior to the growth experiments in wastewater, the strain was pre-cultured in BG-11 medium [25]. Composition of the medium (per litre) is: $NaNO_3$ 1.5 g, K_2HPO_4 0.04 g, $MgSO_4$ 0.075 g, $CaCl_2$ 0.036 g, $C_6H_8O_7$ 0.006 g, and 1 ml trace metal mix (H_3BO_3 2.86 g, MnO_2 1.81 g and $Co(NO_3)_2$ 0.049 g/L). The strain was maintained in BG-11 agar plates (BG-11 with 15 g/L agar) between the experiments. The micro alga was cultivated in a 1-L glass vessel containing 800 mL BG11 medium, at a light intensity of 2000 lux using fluorescent lamps with 0.2 vvm CO_2 (2.5%). The culture was incubated for 15 days at room temperature 27 ± 3°C. Micro algal biomass from the pre-cultures was centrifuged and the developed biomass was used as inoculum to minimize transfer of nutrients from pre-culture medium to the wastewater used in the micro algal growth experiments.

2.2. Municipal wastewater

The raw municipal wastewater used in this study was taken from the Chennai wastewater treatment plant (Perungudi). The raw municipal wastewater was first centrifuged to remove insoluble solids. The supernatant was then filtered through 0.45 µm cellulose membranes to remove the suspended solids, and was then diluted for 5-fold with distilled water before being sterilized (autoclaved at 121°C for 20 min). The characteristics of the raw and pre-treated diluted municipal wastewater are summarized in Table 1. The original municipal wastewater had a high pH value of about 8.95. The initial pH of the pre-treated diluted municipal wastewater was adjusted to around 7.5 with 1 N NaOH prior to its use for micro algae cultivation. There was no pH control during the cultivation of micro algae. Since the COD values are bit higher in the raw wastewater, it inhibited the growth of algae when used as raw wastewater. To avoid the toxicity, the wastewater was pretreated and suitably diluted.

Table 1
Characteristic of municipal wastewater used in this study

Characteristics	Raw wastewater	Pre-treated diluted wastewater
COD (mg L^{-1})	2250	510
NH_3-N (mg L^{-1})	1235	313
NO_3-N (mg L^{-1})	14.6	4.2
PO_4-P (mg L^{-1})	12.9	3.8

2.3. Cultivation of algae in wastewater medium

The micro alga was cultivated in a 5-L transparent polypropylene photo bio-reactor illuminated by external white fluorescent lamps mounted on both sides of the photo bioreactor at a light intensity of 200 W/m and 0.2 vvm CO_2 (2.5%) was supplied continuously. The photo bioreactor used in this study were 60 cm length, had an outer diameter of 11 cm with 0.3 cm thickness. Pretreated and diluted wastewater was used as growing medium. Biomass was centrifuged at 6000 rpm for 10 min, washed with distilled water and dried in an oven to a constant weight at 80°C. The biomass was stored at 4°C for further use. The supernatants after centrifugation was collected and analysed for nitrogen of nitrite, nitrate and ammoniacal nitrogen and total phosphorous.

2.4. Saccharification of algal biomass

Algal biomass is taken for hydrolysis by mild acid or mild alkali with and without sonication. Due to the lack of lignin and the simple cellular structure of micro algae, only mild reactions are required for simultaneous pretreatment to release carbohydrate from the inner cell wall, and hydrolysis reaction to hydrolyse the complex carbohydrate molecules to simple fermentable sugars [26].

2.4.1. Acid hydrolysis

Acid hydrolysis of micro algal biomass was carried out using H_2SO_4 at concentration of 2, 4, 6, 8, 10 and 12% (v/v) at 90°C for 30 min. The reaction time of 30 min at 90°C is sufficient since that increasing the reaction temperature and reaction time beyond the optimum point could predominantly degrade the carbohydrate, resulting in the reduction of the bioethanol yield [27]. The assays were carried out in 250 mL Erlenmeyer flasks. The acid pre-treatments were performed using 100 mL of micro algal biomass at a concentration of 30 g volatile suspended solids (VSS)/L.

