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Effect of anaerobic digestion on algal biomass *Chara species* in biohydrogen generation

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Abstract - *In this present investigation the effect of sulphuric acid pretreatment, steam explosion and its combined effect were evaluated in biohydrogen generation during the anaerobic fermentation on Chara the macro algal species. The results showed that acid pretreatment followed by steam explosion was proved to be the best option among the pretreatment methods studied. The optimum sulphuric acid concentration was found to be at 3% for a reaction period of 90 min at 80°C, whereas the steam explosion exhibited an optimum value of 20 min at 121°C and 15 psi during pretreatment process. Clostridium species was isolated by heat treatment technique from the agriculture soil growing tomatoes. The isolated Clostridium species was effective in hydrogen production using Chara species as the substrate. Irrespective of pretreatment option, the predominant volatile fatty acids were found to be acetate > propionate > butyrate. Anaerobic digestion of acid pretreated biomass coupled with steam explosion resulted in acetate to propionate ratio of 4.3, while acetate to butyrate ratio was 6.7.*

Keywords: *Algal biomass; anaerobic digestion; Chara sp.; Clostridium sp.; biogas; hydrogen; wastewater*

I. INTRODUCTION

In search of renewable energy due to the un-renting thirst in growing energy demand, algal bio-fuel garnered special attention. Algae exhibits fast growing capability compared to terrestrial crop. The algal biomass exhibits higher photosynthetic and carbon dioxide fixation, which results in increased biomass yield when compared to the higher plants. Biofuels derived from biomass of terrestrial crop plants, corps alternative to food source and non-food crops are termed as first, second and third generation biofuels respectively. The third generation biofuels closely resembles fossil fuels of petroleum counterpart. The environmental concern associated with exploitation of fossil fuel, shifted the focus towards algae as a third generation biofuel. With advent in technology know-how once considered algal bloom as a disguise are now been harnessed to derived biofuel either in liquid or gaseous form. Algae plays a major role as a base for food chain in aquatic ecosystem, and it contributes to about 30% of

photosynthetic oxygen produced overall. It also finds application in nutrient removal, carbon dioxide sequestration and as food supplement. Microalgae being the lower plants belong to thallus group can have biomass productivities up to 91 tons ha^{-1} $year^{-1}$ due to photoautotrophic growth through oxygenic photosynthesis (Wolf et al. 2016). Preference of algal biomass as a feedstock in biogas production was attained due to its cell constituents namely high in polysaccharides, low in cellulose and absence of lignin (Zhong et al. 2012).

Algae being an alternative feedstock for biofuel production have an edge to its advantage, as it does not compete with agricultural product as a feed material. The use of algal biomass contributes to carbon neutrality, because algae biofuel only releases carbon dioxide (CO_2) that was recently removed from the atmosphere due to photosynthetic process (Fenton and Ohuallachain, 2012; Sostaric et al. 2012). The algal energy conversion technologies determine the form of end product. In chemical conversion technology it is the biodiesel. In the case of biochemical process different types of product can be derived namely hydrogen (photo-biological), ethanol (fermentation) and biogas (anaerobic fermentation). Thermochemical conversion process results in electricity (combustion), bio-oil (liquefaction), syngas (gasification), bio-oil & syngas (pyrolysis) (Kraan, 2013). Irrespective of the size of algae either micro or macro, both of them are ideal candidature in energy generation. Microalgae exhibits faster growth rate and higher lipid content when compared with macro-algae (Lee et al. 2014). As the algal biomass contains proteins, polysaccharides and lipids, it serves as an excellent raw material in anaerobic fermentation to derive methane or hydrogen. (Demirbas, 2011; Nhat et al. 2018).

