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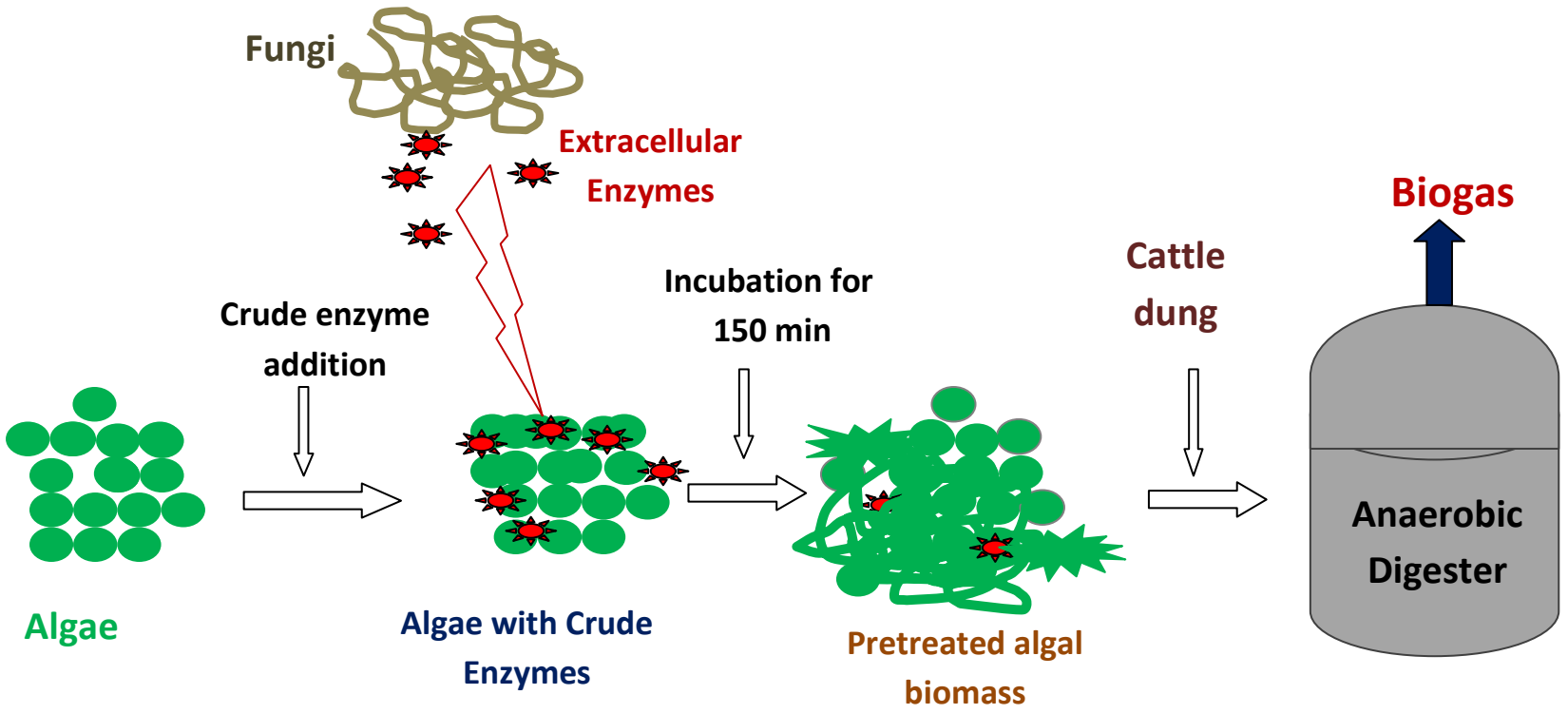

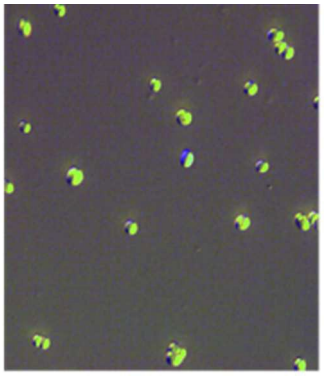

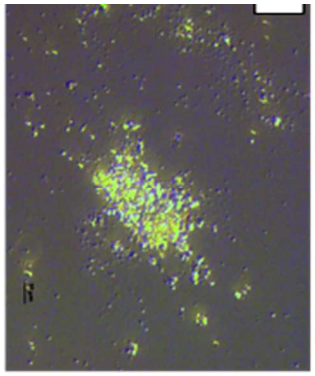

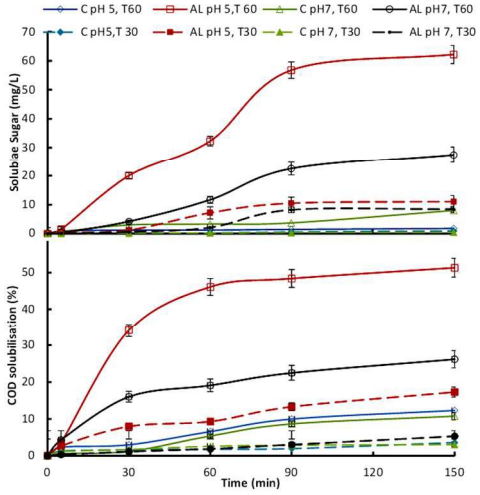

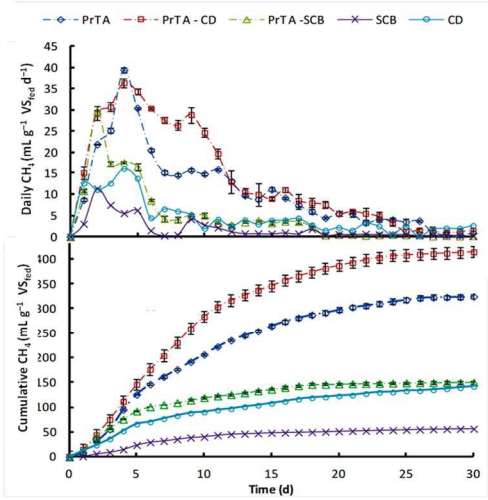

Enhanced methane production from algal biomass through short duration enzymatic pretreatment and codigestion with carbon rich waste

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Abstract

Anaerobic digestion of algal biomass faces problems of low digestibility due to cell wall resistance and improper carbon to nitrogen ratio. In the present work a short duration method involving fungal crude enzyme based pretreatment of algal biomass was disclosed. Effect of fungal crude enzymes on algal biomass was assessed qualitatively through visual and microscopic observations and quantitatively through measuring algal biomass solubilization. Up to 50 % biomass COD solubilization was observed within 150 min of pretreatment under optimal conditions. Subsequent anaerobic digestion of pretreated algal biomass showed production of 324.38 mL CH_4 g^{-1} VS_{fed} as compared to 254.73 mL CH_4 g^{-1} VS_{fed} from untreated algal biomass. Interestingly, methane yield increased up to 413.89 mL g^{-1} VS_{fed} when pretreated algal biomass was codigested with cattle dung. On the other hand, sugarcane bagasse had negative effect on algal biomass codigestion due to its poor digestibility. Overall, present attempt showed promising results by improving methane yield from algal biomass though pretreatment and codigestion.