2.4.2. Alkaline hydrolysis

For the alkaline hydrolysis, the biomass is suspended in 100 mL of NaOH, 1 M–6 M, to set a final concentration of 30 g VSS/L. Afterwards, samples were incubated at 90°C for 30 min with constant agitation with magnetic stirrer at 60 rpm using a 250 mL Erlenmeyer flask. The alkaline hydrolysis parameters in terms of NaOH concentration, temperature and incubation time were adapted from Danquah et al. [8], Castro et al. [20], Stanier et al. [25].

2.4.3. Hydrolysis with ultrasound

The hydrolysis with sonication is carried out by the flask with its content (with acid or alkali content) sonicated at a frequency of 24 kHz and ultrasonic power of 200 W for 15 min. The temperature in the ultrasonic bath was maintained by the recirculation of cold water in the bath throughout the experiment. After the ultrasonic assisted saccharification process, the residue from the liquid is separated by a filter. The supernatant was further filtered through 0.45 µM regenerated cellulose membranes, diluted and analysed for glucose concentration.

2.5. Fermentation of micro algae

Saccharomyces cerevisiae strain used for ethanol fermentation was cultured in Luria Broth (LB) medium containing 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 5 g L^{-1} NaCl. The medium was mixed thoroughly and the pH was maintained at 4.8 using 1 mol L^{-1} NaOH. The yeast suspension of 3 % (v/v) was transferred aseptically into a conical flask containing hundred millilitre of sterilized LB medium. The flask was placed in an incubator set to 200 rpm for 24 h under 30°C [28]. Yeast cells were harvested by centrifuging at 600 × g for 2 min and washed thrice with 1% (v/v) phosphoric acid to eradicate the residual sugars. Later, the fermentation was carried out in 500 mL Erlenmeyer flask containing 100 mL of sterilized sugar-containing liquid medium derived from micro algae pretreatment and the harvested yeast. The flask was sparged with N_2 gas to provide an oxygen-free environment for anaerobic fermentation. The flask was subjected to 200 rpm at 30°C for 72 h using shaking incubator [27].

2.6. Analysis

Every day a volume of 10 mL micro algae suspension was collected from the photo bio-reactor for growth and nutrient analysis. The growth rate of *Chlorella vulgaris* was studied by measuring OD at 660 nm (OD_{660}) for every 24 h by UV-Visible Spectrophotometer. The initial OD was 0.01 and the growth rate was monitored for 15 d. For nutrient analysis, the samples were centrifuged at 6000 rpm for 10 min. The collected supernatant was then filtered through 0.45 µM cellulose membrane. The filtrates were analysed for COD (Hach method), nitrogen of nitrate, nitrite (phenoldisulfonic acid method) and ammonia (Nash-reagent spectrophotometric method) and phosphorous (molybdenum antimony anti-spectrophotometric method). For the micro algal composition analyses, the biomass harvested by centrifugation was washed thrice with reverse osmosis treated water. Carbohydrate content of the extracted algal biomass was determined with the modified quantitative saccharification method [9,29]. The polysaccharides and proteins were detected using phenol-sulphuric method and micro BCA protein assay kit, respectively [30].

3. Results and discussion

3.1. Nutrition and COD removal

The municipal wastewater was treated with *C. vulgaris* for a period of 15 days and characterization of the wastewater was carried out every day. Nutrient utilization by *Chlorella vulgaris* in the wastewater was found remarkable with respect to the micro algal growth. Fig. 1 shows the % removal of all the nutrients in waste water treated by *Chlorella vulgaris*. Ammonia was the main nitrogen source in the diluted municipal wastewater. As can be seen from Fig. 1, there is a substantial reduction in the ammoniacal nitrogen and nitrate nitrogen in the