The microalgae can be grown on non-arable land, wastewaters from livestock farm, sewage and industrial tertiary treatment unit. (Posadas et al. 2015; Molinuevo-Salces et al. 2016). Earlier works had attempted in extracting biogas during the fermentation using algae species as substrates viz: *Scenedesmus*, *Spirulina*, *Chlorella vulgaris*, *Euglena*, and *Ulva* (Ras et al. 2011; Zhong et al. 2012; Saqib et al. 2013). Microalgae species of *Chlamydomonas*, *Chlorella* and *Scenedesmus* when subjected to anaerobic digestion exhibited higher methane yield due to improved C/N ratio resulting in 24 to 26 on weight basis (Klassen et al. 2015). The inhibitory role of ammonia, lipid content and the process parameter were investigated in anaerobic biodegradation of algae (Bux and Chisti, 2016). The ammonia released due to the degradation of algal protein inhibits the methanogens, thus resulting in lower efficiency of anaerobic digestion process. The C/N ratio imbalance can be avoided if co-fermentation was carried out with carbon rich substrates (Zheng et al. 2019). *Chlorella pyrenoidosa* was pretreated by acidifying and steam heating, and its C/N ratio was adjusted to 25.3 by mixing with cassava starch. The resulted feedstock yielded 276.2 mL H_2 g^{-1} volatile solids (Xia et al. 2014). *Taihu blue* algae when co-digested with corn straw at an C/N ratio of 20:1 resulted in a biogas of 325 mL g^{-1} VS^{-1} with 61.69% methane (Zhong et al. 2012). *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* yielded 587 and 287 mL biogas per g of volatile solids destroyed (Mussgnug et al. 2010). In the case of co-digestion of olive mill solid waste with microalgae *Dunaliella Salina* at a C/N ratio 26.7 resulted in a methane yield of 0.063 L g^{-1} VS (Fernández-Rodríguez et al. 2014). The nitrogen replete algal biomass showed less efficiency in biogas production due to failed acidosis, on account of ammonium accumulation. While the microbial community of nitrogen replete was comparatively different from the digester operated under low nitrogen content (Klassen et al. 2017).

The algal type determines the efficiency of pretreatment process due to their different cell wall structures. *Cyanobacterial* biomass differs from microalgae due to the absence of cellulose and complex polymer in the cell wall (Mendez et al. 2015). The focus on algal biomass pretreatment gains importance in fermentation process as the digestion efficiency depends on availability of substrate. Hence disruption of the cellulose fibres present on the algal cell wall helps to hydrolyze the polysaccharides and cellular constitutes. The barrier in algal cell wall resistance can be overcome using various pretreatment options. The algal cell wall disruption was investigated by physical, enzymatic, hydrothermal, acid treatment, autoclave, microwave, ultrasonication and electrolysis (Singh et al. 2022). Pretreatment of microalgal biomass by ultrasound increases the fracture of cell wall leading to increase in hydrolysis during anaerobic fermentation (Park et al. 2013). Mechanically pretreated *Pelvetia canaliculata* when subjected to anaerobic digestion resulted in 283 ml CH_4 g^{-1}VS, which was 45% higher than non-pretreated algae (Rodriguez et

al. 2018). The effect of maceration and thermochemical with glycerol was used as a pretreatmemt technique in enhancing methane generation from macroalgae *Gracilaria vermiculophylla* (Oliveira et al. 2014).

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The present study explores the possibilities in extracting energy in the form of biohydrogen using algal biomass of *Chara* species as a feed stock during anaerobic fermentation. The effect of pretreatment in enhancing digestion efficiency due to the release of cellular constituents, and the role of hydraulic retention time on fermentation efficiency were also investigated.

II. MATERIALS AND METHODS

2.1 Experimental Set-up

The experimental set-up of anaerobic reactor is shown in Figure 1. The dimensions of the reactor are 400 mm height and 100 mm internal diameter, with a working volume of 2 L. The reactor was operated in continuous mode as a suspended growth process.

2.2 Analytical Methods

The *Chara* species served as a feedstock in the anaerobic fermentation process. The total solid was determined by drying the algal biomass at 105°C for 1 h ASTM E1756-08 (2015). The volatile solid was determined by heating the biomass at 550°C for 2 h. The ash content was determined by dry oxidation at 550 to 600ºC. ASTM E1755-01 (2020). The reducing sugar concentration was measured by dinitrosalicylic acid (DNS) method at 540 nm (Miller 1959). Total carbohydrates were estimated by Dubois method (Dubois et al. 1956). The protein content was determined by multiplying with a conversion factor 6.25 on the Kjeldahl nitrogen (Ryan et al. 2010; APHA 2005). The volatile fatty acids constituents were determined by HPLC. The biogas constituent, hydrogen was determined by gas chromatograph equipped with a porapak column (Make: Shimadzu GC).

2.3 Pretreatment Techniques for Algal biomass

The algal biomass was filtered through muslin cloth and then subjected to natural sun drying at 18690±1950 lux for three consecutive days. The dried algal biomass was subsequently grinded and sieved to size less than 3 mm. The characteristics of algal biomass are as shown in Table 1. The pretreatment was carried out by 3 methods viz; (a) acid pretreatment (b) steam explosion (c) acid followed by steam explosion technique. In the case of acid pretreatment, the dried algal biomass of 50 g was treated with 150 ml of concentrated sulphuric acid at varying concentration viz: 1, 2, 3 and 5% (weight basis) for a reaction period of 30, 60, 90 and 120 min respectively at 80°C in a shaker at 50 rpm. The steam explosions were carried out at 121°C at 15 psi for 10, 20, 30 and 40 min. While the third pretreatment option coupled the acid pretreatment technique (H_2SO_4 concentration at 3 and 5%) with steam explosion at 121°C at 15 psi for a period of 30 min. The pretreated algal biomass was stored for a maximum period of 72 h at 20°C. Anaerobic digestion of algal biomass was investigated to determine the biohydrogen generation efficiency using pretreated algal feedstock obtained from acid treatment, steam explosion and a combination of acid treated followed by steam explosion.