Keywords: algae, pretreatment, fungi, energy, codigestion

1 Introduction

Methane produced from anaerobic digestion of biomass is considered as clean, safe and environmental friendly fuel. Among the various biomass, algae have gained significant interest as feedstock for methane production due to high biodegradable content [1–3]. The methane yield from algal biomass depends on the biodegradable organic material which is usually packed inside the rigid cell wall. It is widely reported that poor degradability of algal cell wall is one of the major problems in its anaerobic digestion[4–6]. In this context, pretreatment of algal biomass prior to anaerobic digestion is needed to make algal biomethane viable on commercial scale.

There have been some reports on physicochemical and biological pretreatment of algal biomass for improving its digestibility and biomethane production[7]. However, among the various methods, only thermal pretreatment is reported to show significant improvement in methane yield. For instance, Passos & Ferrer [8] observed up to 70 % enhancement in methane yield with thermal pretreatment (75 – 95 ˚C) of microalgal biomass. Similarly, González-Fernández et al[9] have reported 1.2 fold enhancement in methane yield through thermal pretreatment of *Scenedesmus* biomass at 80 ˚C. However, thermal pretreatment is an energy intensive process. Moreover, formation of any reaction by-products during thermal pretreatment of algae may hamper the methane production by inhibiting growth of anaerobic microflora[10]. Also, there are few reports which showed improvement in algal biomass digestibility and methane yield through enzymatic pretreatment. For example Ehimen et al[11] observed 20 % improvement in methane yield after pretreatment with commercial enzymes. However, these methods are economically unviable due to involvement of either high energy steps (thermal, ultrasonic etc.) or expensive commercial enzymes. Also, as algal cell wall is composed of complex biopolymers, more than one enzyme should be applied to pretreat the algal biomass[11]. Perhaps because of these facts, the

literature on enzymatic pretreatment of algal biomass is scarce. Interestingly, fungi produce mixture of extracellular enzymes. These crude enzymes could provide cheaper alternate for pretreating algal biomass. Our previous study targeting fugal crude enzymes action on algal cell wall showed promising results (up to 44 % algal biomass COD solubilization) in this direction [12]. However, the incubation time required to achieve significant pretreatment by fungal crude enzyme (at 30 ˚C) was considerably large (> 48 h). This could be due to the fact that enzymes act optimally only at specific pH and temperature[13] and the operating conditions were suboptimal for cellulolytic activity of crude enzyme in the above case. Hence, the efficiency of fungal crude enzymes could further be improved by providing specific conditions (60 ˚C with pH 5.0)[14] for optimal enzyme activity.

The carbon to nitrogen (C/N) ratio of algal biomass usually falls in the range below 10, which is another hurdle in its anaerobic digestion [15]. The C/N value between 15 and 25 has been suggested optimal for anaerobic codigestion [16]. Though it was postulated that pretreating the algal biomass with crude enzyme would result in improved methane production, the C/N ratio (< 10) was still a point of concern. In fact, poor performance of anaerobic microflora due to low C/N ratio of algal biomass has been widely reported [17,18]. Codigestion has observed as the best technique to improve C/N ratio during anaerobic digestion [19]. Several attempts have been made on improving anaerobic digestion of algal biomass by codigestion of carbon rich organic waste. For instance, 1.29 times higher methane production was reported during anaerobic codigestion of algae with kitchen waste at C/N ratio of 15/1 [20]. Similarly, up to 28 % enhancement was observed through codigestion of fresh algae with cattle dung during our previous study[21].

Based on the above discussion, it is clear that methane production form algal biomass can be enhanced by pretreatment as well as improving C/N ratio through codigestion with carbon

rich waste. There have been several attempts, separately on both the approaches but, to best of our knowledge no previous study has combined pretreatment (particularly crude enzyme based) with codigestion to improve methane production from algal biomass. Hence the aim of the present study was to evaluate the combined effect of fungal crude enzyme based pretreatment and codigestion of algal biomass on methane yield. In the first phase of the work, a short duration pretreatment of algal biomass was developed. Finally, the pretreated algal biomass was codigested with other substrates to see the effect of improved C/N ratio on methane yield.

2 Material and methods

To begin with, the experimental work was planned and divided into several parallel consecutive steps. The complete experimental design and procedure followed during the work is illustrated in the schematic shown in Figure 1 and same is elaborated in the text given below.

2.1 Fungal strain and crude enzyme production

The fungi, *Aspergillus lentulus* previously isolated from textile effluent collected from Baddi, Himachal Pradesh (India) was used for production of crude enzymes[22]. Freshly revived culture (incubated for 3 d) on potato dextrose agar was used throughout the study. Sugar cane bagasse (SCB) was utilized as substrate for enzyme production under solid state fermentation (SSF) using the methodology reported earlier [14]. Briefly, SCB was grinded in order to obtained particle size of 10 – 50 µm. SSF was carried out in 250 mL conical flask. Dried and grinded SCB (5 g) was added with distilled water containing 2.5 g yeast extract L^{-1} in order to maintain moisture content at 60 %. Flasks were aseptically inoculated with fungal spore suspension (≈6.5 x 10^6 spores mL^{-1}) at inoculum size of 5 % (w/v) of substrate and incubated for 5 d at 30 ˚C [12]. After 5

d incubation, the crude enzyme was extracted using 0.5M sodium citrate buffer (pH 5.0) containing 0.1% Tween 80 and processed for determination of, total protein, cellulase and xylanase activity [23].

2.2 Algal biomass production

Algae, *Chroococcus* sp. previously isolated from drainage line wastewater [24] was used as substrate for fungal crude enzyme based pretreatment and anaerobic digestion. Algal biomass was produced in 20L fabricated photobioreactor under direct sun light and natural dark: light cycle, using tap water medium as reported previously [5]. After proper growth (14 d), the biomass was harvested through pH assisted auto settling [24] in form of concentrated slurry.