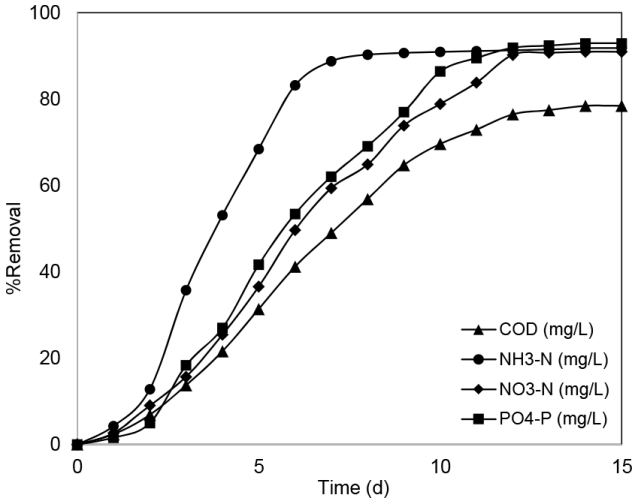

Fig. 1. % removal of various nutrients from waste water inoculated with *Chlorella vulgaris.*

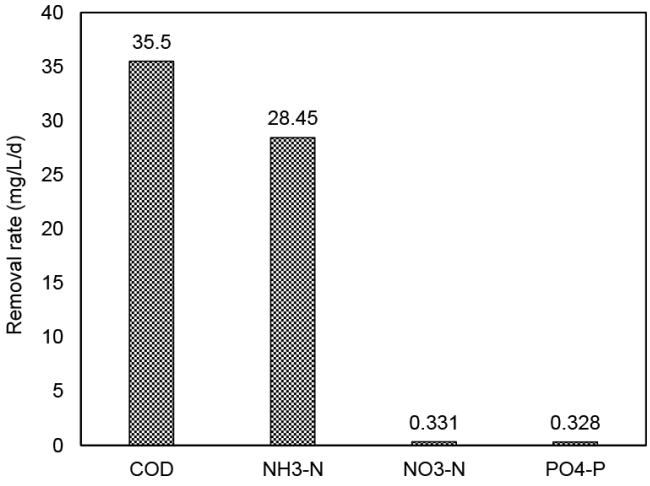

Fig. 2. Nutrient removal rates of various constituents of wastewater by *Chlorella vulgaris.*

diluted wastewater compared with the original wastewater which has also been reported by Han et al. [31]. Similarly, Kshirsagar [32] found that in the retention period of 15 days, the removal of nitrate with *Chlorella vulgaris* and *Scenedesmus quadricauda* was 78.1% and 70.3% respectively. In this study, the removal of ammoniacal nitrogen was 88.8% and 91.8% on the 7th and15th days, whereas for the nitrate it was 59.4% and 91% on 7th and15th day respectively. The removal during the 7th day was less probably due to generation of additional nitrite in the process of nitrate reduction to ammoniacal nitrogen and may be part of nitrite produced is excreted into the media. Phosphate removal is found to be significantly high being 92.9% from diluted wastewater samples with the retention time of 15 days [33]. It also showed 96% removal of phosphorous during the growth of *Chlorella vulgaris*.

Nitrogen is much required for micro alga for the synthesis of nucleic acid, protein and phospholipid, and thus the growth of micro algae is believed to be vital for nitrogen removal via the processes of uptake, decay and sedimentation [34]. It should be noted that CO_2 was supplied as an extra carbon source since it was mixotrophic cultivation. As a result, the ratio of C:N:P was reformed due to the CO_2 supplementation. This could be a crucial factor influencing the performance of NH_3-N removal under mixotrophic growth.

The COD removal was 78.4% after 15 d of continuous treatment with algae. The efficiency of COD removal obtained from micro algal growth is negatively dependent on the initial COD concentration. This was previously shown in the study reported by He et al. [35], in which diluted wastewater was used to cultivate *C. vulgaris* and was found that the COD removal percentage increased from 20.6% to 88%. It was also discovered that the additional supply of CO_2 for the mixotrophic cultivation thus did not affect the COD removal performance.

The removal rates of various nutrient are shown in Fig. 2. These rates will be more helpful in understanding the removal of COD and other nutrients from the system by algae in daily basis.