2.4 Inoculum for Anaerobic Reactor

The inoculum for anaerobic fermentation of algal biomass was obtained from a lab scale biohydrogen reactor actively digesting dairy wastewater anaerobically operated at 28 ±2°C. Initially the *Clostridium* inoculum was isolated by the heat treatment of agricultural field soil cultivating tomatoes.

2.5 Acclimatization of biomass

During the acclimatization phase 2/3rd of the reactor volume was filled with hydrogen producing *Clostridium* species inoculum and 1/3rd with untreated algal biomass. The reactor was allowed to stay in ideal condition for one week with intermittent mixing for 3 min once in every 6 h. Thereafter feeding was carried out in continuous mode using a feed stock of algal and dairy wastewater (composition: pH 6.8; COD 15,800 mg/L; BOD 7,960 mg/L, total suspended solids 3180 mg/L) in the ratio 8:2 having a total solids content of 10% on dry basis for a period of two week. During the further course of acclimatization period 10% macerated untreated algal biomass was feed as substrate till the reactor was stabilized. The inlet substrate pH was maintained at 6.9 ± 0.2. The performance of the reactor was monitored based on the volatile solid destroyed, volatile fatty acids (VFA) content, reactor pH and biogas generation and its composition. The reactor was operated in continuous mode at a hydraulic retention time (HRT) of 5 d.

2.6 Evaluation of Anaerobic Reactor Performance

After the acclimatization period of the reactor, the regular experiments were carried out by using algal feedstock having a total solids content of 10% on dry basis. The anaerobic digestion efficiency of untreated and pretreated algal biomass content in the influent was investigated at a hydraulic retention time of 5 d. Samples were drawn at intermediate period to assertion the efficiency of digestion process. Experiments were carriedout till the reactor stabilized for the given influent.

III. RESULTS AND DISCUSSION

3.1 Algal biomass pretreatment and reducing sugar yield

The effect of pretreatment on *Chara* algal biomass was investigated under varying pretreatment options viz: acid treatment, steam explosion and acid coupled with steam explosion technique.

The acid pretreatment experiments were conducted at varying sulphuric acid concentrations viz: 1, 2, 3 and 5% (w/v) for a reaction period of 30, 60, 90 and 120 min at 80°C respectively. Figure 2 shows the effect of acid dosage on reducing sugar release from algae. For the above said acid pretreatment dosages at 60 min of reaction period resulted in a supernatant reducing sugar content of 70, 125, 174 and 190 mg/g of biomass. Whereas during a reaction period of 120 min the reducing sugar content was 120, 175, 229 and 250 mg/g of biomass. It can be inferred that as the acid concentration increased a gradual increase in reducing sugar was observed due to the hydrolysis of algal polysaccharide, similar trend was also observed with the increase in reaction period.

The purpose of the acid hydrolysis was to disorganize the polysaccharide complex, making algal sugars more amenable to acid hydrolysis,. The hydrolysis of carbohydrate to reducing sugars was related to sulphuric acid concentration. Earlier researchers had stated that during hydrolysis of macroalgae, the sulphuric acid had an edge over the hydrochloric acid in relation to the release of reducing sugar. During hydrolysis the hydrogen ion plays a significant role in the release of reducing sugar (Qian et al. 2005). The acid hydrolysis decreased in the following order H_2SO_4 > HCl > H_2PO_4 (Hamouda et al. 2016). The sulphuric acid concentration should be sufficiently higher than critical concentration for achieving the hydrolysis of carbohydrates into

reducing sugar units. Moreover reduced yield of fermentable sugar content was observed at a sulphuric acid dosage of 6-10% (v/v) (Redding et al. 2010; Wu et al. 2014). The possible reason for the decreasing trend in the amounts of reducing sugars in the acidic condition may be due to the thermal decomposition of sugars into furfural and hydroxymethylfurfural (Jeong et al. 2015). While higher concentrations of acid dosage favour corrosion of equipment and also consumes more neutralizing agent. Hence, 3% (w/v) sulphuric acid concentration was chosen as the optimum during acid pretreatment stage. The reducing sugar yield not only depends on the concentration of the acid but also on the reaction time. In the present investigation a reaction period of 90 min at 80°C was chosen as optimum reaction period for acid digestion of *Chara* species. Further increase in reaction period along with the increase in acid concentration showed a gradual increase in the released sugar.

