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Enzymatic pretreatment of algal biomass for enhanced conversion to biogas

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Abstract. This paper presents a method for the enzymatic pretreatment of algal biomass used as a fermentation substrate in anaerobic bioreactors for biogas production, in order to improve the energy efficiency of the biogas systems. The pretreatment method aims at breaking compact carbohydrates (cellulose and hemicelluloses) macromolecular structures from algal biomass under the action of a hydrolytic enzymes mixture secreted by the fungal species *Trichoderma reesei, Trichoderma versicolor, Penicillinum chrysosporium, Fusarium solani, Chaetomium thermophile* and *Myrothecium verrucaria*, thus facilitating access of anaerobic fermentation bacteria to heavily biodegradable cellulosic fibres, reducing fermentation time length and implicitly increasing the biomethane yield of anaerobic reactors. The laboratory experiments involving the marine macroalgae *Ulva* sp. have proven a significant increase in the concentration and total volume of biomethane in the fermentation gas produced by the enzymatically pretreated sample with the selective fungal mixture, compared to the untreated sample. It is expected that such a non-corrosive pretreatment method can bring higher biomethane production with minimal conditioning costs and fewer process residues, thus increasing the biogas systems profitability.

Keywords: algal biomass, pretreatment, biogas, fungal enzymes, energy efficiency

1. Introduction

Algal biomass is currently considered one of the most promising bioresources for various industrial applications in food technology and pharmaceuticals, but special attention is increasingly granted for the renewable fuels production (bioethanol, biodiesel, biogas). In this regard, several studies highlight the advantages that this aquatic culture could bring in addition to terrestrial crops, including the high

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growth rate and productivity, wide range of valuable chemical compounds and lack of competition with agricultural food and feed production [1,2,3]. Moreover, algae have a high content of cellulose and hemicelluloses and low content of lignin, facilitating the biofuels production as no mechanical grinding pretreatments are additionally required which could make expensive and time-consuming processing. On the other hand, algae are more susceptible to hydrolysis, due to the high-water content. Thereby, the energy balance of algae biogas systems is superior to algae biodiesel production systems, primarily because biogas systems use wet resources, but also because biogas production does not need preliminary extraction of oils as required in the biodiesel production processes.

However, there are some impediments that negatively affect the process efficiency for biogas production. Algal cell walls are composed of macromolecules with low biodegradability and/or low bioavailability such as cellulose and hemicelluloses; such structures are hardly accessible to fermentative bacteria, thus prolonging the hydrolysis stage (rate determining stage) of the anaerobic digestion. Consequently, the digestion time is uneconomically prolonged and the biomethane production is hampered, algal cell walls preventing the bacterial access to the nutrient organic compounds in the cytoplasm [4]. To improve the yield of organic matter decomposition in anaerobic bioreactors and implicitly the biogas production, pretreatment techniques are mandatory biorefining steps for algal biomass solubilisation [5].

Until recently, thermal and mechanical pretreatments have been extensively studied and used, being considered as the most effective in breaking the algal cell wall structures. Thermal techniques have led to superior net energy generation in the biorefining processes compared to energy consumption; however, the energy efficiency is dependent on the type of algal biomass [6]. Instead, mechanical pretreatment techniques are less dependent on algal material, but require much higher energy consumption compared to thermal, chemical and biological methods [7]. Chemical methods are less used than thermal and mechanical pretreatments, but have proven to be effective, especially in combination with thermal methods [8]. Besides associated to large quantities consumption [9], the use of chemicals has also the disadvantage to possibly contaminate the end products and, in the case of biogas production, to influence the biochemical balance and acidify the fermentation environment, thus leading to process difficulties or even failure. In addition, some chemicals used in the pretreatment of algal biomass can be inhibitory or toxic for the fermenting microorganisms, reducing or even irreversibly compromising the biogas production in anaerobic bioreactors.

Biological pretreatment using enzymes secreted by various microorganisms is a promising technique for improving the hydrolysis of algal structures, while being an economical method with low energy consumption [10]. It is considered relatively cheap, environmentally friendly pretreatment for improving the anaerobic biodegradability of macroalgal and microalgal biomass [11].

Compared to the above-mentioned pretreatment techniques, the enzymatic methods have low energy consumption, they do not require the use of chemicals that may

have inhibitory effects on bacterial populations and are conducted under mild environmental conditions.