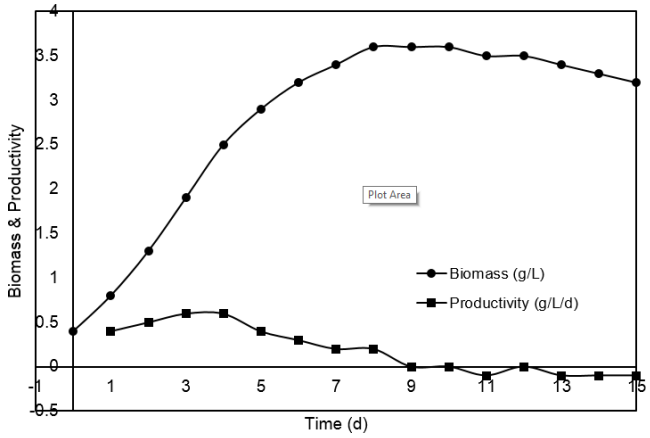

Fig. 3. Biomass and productivity of *Chlorella vulgaris* inoculated in wastewater.

3.2. Growth of algae

The growth characteristics of the *Chlorella vulgaris* in municipal wastewater were investigated. The time-course growth profiles of *C. vulgaris* under mixotrophic conditions are shown in Fig. 3. The results clearly shows that the maximum biomass production was highest during 8th, 9th and 10th day yielding 3.6 g/L. Like other microorganisms, micro algae can undergo four growth phases such as lag, exponential, stationary and lysis. Where as in this study, no significant lag phase was observed, since a large inoculum (the initial micro algal biomass was 0.4 g/L in all batches) size was used. The micro algae grown in diluted municipal wastewater saw rapid growth, with a maximum productivity of 0.4 g/L/d and attained the highest cell output under the mixotrophic cultivation. The longer exponential growth phase resulted in a higher biomass concentration. This suggests that providing sufficient nutrients (5-fold dilution) is vital in achieving a higher maximum cell concentration for subsequent biomass of *C. vulgaris*. The productivity was reduced after 6 days of cultivation since there was a signifi-

cant reduction of nutrients in the medium. Though the productivity was less, the micro algae would have undergone a strong metabolic mechanism during which most of the important biomolecules will be synthesized by digesting the absorbed nutrients.

3.3. Carbohydrate content and productivity

The amount of carbohydrates produced during the growth of micro alga in municipal wastewater was estimated every day and the results obtained are shown in Fig. 4. From the results, it is evident that the amount of carbohydrates produced during the initial stage of growth was very less. A maximum carbohydrate content of about 59% (by dw) was achieved after 14 days of growth. This productivity is higher than that obtained by other species reported by many researchers as shown in Table 2. The carbohydrate content shows increasing trend even after the declination of biomass productivity because carbohydrate tends to increase while cultivation micro algae in nitrogen-deficient conditions [9,36].

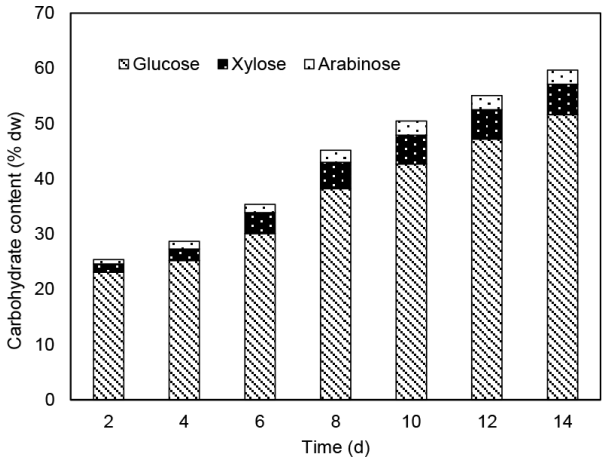

Fig. 4. Availability of carbohydrate content in *Chlorella vulgaris* during growth period.

The composition of various monosaccharides was also studied in detail, which is also shown in Fig. 4. Based on the results obtained, Glucose was predominantly produced during the process. During all the observations, the concentration of glucose was about 86% of the total monosaccharides produced. Since organic nutrients are abundant in the municipal wastewater medium and the process being a mixotrophic one, the availability of carbon was ample enough for the micro algae to synthesize carbohydrates in higher composition. If carbon is scarce in the medium, synthesize of penta-carbon mono saccharides would be triggered, but in this system, nitrate was simultaneously removed from the system and carbon was stored in the form of carbohydrate.