Figure 3 shows the effect of steam explosion on reducing sugar concentration in the supernatant. The steam explosion for algal biomass was carried out at varying period viz: 10, 20, 30 and 40 min at 15 psi at 121°C. The results showed that as the steam explosion period increased from 10 to 40 min the reducing sugar concentration was found to be 130 and 295 mg/L respectively. Hydrolysis of polysaccharide complex was achieved during steam explosion, resulting in the release of sugar thus favouring the anaerobic digestion process in the subsequent stage of treatment.

Figure 4 shows the reducing sugar content in the supernatant during combined pretreatment technique based on the optimum value of acid pretreatment and steam explosion. The acid pretreatment was carried out using 3 and 5% sulphuric acid for 90 min at 80°C, which was followed by steam explosion at 121 psi for 30 min. The results showed a reducing sugar value of 470 and 490 mg/g biomass.

3.2 Acclimatization of biomass

The anaerobic reactor was started-up by mixing algal feedstock with dairy wastewater in the ratio 8:2 having a total solids content of 10% on dry basis. The inlet substrate pH was maintained at 6.9 ± 0.2, while the reactor pH was found to be 7.1 ± 0.3 during the started up period. The performance of the reactor was monitored based on the MLVSS concentration, volatile solids destroyed, VFA content, reactor pH and biogas generation. The reactor was operated in continuous mode at 5 d HRT. The volatile fatty acid content in the reactor was 2280±160 mg/l. During anaerobic digestion process the first phase is hydrolysis where long chain carbon polymers are broken down into short chain carbon compounds. Hydrolysis is followed by acidification were acidogens convert short chain carbon substrate is converted into ATP, reducing agent and monomers. The acetogens produces fatty acids through glycolytic pathway. The results showed that the major VFA constituents as acetate > propionate > butyrate. The acetate to propionate ratio was 4, while acetate to butyrate ratio was 6. The accumulation of volatile fatty acid illustrates the relationship between the acid producers and its consumers and the extent of stress situations in the reactor. The VFA accumulation in the reactor serves as an process indicator in determining the effective digestion of the substrate. Earlier researchers had stated various levels of VFA content of the reactor, which again depends on the substrate and operating condition (Ahring et al. 1995). The MLVSS concentration in the reactor showed a steady rise during the acclimatization period. The MLVSS concentration in the anaerobic digestion stabilized at 2938 ± 159 mg/L during the end of acclimatization period. As the MLVSS concentration was more or less stable it could be considered that the reactor has reached the steady state.

The biogas generation showed a rise in value during the acclimatization period as the *Clostridium sp.* microflora was adapted in digesting the algal biomass. As shown in Figure 5 during the end of 20 and 40 days of operation the cumulative biogas generation was found to be 16.1 and 37.9 L with a hydrogen content of 53%. Further increase in acclimatizing phase showed a steady biogas generation, thus confirming the completion of acclimatization stage.

3.3 Effect of anaerobic digestion on acid pretreated algal biomass

The effect of anaerobic digestion was investigated using 10% of dried and shredded acid pretreated algal biomass on weight basis. The anaerobic digestion experiments were conducted using 3 and 5% sulphuric acid pretreated biomass. Figure 6 shows the corresponding average biogas production was found to be 4.6 and 5.1 L/d with a hydrogen content of 55% for a hydraulic retention time of 5 d. In the case of volatile solids destruction efficiency it was 26 and 28%. The acetate to propionate ratio was 4.0, while acetate to butyrate ratio was 5.8. The reactor was operated for a period of 4 weeks in order to determine the stability of anaerobic digestion process.

3.4 Effect of anaerobic digestion on steam exploded algal biomass

The effect of anaerobic digestion was investigated using steam exploded algal biomass for a period of 30 and 40 min as a substrate. The results showed that anaerobic digestion of steam exploded biomass at 30 and 40 min the resulted biogas generaion of 7.9 and 8.6 L/d (Figure 7), with a hydrogen content of 55%. The volatile solids destruction efficiency was found to be 42 and 46% respectively. The VFA content of the reactor was found to be 2700 ± 127 mg/L with a pH value of 7.4± 0.2. The acetate to propionate ratio was 4.2, while acetate to butyrate ratio was 6.4. The VFA and pH value was well with the level of healthy functioning anaerobic process.

3.5 Effect of anaerobic digestion on acid pretreated and steam exploded algal biomass

The optimized value of algal pretreatment was found to be 3% H_2SO_4. The acid pretreated algal biomass was subjected to steam explosion for 30 and 40 min, which was used a substrate for anaerobic digestion. The average biogas generation was found to be 10.3 and 10.7 L/d (Figure 8) with a hydrogen content of 57% for the above said steam explosion period. The volatile solids destruction efficiency was found to be 53 and 57% respectively. Irrespective of pretreatment option, the major volatile fatty acid constituent were found to be acetate > propionate > butyrate. The acetate to propionate ratio was 4.3, while acetate to butyrate ratio was 6.7.