2.3 Pretreatment of algal biomass with fungal crude enzymes

For algal biomass pretreatment, falcon tubes containing algal suspension (20 mL, having biomass concentration ≈ 2.0 g L^{-1}) were divided in to two sets; one with actual pH (> 7.0) and another with balanced pH (≈ 5.0). For pH adjustment, algal suspension (from falcon tubes) was centrifuged (at room temperature and ≈ 8000 rpm) and supernatant was discarded. The pellets were then washed with distilled water and resuspended in equal volume (20 mL) of 0.5M sodium citrate buffer (pH 5.0). Each tube was supplemented with crude enzyme of *A. lentulus* at previously optimized loading equivalent to cellulase activity of ≈ 21.0 FPU g−1 dry algae [12] . Falcon tubes (from each set) were then incubated separately, at 30 ˚C and 60 ˚C with continuous shaking (150 rpm). An aliquot (0.5 mL) was withdrawn from each tube after every 30 min and analyzed for microscopic observations as well as sugar and COD solubilisation from algal biomass as reported previously [12]. Microscopic examination of algal cells was carried out under

differential interference contrast (DIC) and fluorescent modes using inverted research microscope (ECLIPSE Ti-U, Nikon)

2.4 Anaerobic digestion of enzyme pretreated algal biomass

The actual pretreatment of algal biomass for anaerobic digestion was carried out under optimal conditions (pH 5.0 and 60 ˚C) using thick algal slurry. Algal biomass was centrifuged (at room temperature and ≈ 6000 rpm), pellets were washed with distilled water and then resuspended in 0.5M sodium citrate buffer (pH 5.0) to obtain thick slurry containing 40.0 g dry algal biomass L^{-1}. Algal slurry was added with fungal crude enzyme at a dose equivalent to ≈ 21.0 FPU g−1 dry algal biomass and incubated at 60 ˚C with continuous shaking at 150 rpm for 150 min. Intermittent manual mixing (using sterile glass rod) was also done after every 15 min in order to ensure proper contact of algal biomass with enzyme. COD solubilisation was monitored at regular intervals to evaluate the algal biomass pretreatment due to enzyme action.

To confirm the effect of fungal crude enzyme on methane yield and digestibility of algal biomass, biochemical methane potential (BMP) assays were performed following standard protocols [25,26]. Amber color reagent bottles (500 mL) were used as batch anaerobic digester. Pretreated algal slurry (≈ 1.5 g VS) was added to each bottle containing inoculum (≈ 4.5 g VS) collected from actively running cow dung based lab scale anaerobic digester (effective I/S ratio of 3/1[26]). The volume of each bottle was then made up to 300 mL using distilled water (final substrate concentration of 5 g VS L^{-1}). The bottles containing inoculum only (without algal biomass) were used as control. Anaerobic digestion was carried out at 37 ˚C under stationary conditions for 30d. Volume of daily gas produced was measured through acidic water (pH < 2.0)

displacement method [26] and methane content was determined through Gas Chromatograph equipped with stainless steel column packed with Porapack-Q 80/100 mesh (Supelco) and thermal conductivity detector [25] .

2.5 Codigestion of pretreated algal biomass

The imbalanced C/N ratio is another major problem in anaerobic digestion of algal biomass. In order to improve the C/N ratio, two wastes materials containing relatively higher carbon *viz.,* cattle-dung (C/N ≈ 30) and spent sugar cane bagasse (SCB) having C/N ≈ 60 were used. The cattle-dung was collected from the cattle dairy shed situated at R. K. Puram, New Delhi, India. The spent SCB was obtained as waste from the solid state fermentation (SSF) in fungal crude enzyme production (section 2.1). Freshly collected dung and spent SCB were processed for determination of elemental composition and volatile solids (VS) content following the standard protocols reported elsewhere [25]. For biomethane potential estimation, the pretreated algal biomass was mixed with each cosubstrate separately at 1:1 ratio (on VS basis) and subjected to anaerobic digestion at substrate concentration of 5 g VS L^{-1} through BMP protocols. Other process conditions and methodologies were similar as reported above (Section 2.4).

2.6 Computation of daily and cumulative methane yields

Methane yield from the algal biomass was calculated by multiplying the daily biogas yield with respective CH_4 content and reported in mL CH_4 g^{-1} VS_{fed}. Daily and cumulative biomethane yield was then calculated using Equation 1 and 2, respectively.

$$B_{net} = B_{exp} - B_0 \quad (1)$$

$$M_i = \sum_{i=1}^{i=i} B_i \quad (2)$$

where, B_0 and B_{exp} are the daily biomethane produced (mL CH_4 g^{-1} VS added d^{-1}) from control and experimental flask, respectively; B_{net} is the net daily biomethane produced from the algal biomass; B_i and M_i are net and cumulative biomethane yield (mL CH_4 g^{-1} VS added) on i^{th} d.

The cumulative methane data was then fitted with the Gompertz equation for estimating the improvement in ultimate methane yield (P), maximum rate of CH_4 production (R_m) & lag phase (λ) in the gas production profile. The used Gompertz equation, adopted from Lay et al. [27] is given as

$$M = P \times exp\left\{-exp\left[\frac{R_m \times e}{P}(\lambda - t) + 1\right]\right\} \quad (3)$$

where M is the cumulative biomethane yield (mL CH_4 g^{-1} VS added) and $e = 2.718$.

Further, first order kinetic equation of anaerobic digestion was also applied to the cumulative methane data in order to predict the enhancement in the hydrolysis constant (k_h). It was assumed that the hydrolysis in the algal digestion follows first order kinetics and k_h was then computed by fitting the cumulative biomethane data to the first order hydrolysis kinetics model adopted from Angelidaki et al. [26].

$$M = P\{1 - exp(-k_h t)\} \quad (4)$$

MATLAB (7.0) was used as the software platform to fit the experimental data in the modes.

2.7 Statistical analysis

All the experiments were performed in triplicate unless otherwise stated. Results are reported as either mean ± SD or error bars in graphs. Same statistical analysis was followed throughout the manuscript, unless stated otherwise.

3 Results and discussion

Crude enzyme produced from *A. lentulus* under SSF was eluted in 0.5M sodium citrate buffer (pH 5.0). The cellulase and xylanase activity of the final elute (crude enzyme) was 0.169 FPU mL^{-1} and 0.658 IU mL^{-1}, respectively, whereas the protein content was 4.005 mg mL^{-1}. The crude enzyme was stored at – 20 ˚C till used for algal biomass pretreatment [12].

3.1 Algal biomass pretreatment at optimal temperature and pH

The optimal temperature (≈ 60 ˚C) and pH (≈ 5.0) for the action of *A. lentulus* crude enzyme have been previously determined [14,28]. In an attempt to reduce the process time and enhance the efficiency, pretreatment was conducted at optimal temperature and pH and the results were compared qualitatively through visual/microscopic observations and quantitatively in terms of sugar and COD solubilisation.