Several laboratory research papers have highlighted the improvement of hydrolysis of recalcitrant chemical structures in algal cell walls, by using pure enzymes secreted by various fungal species such as *Phanerochaete chrysosporium*, *Trametes versicolor*, *Ceriporiopsis subvermispora, Pleurotus ostreatus*, etc. [12,13,14]. Experimental research has shown that in case of biogas production from algal biomass, better biodegradability results have been obtained by using a mixture of different types of enzymes, and not by using single species [10]. This is explained by a chain biodegradation behaviour in which the hydrolysis of one component improves the bioavailability of other components to be hydrolysed [4].

Many of the enzymatic pretreatment processes have involved mostly pure enzymes which were selected according to the chemical composition of the substrate comprising cellulose, hemicelluloses, pectins, glycoproteins, lignin, etc. [15]. So far, the most commonly used enzymes in the treatment of algal biomass were commercial enzymes like α-amylase, amiglucosidase, cellulase, xylanase, lipase and protease [10]. On the other hand, commercial enzyme mixtures led to better biogas yields compared to the case when using a single enzyme with specific activity [16].

This paper presents an innovative enzymatic method for the pretreatment of an algal biomass organic substrate, using a selective fungal mixture. The efficacy of the method was assessed by measuring the cumulative biogas production and the biomethane content in two parallel anaerobic digestion batch experiments of enzymatically-pretreated, respectively untreated algal biomass.

2. Challenges associated to biomass biodegradability

The complexity of biomacromolecules chemical structures and properties make them resistant to enzymatic attack in biochemical processes. Cellulose and hemicelluloses are cemented together by lignin which is responsible for integrity and structural rigidity of lignocelluloses. Glucose is the simplest sugar that can be readily fermented into biogas if it can be accessed.

Some physical and chemical pre-treatment processes (e.g. ultrasonic treatment, dilute acid or base treatment etc.) remove part of hemicelluloses, eliminating or reducing the need for use of enzyme mixtures for degrading biomass. Deconstructing polysaccharides structures in biomass to accessible sugars followed by chemical or fermentation processes is expected to be the most practical pathway to biogas production.

Not only terrestrial biomass is made up of complex polysaccharides but also macroalgae, that have become a valuable bioresource widely exploited for many industrial applications, including for the biofuels industry.

Seaweeds contains specific polysaccharides that make up the composition of the highly complex fibrillar and the matrix polymers of each cell wall [17]. Green macroalgae cell wall polysaccharides represent around 38–54% of the dry algal

matter [18]. Green marine macroalgae belonging to the *Ulva* genus, used as a fermentation substrate in the present research, contain starch as the storage material and several types of structural disaccharides and polysaccharides such as cellulose, xyloglucan, β-mannans, β-xylans, sulfated glucuronoxychloramans and glucuronoxychloramnogalactans [17]. Structure of the main repeating disaccharides found in *Ulva* species (ulvanobiuronic acids) is shown in Figure 1 [19].

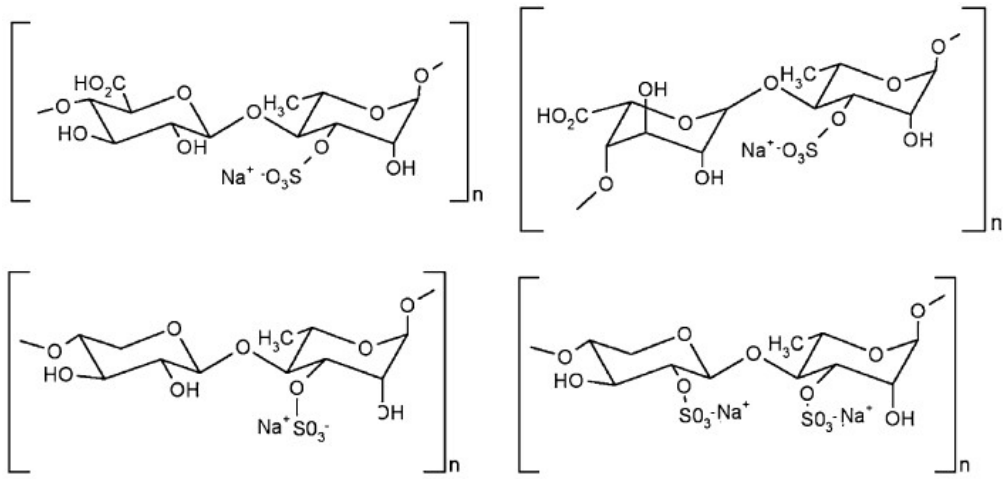

Fig. 1. Polysaccharides structures found in Ulva sp. cell walls.