3.4. Hydrolysis of cellulose

3.4.1. Acid saccharification

Saccharification of cellulose available in *Chlorella vulgaris* was done by acid hydrolysis method combined with ultrasonication. To emphasize the effect of ultrasound, experiments were also carried out with out ultrasonication. The results obtained are shown in Fig. 5. The experiments were carried out with varying acid concentrations of 2–12%. The sugar yield was increased from 35 mg/g dw to 99 mg/g dw when acid concentration was increased from 2% to 10%, whereas for the acid concentration of 12% the yield slightly decreased to 99 mg/g dw. This can be attributed to the production of furfural in the acid hydrolysis process. During the treatment of biomass with higher acid concentrations (>10%), the glucose formed would be converted to furfural hence reducing the yield of glucose. If the acid concentration is increased further, the formation of furfural will also be increased. Under ultrasonic conditions, the yield of glucose increased significantly because of thermal and mechanical effects. Ultrasound facilitates the hydrolysis by disintegrating the structural rigidity of the cellulose and increases the solubility of saccharide-like substance in the biomass. Also, ultrasound-assisted acid hydrolysis accelerate the hydrolysis process with a shorter

Table 2
Comparison of the carbohydrate production and glucose content of *C. vulgaris* and other micro algae species

Strains	Biomass productivity (mg L^{-1} d^{-1})	Carbohydrate production (%)	Glucose (% of total carbohydrate)	References
Scenedesmus obliquus	840.57	46.65	79	[9]
Scenedesmus obliquus	554.5	49.4	80	[38]
Scenedesmus obliquus	NA	31.8	46.22	[39]
Chlorococcum infusionum	NA	32.52	46.8	[40]
Chlorella vulgaris	1363	50.39	93.1	[26]
Chlorella vulgaris	NA	22.4	48.21	[41]
Chlorella sp.	NA	36.1	82.8	[42]
Chlorella vulgaris	310	16.74	NA	[43]
Botryococcus braunii	610	2.38	NA	[43]
Spirulina platensis	730	11	NA	[43]
Dunaliella tertiolecta	420	13.95	NA	[43]
Chlorella vulgaris	400	59.7	86.43	This study

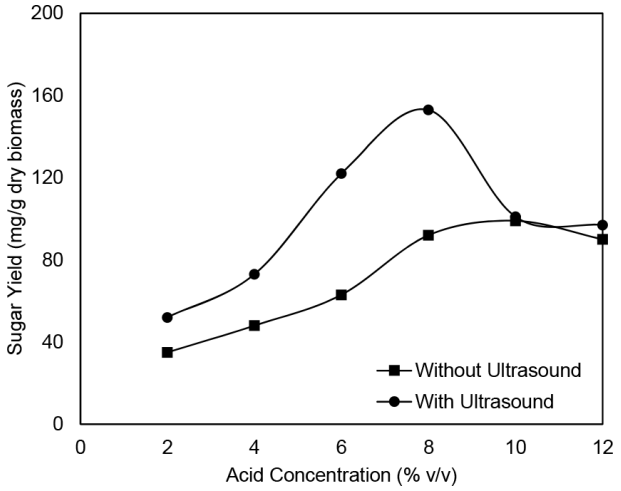

Fig. 5. Amount of glucose produced by acid saccharification of *Chlorella vulgaris.*

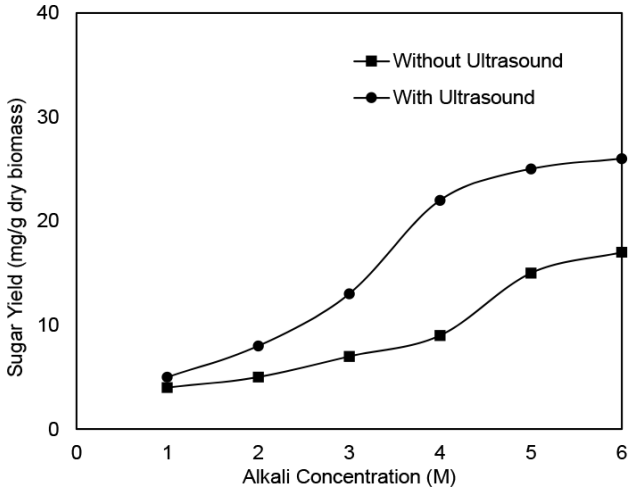

Fig. 6. Amount of glucose produced by alkali saccharification of *Chlorella vulgaris.*