The visual observations revealed that optimal pH and temperature conditions significantly improve the algal biomass treatment. As can be seen from the Figure 2, highest pretreatment of algal biomass occurred at optimal pH and temperature combination within 150 min of incubation. Further, optimal pH was found crucial as pretreatment observed with sample treated at 60 ˚C but without pH adjustment was relatively poor (Fig. 2). No such pretreatment was noticed with algal biomass in control tubes incubated (without enzyme at optimal pH and

temperature) under similar conditions. The current observations have proved the active role of crude enzyme of *A. lentulus* in pretreatment of algal biomass.

In order to further confirm visual observations, microscopic examination of crude enzyme treated algal cells was done. DIC and fluorescent images of crude enzyme treated algal cells are shown in Figure 3. As reflected from DIC images, significant disruption of algal cells was observed within 150 min under optimal conditions (Fig. 3 B-C). Similarly, the fluorescent image (Fig. 3 D) also confirms the cell wall disruption of algae treated with crude enzyme as most of the cells were stained with Sytox-green due to ruptured cell wall. Hence, from the microscopic observation it is clear that crude enzyme of *A. lentulus* has good ability to disrupt algal cell wall. However, further analysis was needed to quantify the resulting pretreatment due to crude enzyme action on algal cell wall.

Quantification of enzyme efficiency in algal biomass pretreatment was done in terms of sugar released and COD solubilisation. The profiles of sugar and COD solubilisation with time are shown in Figure 4. Highest sugar release (up to 60 mg L^{-1}) and COD solubilisation (≈ 50 %) was achieved under optimal conditions (60 ˚C and pH 5.0). Further, soluble sugar concentration and COD solubilisation in case of sample incubated at 60 ˚C but with neutral pH was ≈ 27 mg L^{-1} and 25 %, respectively. Similarly, relatively lower pretreatment was observed with the samples incubated at 30 ˚C with or without pH adjustments. Algal biomass pretreatment in control tubes was insignificant with < 8.0 mg L^{-1} sugar concentration and < 10 % COD solubilisation. Hence, it is obvious that provision of optimal pH and temperature conditions is must to efficiently pretreat the algal biomass in short incubation time. Further, it is worth mentioning that the pretreatment achieved under optimal conditions (within 150 min) was relatively higher than that observed in relatively longer incubation (48 h) under non-optimal conditions [12]. Hence, the

pretreatment time was reduced by almost 19 times under optimal conditions with regard to non-optimal conditions. Recent studies also reported significant enhancement in activity of fungal crude enzyme under optimal conditions [29–31].

It is worth noticing that the observed COD solubilization with fungal crude enzyme was higher than the previously reported value during thermal pretreatment of *Chlorella vulgaris*[32]. Similarly, relatively lower biomass solubilization (< 29 % COD) was reported during biological pretreatment of algal biomass by Alzate et al[2] . Moreover, the observed values are at par with those reported from ultrasonic (62 % COD solubilisation) and thermal (63 % COD solubilisation) pretreatment of algal biomass [2]. Hence, the fungal crude enzyme based pretreatment is efficient enough to replace the conventional biological or physicochemical methods.

Present investigation revealed promising application of fungal crude enzymes in algal biomass pretreatment for biofuel production. Furthermore, pretreatment time was significantly reduced by providing optimal conditions for efficient enzyme action.

3.2 Effect of enzyme pretreatment on BMP and digestibility of algal biomass

3.2.1 Daily and cumulative methane profiles

The COD solubilisation in algal slurry pretreated with crude enzyme for BMP was around 43 – 45 %. The pretreated algal biomass was then subjected to anaerobic digestion in order to determine actual enhancement in algal biomass digestibility and BMP. Variation of daily and cumulative methane profiles with digestion time is shown in Figure 5a.

Effect of enzymatic pretreatment can be clearly seen from the daily methane profile (Fig. 5). The daily methane production from the pretreated algal biomass reached to maxima (up to 39.36 ± 1.33 mL CH_4 g^{-1} VS_{fed} d^{-1}) within first 4 d of the anaerobic digestion. In contrast to this, the first peak (on 3rd d) in case of untreated algal biomass was significantly lower (≈ 13.46 ± 0.54 mL CH_4 g^{-1} VS_{fed} d^{-1}). Further, the biomethane production from untreated biomass remained between 5 – 10 mL CH_4 g^{-1} VS_{fed} d^{-1} during 4 -10 d and again increased to its maxima at 19.06 ± 0.17 mL CH_4 g^{-1} VS_{fed} d^{-1} on 11th d. Beyond this, it started decreasing continuously and remained at lowest value of 5 mL CH_4 g^{-1} VS_{fed} d^{-1} from 17th d onward. Whereas the maximum methane production from pretreated biomass was observed during first 10 d of anaerobic digestion. Significantly higher methane production during the early stage of anaerobic digestion clearly reflects the availability of released cellular constituents of algal biomass due to enzymatic pretreatment. Further, during the last phase (20 -30 d), methane production from pretreated algal biomass was relatively lower than that of untreated biomass. This revealed slower hydrolysis of untreated biomass as compared with pretreated biomass.

Similarly, the cumulative methane profile of pretreated biomass was significantly improved as compared to untreated biomass. The cumulative methane yields from untreated and pretreated algal biomass were 254.73 ± 2.25 and 324.38 ± 4.36 mL CH_4 g^{-1} VS_{fed}, respectively. Hence, there is around 28 % enhancement in the cumulative methane yield with the fungal crude enzyme based pretreatment of algal biomass. Further, as depicted from the cumulative methane profiles, the rate of methane production was much higher for pretreated biomass. In fact the methane yield from pretreated biomass during the first 14 d of the anaerobic digestion (253.57 ± 1.31 mL CH_4 g^{-1} VS_{fed}) was at par with the value obtained for untreated biomass during 30 d.

Hence, it can be concluded that the digestion time for *Chroococcus* sp. biomass may be decreased to 14 d through its pretreatment with fungal crude enzymes.

In a recent study, range of commercial enzymes including lipase, cellulase, xylanase and protease (single or in mixture) were used for pretreatment of algal biomass [11]. Interestingly, the observed improvement in methane yield (28 %) in the current study was higher than that reported with commercial enzymes (20%). Similarly, 24 % improvement in the methane yield has been reported for mechanically (ultrasonic) pretreated microalgae mixture [2]. Hence, it is obvious that the improvement in the methane yield is either higher or at par with the reported values for enzymatic or mechanical pretreatment. However, energy intensive thermal pretreatment was reported to have highest increase in methane yield (46 – 62 %) from the algal biomass [2]. Gonzalez-Fernandez et al. [33] also reported up to 57 % enhancement in the methane yield by thermal pretreatment of *Scenedesmus* biomass. Hence, it is obvious that thermal pretreatment has higher potential to improve biomethanation of algal biomass. However, this may not be true in every case and especially during large scale continuous anaerobic digestion of algal biomass. For instance, Mendez et al. [32] have experimentally proven that although the thermal pretreatment resulted in higher biomass solubilisation, the methane production was hampered. The decrease in biomethane production could be attributed to the reaction by-products such as furanic and phenolic compound [10]. Further, the high energy expenditure in thermal pretreatment makes this method economically unviable. Interestingly, Mahdy et al. [34] recently reported up to 90 % algal biomass solubilisation through pretreatment with commercial hydrolytic enzymes but the enhancement in methane yield was only 14 %. In contrast, present study revealed that the fungal crude enzymes were efficient for algal biomass solubilisation and a significant enhancement (up to 28 %) in methane yield was also observed.