These macro-compounds are quite resistant to enzymatic attack due to their structural constitution, as shown in Figure 2. The cellulose chains are packed by hydrogen bonds in microfibrils that are attached to each other by hemicelluloses, amorphous polymers of different sugars, as well as other polymers such as pectin, and covered by lignin in case of lignocellulosic biomass. The microfibrils are associated in macrofibrils. This special and complicated structure makes cellulose resistant to both biological and chemical treatments [20]. Special pre-treatment techniques serve to remove or alter recalcitrant hemicelluloses and lignin, remove acetyl groups from hemicelluloses, decrystallize cellulose, and open the long-chain structures to give the enzymes proper accessibility to work.

The primary challenge in the biomass conversion to valuable products, including biogas, is that the glucose in cellulose is joined by beta bonds in a crystalline structure that is far more difficult to depolymerize than the alpha bonds in starch. Also, hemicellulose is an amorphous polymer that is more easily hydrolysed into its component sugars than is cellulose. However, native organisms do not efficiently ferment this range of sugars to products [21].

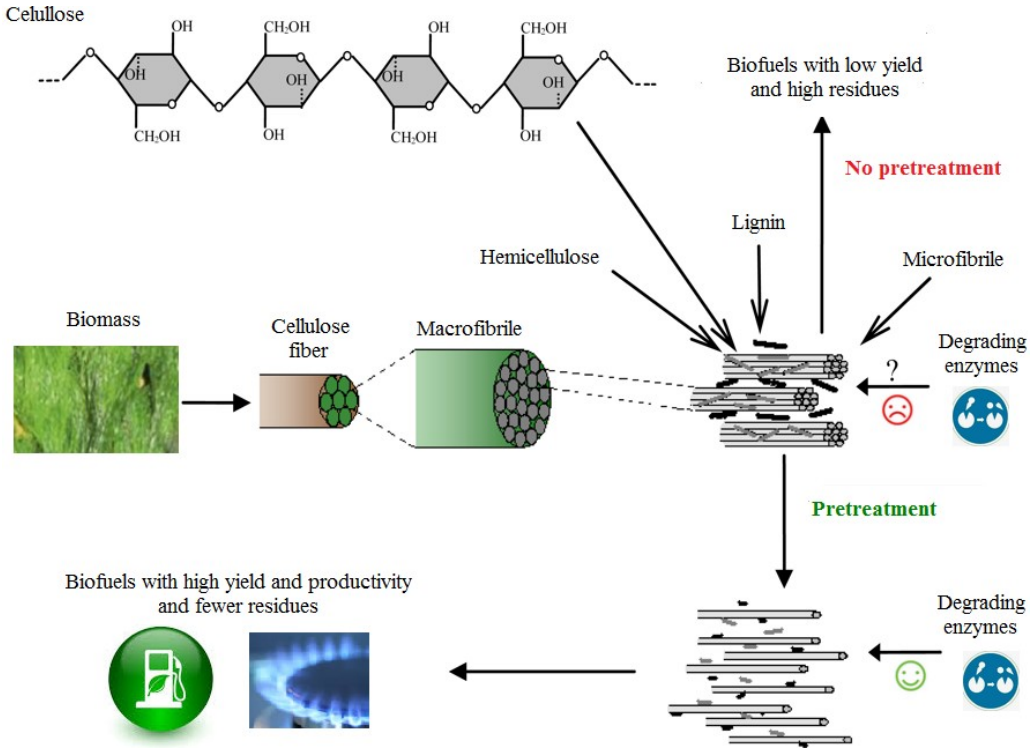

Fig. 2. Effect of enzymatic treatment on cellulosic fibres (image adapted from [20]).