IV CONCLUSION

Deriving energy from algal biomass proved to be a promising way of controlling excess algal growth in the environment, as well as an effective way of utilizing the biomass. Algae being a third generation biofuel underlines its carbon neutrality thus considering it as the best alternative bio-resource. The present study demonstrated that macro algae *Chara sp.* as an ideal candidature towards generating hydrogen through anaerobic process. The role of pretreatment techniques viz: sulphuric acid, steam explosion and a combination of both processes in hydrolysing the algal biomass was also investigated. The hydrolysed biomass was easily amenable substrate to the anaerobic digestion process, thus resulting energy in the form of hydrogen.

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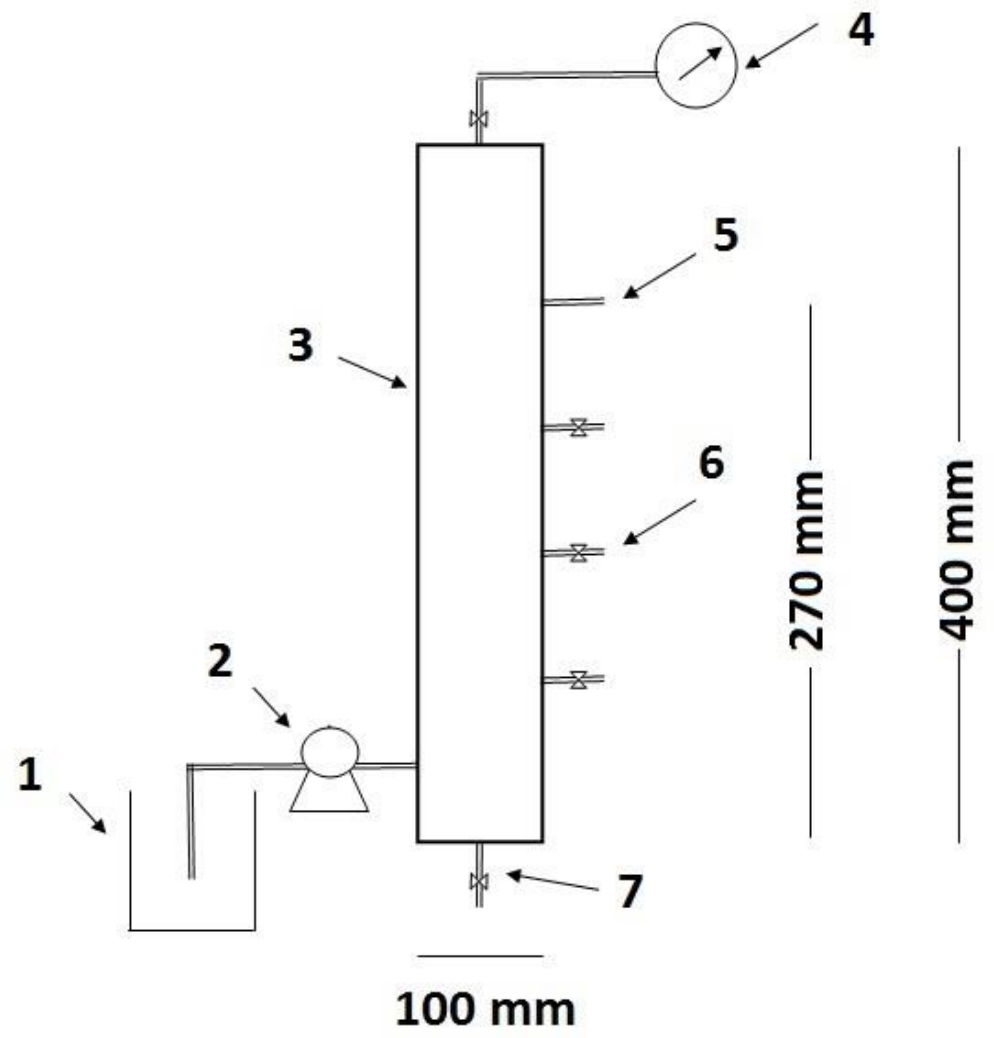