3.2.2 Assessing the effect of pretreatment on BMP through Gompertz model

The cumulative methane data of enzymatically pretreated biomass was fitted with the Gompertz model (Fig. 5b) and the outputs were compared with the values obtained for untreated algal biomass (Table 1). The data showed good fitting with the model equation (R^2 = 0.9915). As a result of enzymatic pretreatment, the ultimate methane yield (P) increased significantly to 321.8 mL CH_4 g^{-1} VS (pretreated biomass) from 267.6 mL CH_4 g^{-1} VS (untreated biomass). Further, the maximum rate of CH_4 production (R_m) reported as mL CH_4 g^{-1} VS d^{-1} was also doubled (from 11.62 for untreated to 22.30 for pretreated biomass) due to enzymatic pretreatment. The observed lag phase (τ) in the gas production profile was also shortened (0.24 d instead of 0.7 d) as a result of enzymatic pretreatment. Apart from the Gompertz equation variable, the first order hydrolysis constant was also improved from 0.0308 d^{-1} to 0.0838 d^{-1}. Moreover, the estimated digestibility was 49.51 % for pretreated biomass incontrast to only 35 % in case of untreated biomass. Similarly, enhancement in the volatile solid reduction (VSR) was also observed. VSR for pretreated and untreated algal biomass was around 66.12 and 48.12 %, respectively. The present observation further validated the observed improvement in the algal biomass digestibility and methane yield due to enzymatic pretreatment. Hence, it is clear that the investigated pretreatment method has tremendous potential to be explored as a suitable approach in algal biomass based biomethane production.

3.3 Codigestion with cattle dung further improves the methane yield

As discussed above, pretreatment of algal biomass with fungal crude enzymes significantly enhances its digestibility and biomethane yield. However, it is to be noticed that the C/N ratio (≈ 9.00) was not optimal in case of pretreated algal biomass (PrT-A). Therefore, it was very likely that the biomethane yield from PrT-A can further be enhanced through its codigestion with carbon rich waste. Hence, anaerobic codigestion studies were conducted with pretreated algal biomass using cattle dung and spent SCB as carbon rich waste. The effect of codigestion on pretreated biomass is illustrated through daily and cumulative biomethane profiles (Figure 6). As observed from the daily methane production profile, the codigestion of pretreated algae with cattle dung (PrT A-CD) resulted in further improvements in gas production.

The methane production from PrT A-CD (C/N ≈ 13.0) was significantly higher than the other test setups. Daily methane production reached up to 30 mL d^{-1} within first 2 d and eventually increased to its maxima at 35 mL d^{-1} on 4th day. The biomethane production remained between 30 to 35 mL d^{-1} during first 9 d of experiment and subsequently decreased to10 mL d^{-1} (on 14th d). Beyond this, the methane production decreased at relatively slower rate. Similarly, methane production profile during codigestion of pretreated algae with spent SCB (PrT A–SCB) attained to its maxima (≈30 mL d^{-1}) on 2nd d and subsequently decreased drastically to 2 – 5 mL d^{-1}. Hence, the SCB had negative effect on anaerobic digestion of pretreated algae. This could be attributed to the poor digestibility [35,36] and methane production from spent SCB alone, as depicted from its daily and cumulative methane profile (Fig.6).

Similarly, the cumulative methane profile for PrT A-CD was better than the other sets. The cumulative methane from PrT A-CD was up to 413.89 mL g^{-1} VS_{fed}. In contrast, the methane production from pretreated algae alone was 324.38 mL g^{-1} VS_{fed}. It is also noticeable that the

methane yield from codigestion of untreated algae with cattle dung was around 291 mL g^{-1} VS_{fed} during our previous study [21]. Therefore, the codigestion of pretreated algal biomass resulted in ≈ 63 % enhancement in methane yield as compared with only 30 % enhancement during fresh algae codigestion with cattle dung [21]. This could be attributed to the improved digestibility of algae having damaged cell wall as well as better activity of anaerobic microflora at relatively improved C/N ratio. Another possible explanation for higher methane production during codigestion of algae with cattle dung could come from the fact that cattle dung is usually rich in anaerobic bacteria and hence provides additional inoculum for fast digestion of algal biomass[21].

To the best of our knowledge, this is the first report which combines crude enzyme based pretreatment and codigestion of algal biomass for improving methane yield and digestibility. Though, codigestion of untreated algal biomass with different organic waste has been attempted previously, the observed enhancement in methane yield was comparatively low. For instance, 23 % and 29 % enhancement in methane yield was observed during codigestion of algae with sewage sludge [18] and codigestion of blue green algae with kitchen waste[20] , respectively.

Overall results indicate that combining the crude enzyme based pretreatment and codigestion of algal biomass with cattle dung could be the most suitable approach for overcoming the hurdles associated with commercialization of algal based biomethane.

3.4 Improved digestibility of pretreated algal biomass under codigestion

To quantify the improvement in algal biomass digestibility and biomethane yield due to pretreatment and codigestion, the cumulative data was further fitted with Gompertz model (Eq. 3) and first order hydrolysis kinetics (Eq. 4) apart from the experimental VSR determination. The

outputs of Gompertz model, hydrolysis constant and VSR values for various BMP substrates are listed in Table 1. The cumulative methane yield for all sets of experiments had good fitness with the theoretical models (R^2: 0.95 – 0.99). The estimated ultimate methane yield (P) values showed that PrTA–CD is the best combination for algal biomethane production. Further, the maximum rate of biomethane production (R_m) from this combination was almost three times of that for the fresh algae.

The R_m value for pretreated algae codigestion with SCB was also improved significantly. Moreover, for PrTA–CD, R_m value increased significantly whereas increase in hydrolysis constant (K_h) was insignificant as compared with PrT-A. It is worth mentioning that although methane yields from bottles having SCB as (co)substrate were poor, the respective K_h values were relatively higher. This is because K_h represents the fraction of ultimate methane yield being converted to actual methane yield. Therefore, it can only be used to study the effect of different treatments of same substrate rather than inter substrate comparisons.