reaction time by enhancing phase transfer and mixing, and by facilitating the diffusion of chemicals through the cell membranes of algae [37]. For the acid concentration of 8%, the yield was about 153 mg/g dw which was 55% higher than the maximum yield during the hydrolysis without ultrasonication. Also, the maximum yield obtained in the previous process was for the acid concentration of about 10% whereas for the latter it was for 8%. Furthermore, the increased concentrations (10% and 12%) reduced the yield of glucose, which is attributed to the formation of furfural. Since, ultrasonication is an intensification technique; the formation of furfural was triggered for smaller acid concentrations.

3.4.2. Alkali saccharification

Saccharification was also carried out by alkali hydrolysis with and without application of ultrasound. Fig. 6 shows the results obtained during alkali saccharification. Of all the concentrations studied from 1 to 6 M NaOH, the yield increased from 4 mg/g dw to 17 mg/g dw, which were pretty lower compared with the yield values obtained in acid hydrolysis. With the application of ultrasound the yield values were 5 mg/g dw to 26 mg/ g dw, which were slightly higher than the values obtained by alkali hydrolysis without ultrasonication and much lower than the acid hydrolysis. This shows that the acid hydrolysis is highly efficient for saccharification of algal biomass.

3.5. Micro algal fermentation

During fermentation process, the yeast consumes glucose and other fermentable sugars as a carbon sources to produce bioethanol. The released sugar concentration and bioethanol production over a time during fermentation process were monitored. The results shows that there is a slower declining trend for released sugar concentration after 24 h. In contrast, bioethanol production displayed an increasing trend up to 30 h followed by declining phase. This suggest that yeast consumes released sugar for growth and results in extracellular bioethanol production. The decline in bioethanol production is mainly due to the depletion of nutrients (simple sugars) for yeast growth after a certain period of time. The ultrasound assisted acid pre-treated system gave 153 mg of glucose/g of algae that is fermented using *S. cerevisiae* to yield 68.8 mg of bioethanol/g of algae (248 mg bioethanol/L of wastewater).

4. Conclusions

Chlorella vulgaris can efficiently utilize the nutrients and COD in diluted municipal wastewater for cell growth (3.6 g/L) and carbohydrate accumulation (59.7%), with high COD (78.4%), nitrogen (91%) and phosphate (92.9%) removal efficiencies. Furthermore, saccharification of algae was carried out by acid and alkali hydrolysis with the application of ultrasonication. The yield of glucose was higher (153 mg/g dw) during ultrasound assisted acid hydrolysis with acid concentration of 8%. Subsequently, the uptake of glucose by the fermenting yeast resulted in the production of 68.8 mg of bioethanol/g of algae. These findings suggest that municipal wastewater with sufficient nutrients supplement can be directly used for mass cultivation of micro algae, which is an essential source of carbohydrate for bioethanol production.

References

[1] B. Fatih, World Energy Outlook 2015, International Energy Agency, 2015.
[2] L. Doman, EIA projects 48% increase in world energy consumption by 2040, in, Today in Energy, U.S. Energy Information Administration (EIA), 2016.
[3] BP Energy Outlook, in, 2017.
[4] P.S. Nigam, A. Singh, Production of liquid biofuels from renewable resources, Prog. Energy Combust. Sci., 37 (2011) 52–68.
[5] M. Tutt, T. Kikas, J. Olt, Influence of different pretreatment methods on bioethanol production from wheat straw, Agron. Res., 10 (2012) 209–276.