Figure 1. Experimental set-up of anaerobic reactor

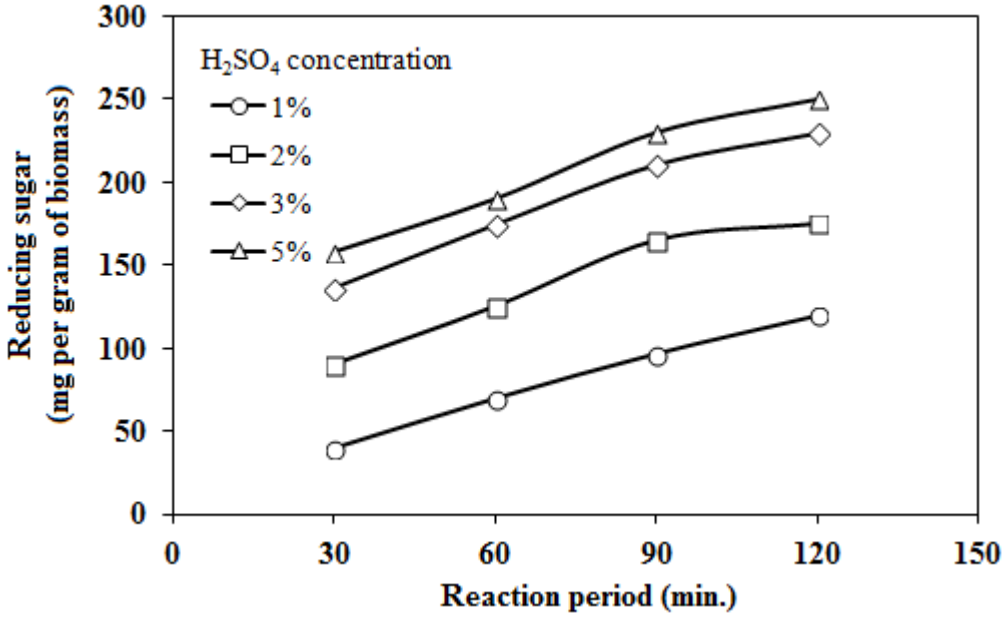

Figure 2. Effect of sulphuric acid pretreatment on reducing sugar yield at 80°C from *Chara* algal biomass.

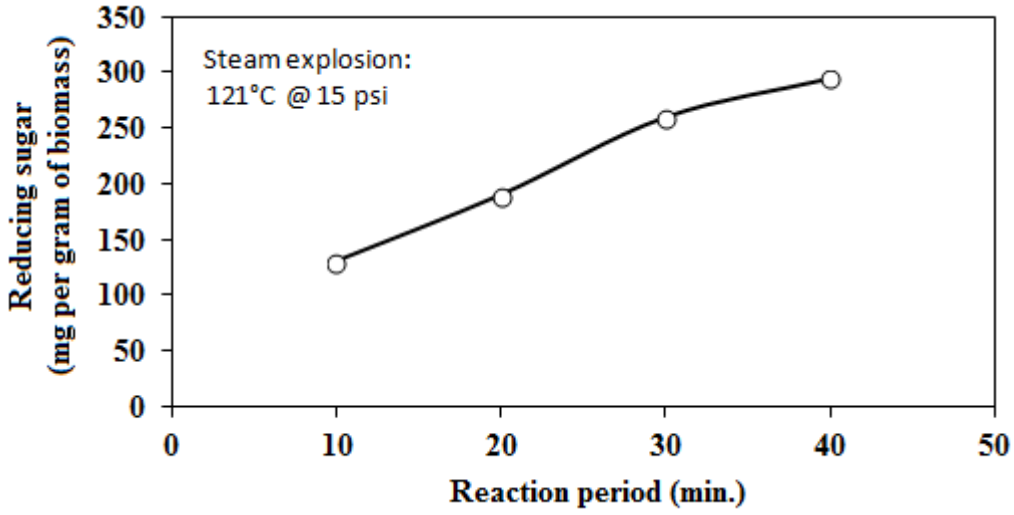

Figure 3. Effect of steam explosion on reducing sugar yield from *Chara* algal biomass.