In the more direct approach, the VSR may give better estimate of digestibility. It is interesting to notice that VSR increased to ≈ 66 (PrT-A) and 89 % (PrTA-CD), as compared with only 48 % for Fresh-A. Overall, the algal biomass pretreatment and its codigestion with cattle dung improve not only the biomethane production but also the digestibility. Hence, the current observations have proven that the fungal crude enzymes are excellent tools for pretreatment of algal biomass leading to improved methane production. No previous report exist which utilizes fungal crude enzymes for algal biomass pretreatment to improve the digestibility and methane yield. Some researchers have used range of commercial enzyme for algal biomass pretreatment but in specific case of methane production, use of commercial enzymes seems highly uneconomical. Furthermore, as evident from the literature reports use of single enzyme is not

sufficient to obtain desired degree of biomass solubilization and hence two or more pure enzymes are used in a mixture[11]. In contrast, fungal crude enzymes, which are already a mixture of enzymes, provide cheap alternative for these expensive commercial enzymes. Moreover, utilization of cattle dung as cosubstrate further enhances the methane yield and hence increases the feasibility of algal biomethanation process.

4 Conclusion

The present study was aimed to explore possibilities of utilizing fungal crude enzyme based pretreatment for improving algal biomass digestibility and methane yield under anaerobic digestion. Crude enzymes obtained from *A. lentulus* showed phenomenal solubilization (up to 50 % COD solubilisation within 150 min) of algal biomass and the results were comparable with the conventional physicochemical pretreatment methods. Significant enhancement (≈ 28 %) in methane yield was observed from pretreated algal biomass upon anaerobic digestion. Further attempts were made to improve methane yield by balancing C/N ratio of pretreated algae. Interestingly, codigestion with cattle dung showed commendable results with more than 63 % enhancement in methane yield. However, codigestion with SCB had negative effect on methane yield, probably due to its poor digestibility. The present investigations revealed that methane yield from algal biomass can be significantly enhanced by coupling pretreatment and codigestion with cattle dung. The investigated process has potential to improve the viability of algal biomethane by enhancing methane yield. The present findings warrant further exploration and scale up validation of the process.

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469 Table 1: Value of different parameters estimated from theoretical model and volatile solid
470 reduction (VSR) obtained for various BMP substrates combinations

Substrate	P ($mL\ g^{-1}$ VS)	R_m ($mL\ g^{-1}\ d^{-1}$)	τ (d)	K_h (d^{-1})	R^2	VSR (%)
Fresh A	267.60	11.62	0.70	0.0308	0.99	48.42
PrT-A	321.80	22.30	0.24	0.0838	0.99	66.11
PrT A - SCB	145.30	16.32	0.02	0.1629	0.97	35.50
PrT A – CD	409.50	31.47	0.66	0.0883	0.99	88.90
SCB	54.57	5.402	0.02	0.1401	0.9592	14.80
CD	131.5	10.05	0.000	0.1019	0.9679	49.33

471 (Abbreviations: P: ultimate methane yield; R_m: maximum rate of CH_4 production; τ : lag phase and K_h : first order hydrolysis constant)

Figure Captions

Figure 1: Schematic illustration of the steps involved in the experimental procedure.

Figure 2: Visual observations of effect of crude enzyme on *Chroococcus* sp cells under optimal pH and temperature. (A) Enzyme treated sample at 60 °C and pH 5.0 (B) control (without enzyme) at 60 °C and pH=5.0; (C) Enzyme treated sample at 60 °C and pH=7.0 and (D) control (without enzyme) at 60 °C and pH=7.0

Figure 3: Differential interference contrast and fluorescent images (40X) of algal cells treated with fungal crude enzyme from *A. lentulus* at optimal pH (5.0) and temperature (60 °C): DIC images of samples collected at (A) 0 h; (B) 1 h; (C) 2.5 h and (D) fluorescent images at 2.5 h (Red: live cells; Green: dead cells)

Figure 4: Variation of sugar and COD solubilisation from algal biomass with enzymatic pretreatment under optimal pH and temperature with elapsed time. (AL-crude enzymes from *A. lentulus*, C-control; buffer only)

Figure 5: (a) Daily and cumulative biomethane production profiles of untreated and enzymatically pretreated algal biomass and (b) fitting of the cumulative methane data with the Gompertz equation

493 Figure 6: Variation of (a) daily and (b) cumulative methane production with elapsed time for
494 pretreated algal biomass (PrT-A), spent sugarcane bagasse (SCB), cattle dung (CD) and their
495 different combinations.