[6] Y. Chisti, Biodiesel from micro algae, Biotechnol. Adv., 25 (2007) 294–306.
[7] R.P. John, G. Anisha, K.M. Nampoothiri, A. Pandey, Micro and macro algal biomass: a renewable source for bioethanol, Bioresour. Technol., 102 (2011) 186–193.
[8] M. Danquah, B. Liu, R. Harun, Analysis of process configurations for bioethanol production from micro algal biomass, in: Progress in Biomass and Bioenergy Production, InTech, 2011.
[9] S.-H. Ho, C.-Y. Chen, J.-S. Chang, Effect of light intensity and nitrogen starvation on CO_2 fixation and lipid/carbohydrate production of an indigenous micro alga *Scenedesmus obliquus CNW-N*, Bioresour. Technol., 113 (2012) 244–252.
[10] L. Brennan, P. Owende, Biofuels from micro algae—a review of technologies for production, processing, and extractions of biofuels and co-products, Renew. Sust. Energ. Rev., 14 (2010) 557–577.
[11] J.-S. Deschênes, A. Boudreau, R. Tremblay, Mixotrophic production of micro algae in pilot-scale photo bioreactors: Practicability and process considerations, Algal Res., 10 (2015) 80–86.
[12] R.L. White, R.A. Ryan, Long-term cultivation of algae in open-raceway ponds: lessons from the field, Ind. Biotechnol., 11 (2015) 213–220.
[13] W.J. Oswald, H. Gotaas, H.F. Ludwig, V. Lynch, Algae symbiosis in oxidation ponds: III. Photosynthetic oxygenation, Sewage Ind. Waste, (1953) 692–705.
[14] R. Boonchai, G.T. Seo, C.Y. Seong, Micro algae photo bioreactor for nitrogen and phosphorus removal from wastewater of sewage treatment plant, Int. J. Biosci. Biochem. Bioinforma., 2 (2012) 407.
[15] W.J. Oswald, H.B. Gotaas, Photosynthesis in sewage treatment, Trans. Am. Soc. Civ. Eng., 122 (1957) 73–105.
[16] J. Kim, B.P. Lingaraju, R. Rheaume, J.-Y. Lee, K.F. Siddiqui, Removal of ammonia from wastewater effluent by *Chlorella vulgaris*, Tsinghua Sci. Technol., 15 (2010) 391–396.
[17] W. Zhou, Y. Li, M. Min, B. Hu, P. Chen, R. Ruan, Local bioprospecting for high-lipid producing micro algal strains to be grown on concentrated municipal wastewater for biofuel production, Bioresour. Technol., 102 (2011) 6909–6919.
[18] P. Singh, S.K. Gupta, A. Guldhe, I. Rawat, F. Bux, Micro algae Isolation and Basic Culturing Techniques, in: Handbook of marine micro algae, Elsevier, 2015, pp. 43–54.
[19] J.U. Fangel, P. Ulvskov, J.P. Knox, M.D. Mikkelsen, J. Harholt, Z.A. Popper, W.G.T. Willats, Cell wall evolution and diversity, Front. Plant Sci., 3 (2012) 152.
[20] Y.A. Castro, J.T. Ellis, C.D. Miller, R.C. Sims, Optimization of wastewater micro algae saccharification using dilute acid hydrolysis for acetone, butanol, and ethanol fermentation, Appl. Energy, 140 (2015) 14–19.
[21] F.M. Gírio, C. Fonseca, F. Carvalheiro, L.C. Duarte, S. Marques, R. Bogel-Łukasik, Hemicelluloses for fuel ethanol: a review, Bioresour. Technol., 101 (2010) 4775–4800.
[22] S.W. Kim, C.-H. Hong, S.-W. Jeon, H.-J. Shin, High-yield production of biosugars from *Gracilaria verrucosa* by acid and enzymatic hydrolysis processes, Bioresour. Technol., 196 (2015) 634–641.
[23] C. Ofori-Boateng, K.T. Lee, Ultrasonic-assisted simultaneous saccharification and fermentation of pretreated oil palm fronds for sustainable bioethanol production, Fuel, 119 (2014) 285–291.
[24] B.D. Kaushik, Laboratory methods for blue-green algae, Associated Publishing Company, New Delhi, 1987.