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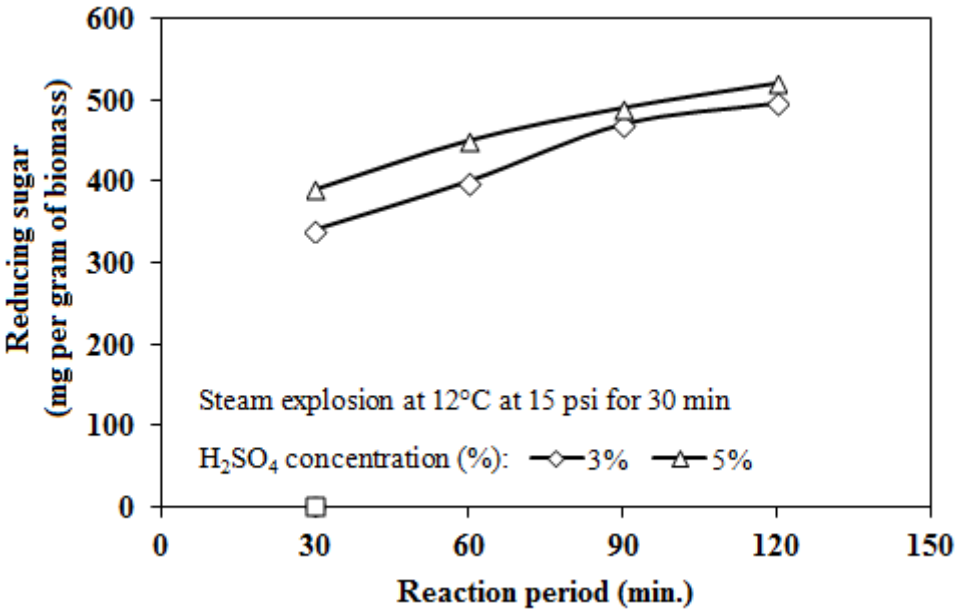

Figure 4. Effect of sulphuric acid pretreatment followed by steam explosion on reducing sugar yield from *Chara* algal biomass.

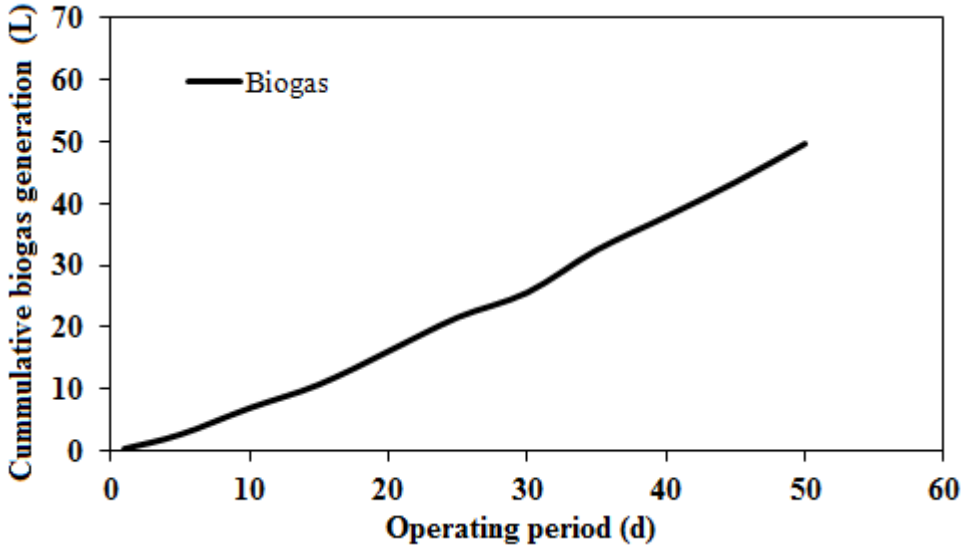

Figure 5. Biogas generation during the acclimatization phase of the anaerobic reactor

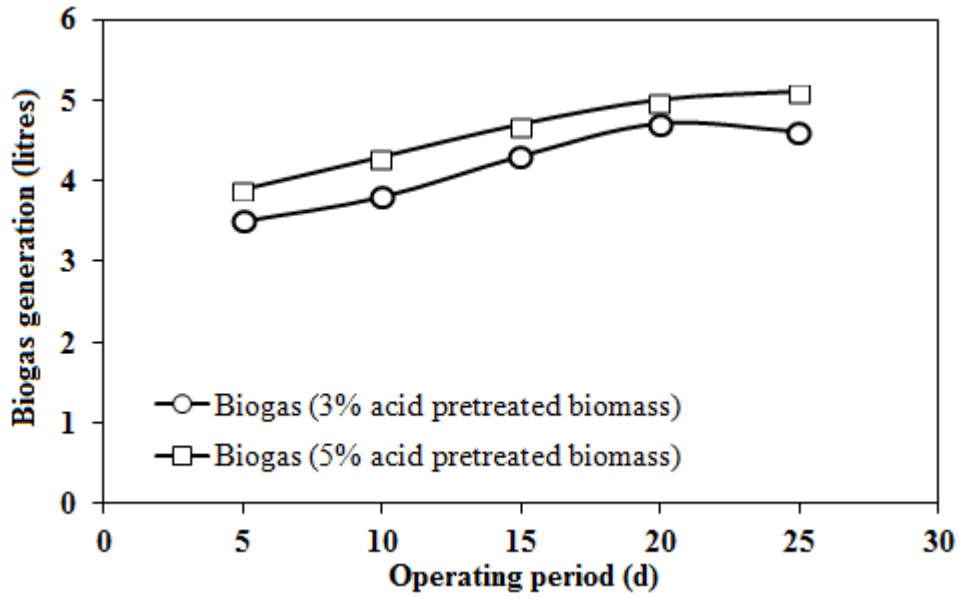

Figure 6. Biogas generation during the anaerobic digestion of acid pretreated *Chara* algal biomass