Besides biomass composition, there are many other physical, chemical and physiological factors that affect biodegradation of organic materials in anaerobic digesters and influence biogas production. Thus, redox conditions, temperature, pH, hydraulic retention time, organic loading rate, mass mixing, C/N ratio represents important process issues to consider for an efficient conversion of organic waste to biogas [22]

2. Materials and methods

The fermentation organic substrate, represented by *Ulva intestinalis* marine macroalgae, was supplied by INCDDD, Tulcea, after being harvested from the Black Sea shore in the Danube Delta Biosphere Reserve. The algal biomass was stored in freezer prior to pretreatment and anaerobic digestion experiments.
The pretreatment method consisted of exposing the macroalgae sample to the biological action of an enzymatic fungal mixture secreted by the following 6 types of filamentous fungal species: *Trichoderma reesei, Trichoderma versicolor, Penicillinum chrysosporium, Fusarium solani, Chaetomium thermophile* și *Myrothecium verrucaria*. The fungal exposure was maintained for 24 hours and accomplished before the initiation of the anaerobic digestion tests and aimed at

improving the hydrolysis of recalcitrant compounds in the algal cell walls, reducing the fermentation time length and increasing the substrate biomethane potential.

The preparation of the enzymatic mixture consisted in the cultivation in nutritive salts solution of the fungal species *Trichoderma reesei*, *Trichoderma versicolor*, *Penicillinum chrysosporium, Fusarium solani, Chaetomium thermophile* and *Myrothecium verrucaria*; the fungal species used in the experiment had an average maturity of 14 days from the date of sowing. The nutritive solution was obtained according to the method described in SR CEI 68-2-10 standard, using the following compounds: 0.7 g/L KH_2PO_4, 0.3 g/L K_2HPO_4, 0.5 g/L $MgSO_4x7H_2O$, 2 g/L $NaNO_3$, 0.5 g/L KCl, 0.01 g/L $FeSO_4x\ 7H_2O$, 30 g/L sucrose [23]. Fungal suspensions from each species were prepared with 10 mL distilled water, after which the aliquots were combined and nutritional solution was added up to a volume of 500 mL.

The algal biomass was inoculated with a native mixed fermenting bacteria from cattle manure. A volume of 50 ml fungal enzymatic mixture was added to 125 mL of fermentation substrate and conditioned at 28±1°C for 24 hours. The total water content of the fermentation slurry was 90%. After this conditioning process, the enzymes-treated organic substrate was subjected to batch anaerobic digestion tests, in mesophilic temperature regime at 37±1°C, for a total fermentation time length of 30 days. Anaerobic digestion tests were carried out in 500 mL serum bottles, sealed with butyl rubber stoppers and connected via Teflon tubes to 5 L Supel-Inert Multi-Layer Foil gas bags. Anaerobic conditions were created by flushing nitrogen for 3 minutes to remove the air in the headspace, after which the bottles were immediately closed with rubber stoppers and sealed with silicone tape. The bottles were manually homogenized twice a day.

In order to determine the influence of the fungal mixture on the substrate biodegradability, parallel testing was performed both for the substrate consisting of untreated algae and the substrate with enzymatically treated algal biomass. Measurements of biogas production and methane concentration in biogas were carried out periodically after the digestion test initialization, on the experiment days 7, 14, 18, 22, 26 and 30, respectively.

The biogas analysis was performed using a Varian 450 gas chromatograph coupled to a flame ionization detector (FID). Methane standard 5.5 supplied by Linde was used to calibrate the chromatograph before determining the methane content in biogas.

The calibration curve was obtained by measuring the resulting peak area for the following ratios of CH_4/air: 100:0; 75:25; 50:50; 0:100. Methane showed a retention time of 4.774 minutes. For each concentration ratio, measurements were done in triplicate.

3. Results and discussions

The experimental results for the enzymatically treated organic substrate sample and the control sample are plotted in Figure 3. The experiment was conducted over 30 days, which represented the significant gas production period.