[25] R. Stanier, R. Kunisawa, M. Mandel, G. Cohen-Bazire, Purification and properties of unicellular blue-green algae (order *Chroococcales*), Bacteriol. Rev., 35 (1971) 171.
[26] S.-H. Ho, S.-W. Huang, C.-Y. Chen, T. Hasunuma, A. Kondo, J.-S. Chang, Bioethanol production using carbohydrate-rich micro algae biomass as feedstock, Bioresour. Technol., 135 (2013) 191–198.
[27] R. Harun, M.K. Danquah, Influence of acid pre-treatment on micro algal biomass for bioethanol production, Process. Biochem., 46 (2011) 304–309.
[28] R. Harun, M.K. Danquah, G.M. Forde, Micro algal biomass as a fermentation feedstock for bioethanol production, J. Chem. Technol. Biot., 85 (2010) 199–203.
[29] G. Moxley, Y.-H.P. Zhang, More accurate determination of acid-labile carbohydrates in lignocellulose by modified quantitative saccharification, Energy Fuels., 21 (2007) 3684–3688.
[30] S. Taylor, Marine medicinal foods: implications and applications, macro and micro algae, Academic Press, 2011.
[31] X. Han, Y.S. Wong, M.H. Wong, N.F.Y. Tam, Biosorption and bioreduction of Cr (VI) by a micro algal isolate, Chlorella miniata, J. Hazard. Mater., 146 (2007) 65–72.
[32] A.D. Kshirsagar, Bioremediation of wastewater by using micro algae: an experimental study, Int. J. Life Sci. Biotechnol. Pharma., 2 (2013) 339–346.
[33] M. Garrett, M. Allen, Photosynthetic purification of the liquid phase of animal slurry, Environ. Poll., 10 (1976) 127–139.
[34] E.J. Olguín, S. Galicia, G. Mercado, T. Pérez, Annual productivity of *Spirulina (Arthrospira)* and nutrient removal in a pig wastewater recycling process under tropical conditions, J. Appl. Phycol., 15 (2003) 249–257.
[35] P. He, B. Mao, F. Lü, L. Shao, D. Lee, J. Chang, The combined effect of bacteria and *Chlorella vulgaris* on the treatment of municipal waste waters, Bioresour. Technol., 146 (2013) 562–568.
[36] G. Dragone, B.D. Fernandes, A.P. Abreu, A.A. Vicente, J.A. Teixeira, Nutrient limitation as a strategy for increasing starch accumulation in micro algae, Appl. Energy, 88 (2011) 3331–3335.
[37] L. Korzen, I.N. Pulidindi, A. Israel, A. Abelson, A. Gedanken, Single step production of bioethanol from the seaweed *Ulva rigida* using sonication, RSC Adv., 5 (2015) 16223–16229.
[38] S.-H. Ho, A. Kondo, T. Hasunuma, J.-S. Chang, Engineering strategies for improving the CO_2 fixation and carbohydrate productivity of *Scenedesmus obliquus CNW-N* used for bioethanol fermentation, Bioresour. Technol., 143 (2013) 163–171.
[39] J. Miranda, P.C. Passarinho, L. Gouveia, Pre-treatment optimization of Scenedesmus obliquus micro alga for bioethanol production, Bioresour. Technol., 104 (2012) 342–348.
[40] R. Harun, W. Jason, T. Cherrington, M.K. Danquah, Exploring alkaline pre-treatment of micro algal biomass for bioethanol production, Appl. Energy, 88 (2011) 3464–3467.
[41] K.H. Kim, I.S. Choi, H.M. Kim, S.G. Wi, H.-J. Bae, Bioethanol production from the nutrient stress-induced micro alga *Chlorella vulgaris* by enzymatic hydrolysis and immobilized yeast fermentation, Bioresour. Technol., 153 (2014) 47–54.
[42] O.K. Lee, Y.-K. Oh, E.Y. Lee, Bioethanol production from carbohydrate-enriched residual biomass obtained after lipid extraction of *Chlorella sp. KR-1*, Bioresour. Technol., 196 (2015) 22–27.
[43] E.B. Sydney, W. Sturm, J.C. de Carvalho, V. Thomaz-Soccol, C. Larroche, A. Pandey, C.R. Soccol, Potential carbon dioxide fixation by industrially important micro algae, Bioresour. Technol., 101 (2010) 5892–5896.