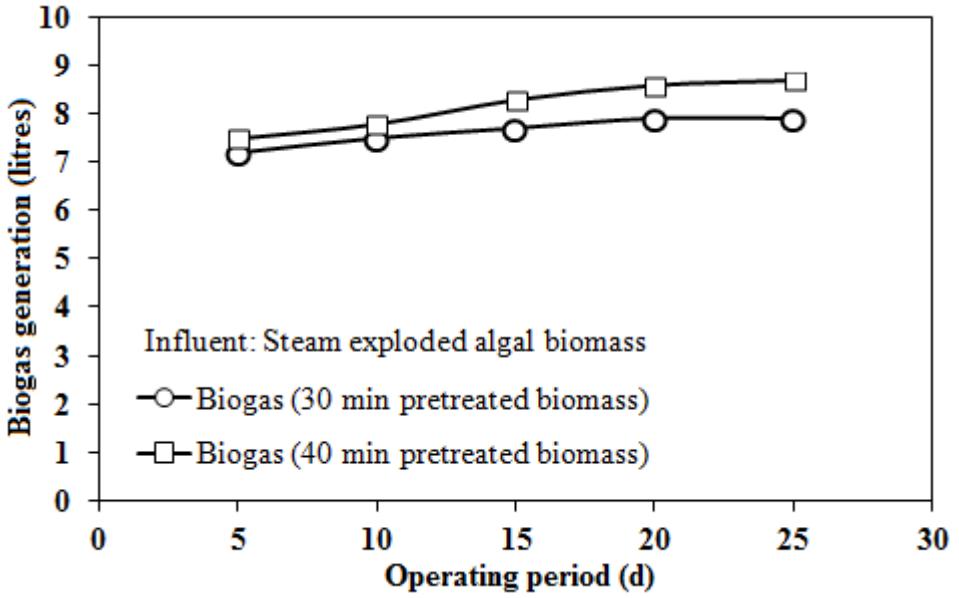

Figure 7. Biogas generation during the anaerobic digestion of steam exploded Chara algal biomass

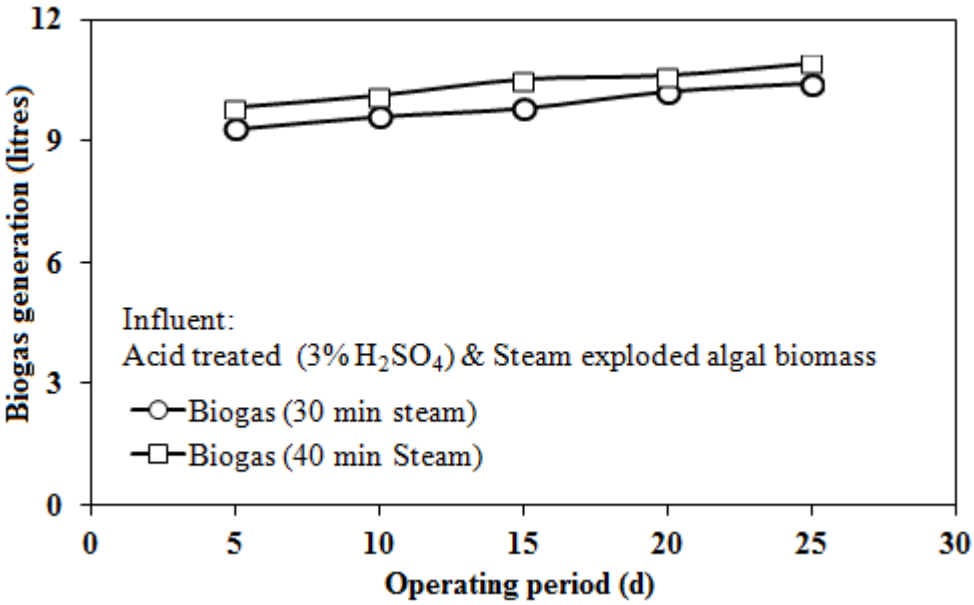

Figure 8. Biogas generation during the anaerobic digestion of acid pretreated followed by steam explodec Chara algal biomass

Table 1: Characteristics of *Chara* species algal biomass

Proximate composition	**Relative percentage based on dry weight of algae**
Total solids	15±3.2
Volatile solids	92±0.3
Carbohydrate	56±2.5
Protein	21±1.3
Lipids	6±0.8
Ash content	7±0.2