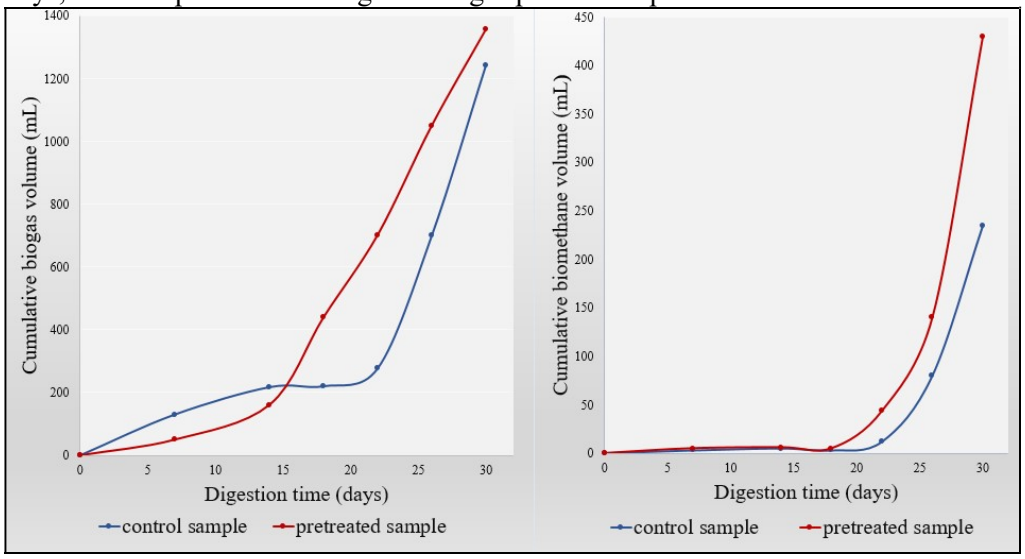

Fig. 3. Cumulative biogas (left) and biomethane (right) production in digestion assays.

Figure 3 shows that during the first 15 days of fermentation the biogas production for the control sample started faster compared to the enzyme-treated sample. This behavior can be attributed to the microorganisms need to accommodate with the substrate containing the added fungal mixture. After the accommodation stage, the control sample showed a cease in biogas production that might be related to the longer lasting hydrolysis stage after the easily accessible organic compounds in the substrate had already been digested. The sharp increase in biogas production for the control sample demonstrated the process progress towards the gas-producing biochemical stages (acetogenesis, metanogenesis), subsequent to the conversion of macromolecules into intermediate organic fragments such as organic acids, alcohols, etc. (acidogenesis).

Instead, the pretreated sample showed a much lower hydrolysis time length, indicating the biomacromolecules decay efficiency in the substrate under the action of the selected fungal suspension. An increase in cumulative biogas volume from 1242 ml for the control sample to 1356 ml for the enzymatic pretreated sample was noticed, which represents an improvement in the total biogas production of 9.2%. Regarding the cumulative biomethane volume in biogas at 30 days (as seen in Figure 3, right), an increase from 235 ml CH_4 for the control sample to 430 ml CH_4 for the enzymatic pretreated sample was observed; this means a rise in biomethane production of 83%.

Biomethane production curves for both tests showed that in the first 18 days, the fermentation gases contained very low methane concentrations. At this point, the resulting biogas consisted mainly of carbon dioxide along with small concentrations of other gases (H_2S, NH_3, H_2O, H_2 etc.) which indicated that the process was before the final stage of anaerobic digestion (metanogenesis). After approx. 20 days of experiment, the methane concentration increased sharply for both samples; the stimulatory effect of the enzymatic fungal mixture was obvious in the pretreated sample which reached a maximum methane concentration of 59%. This result is consistent with literature data for marine microalgae, but the content is lower than the methane level generated by freshwater microalgae. The poor performance of macroalgae in producing high methane-content biogas could be explained by the inhibitory effect of salinity, discussed in many experimental studies [24,25].
Therefore, this experiment demonstrated that the enzymatic pretreatment has induced not only the increase of the biogas volume, but also an improvement by 145% of its energetic value in terms of methane content [26].

4. Conclusions

Biomass pre-treatment techniques aim at improving the biodegradability of recalcitrant structures in cell walls (cellulose, hemicellulose, lignin) in order to facilitate access of microorganisms to cell nutrients and to increase biogas production.
The experimental results have shown both increase in the cumulative biogas volume for the enzymatic pretreated biomass sample with 9,2%, compared to the control sample and increase in the methane total production with 83%. This behaviour may be due to the stimulatory effect of the enzymes secreted by the selected fungal species on the biodegradability of compact organic structures in the cell walls of the algal biomass.
The proposed innovative pretreatment method can be applied to biomass samples including macroalgae, filamentous algae and microalgae which may be used as an organic fermentation substrate in biogas reactors.
In order to elucidate the mechanisms by which this pretreatment technique acts at the cellular level, additional research is needed, both on laboratory models and on pilot-scale bioreactors.

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