Figure 1

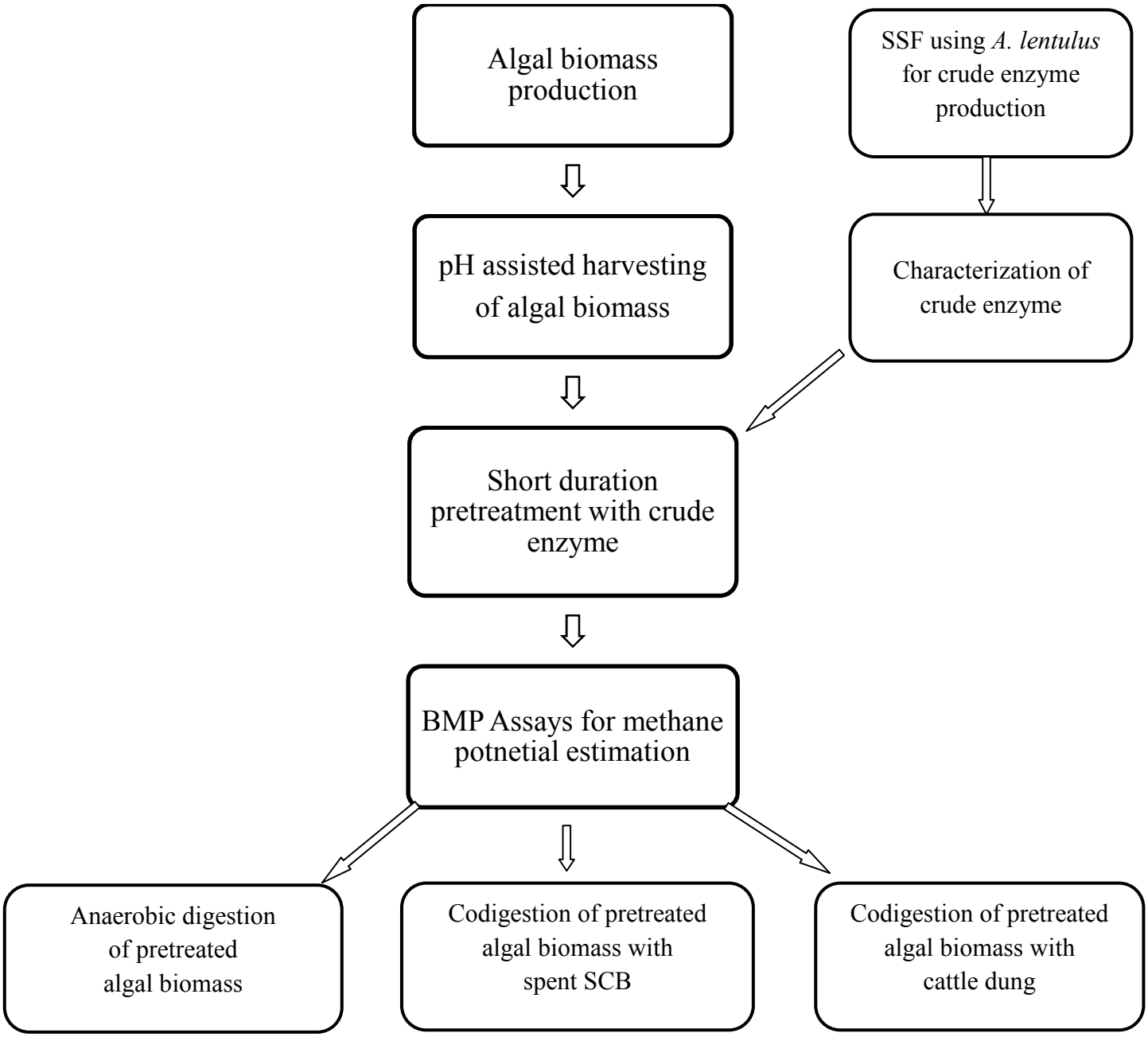


Figure 2

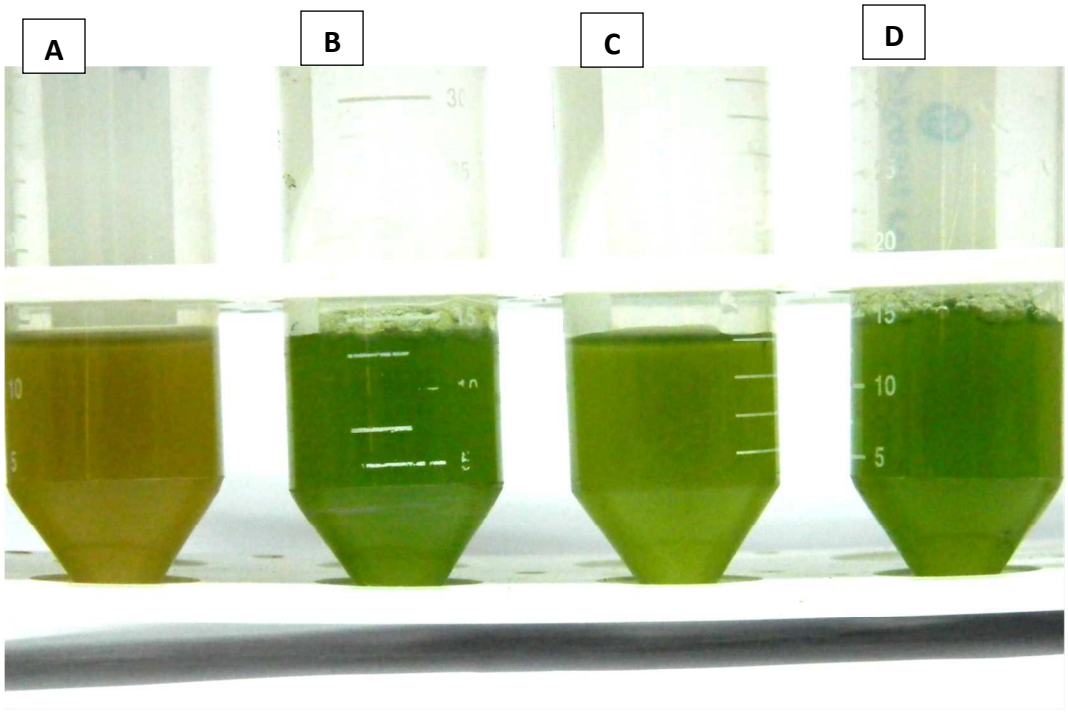


Figure 3

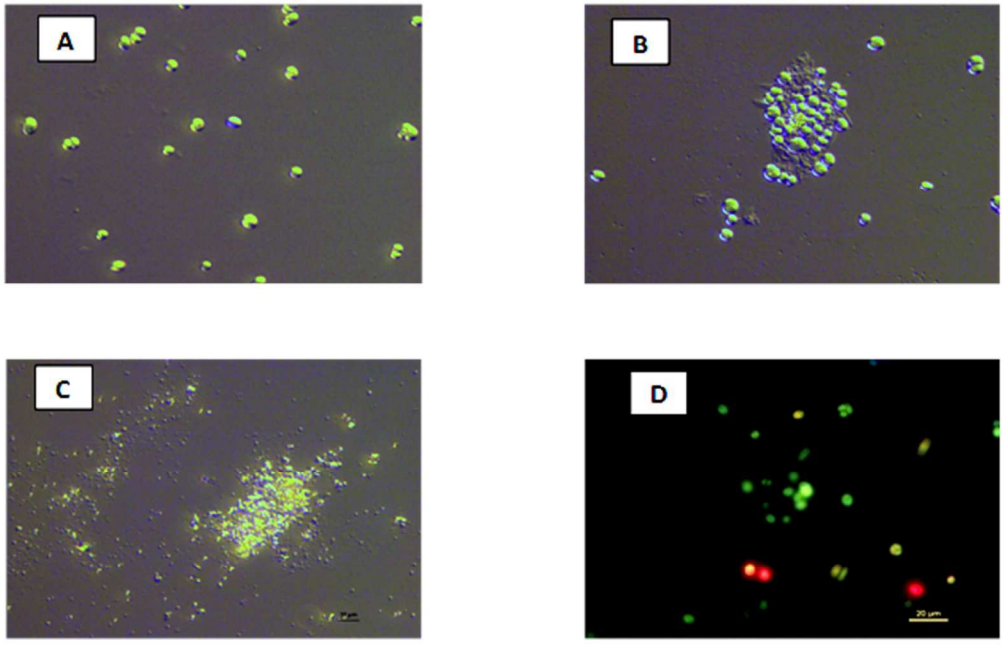


Figure 4

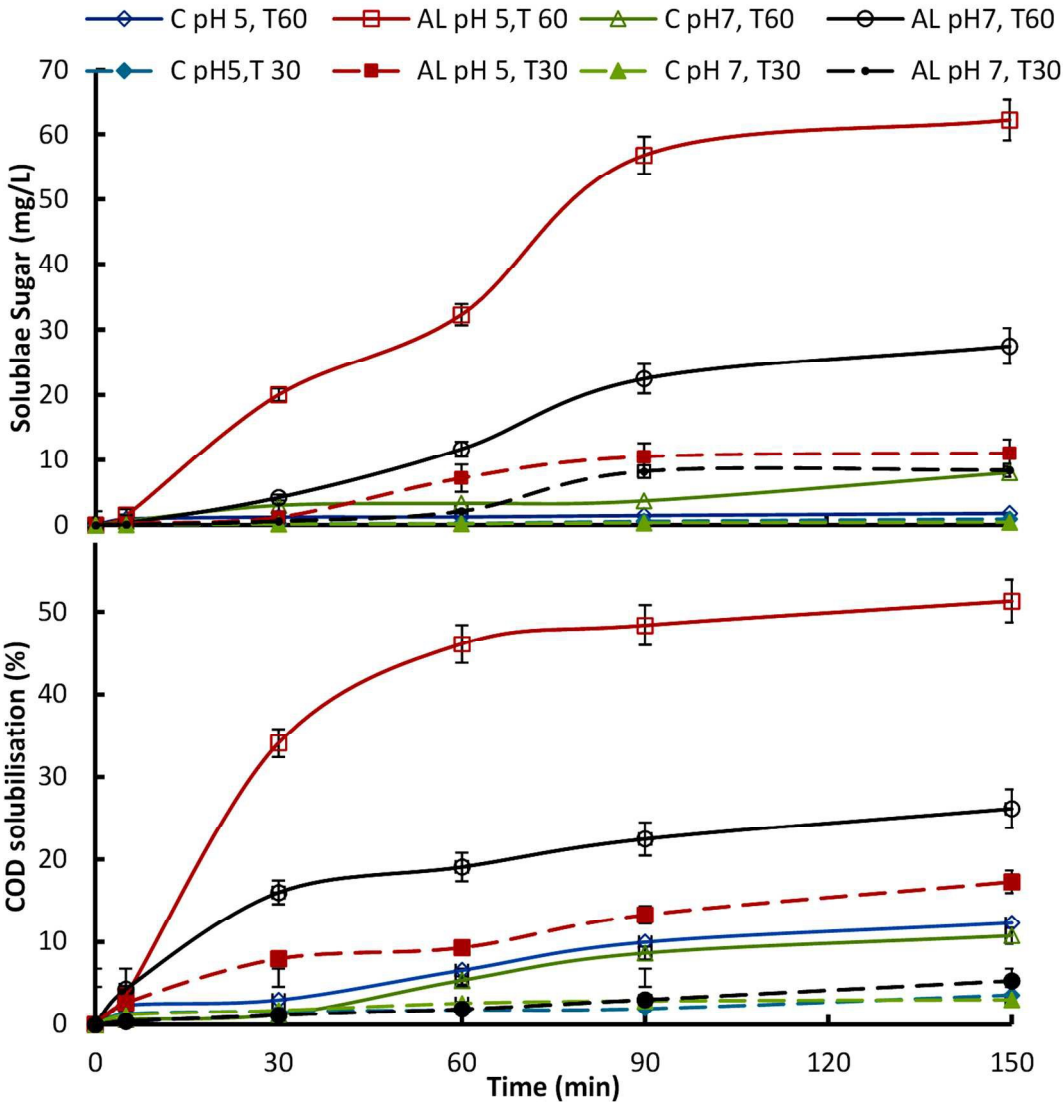
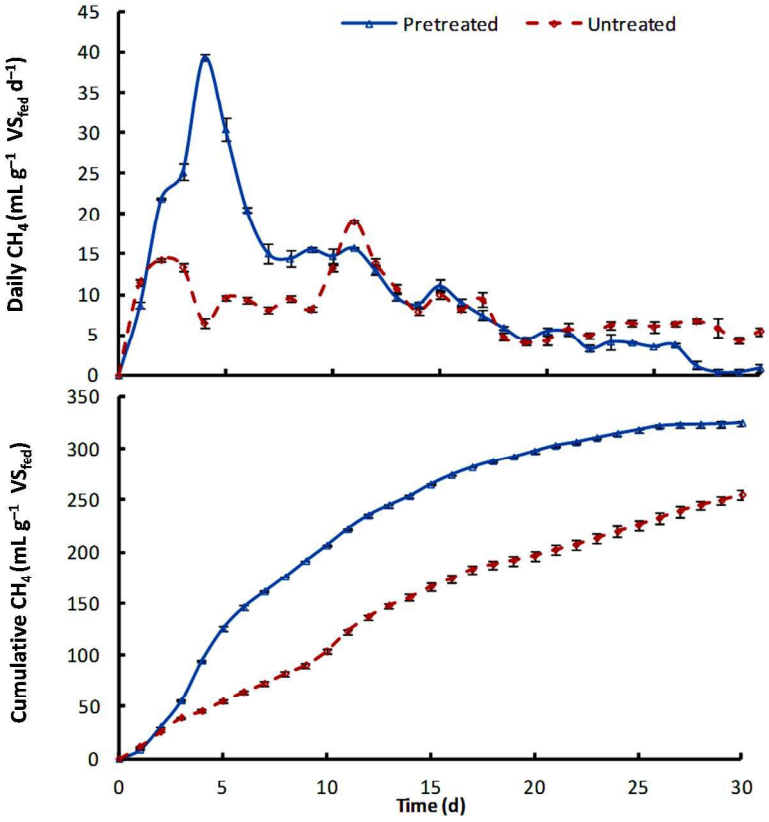
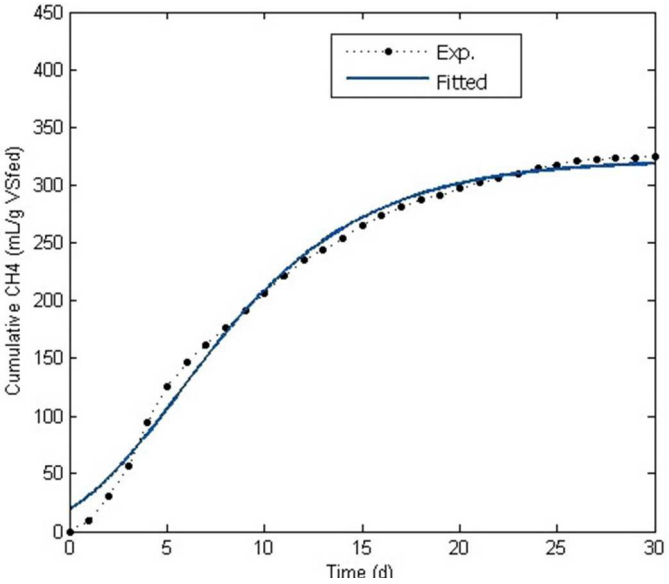


Figure 5



a



b

Figure 6