ENHANCEMENT OF BIOGAS PRODUCTION FROM MICROALGAE BY ENZYMATIC PRETREATMENT

ENZİMATİK ÖN ARITIM İLE MİKROALGLERDEN BİYOGAZ ÜRETİMİNİN ARTTIRILMASI

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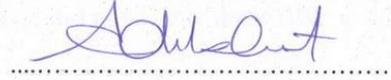Assoc. Prof. Dr. S. Ferda MUTLU
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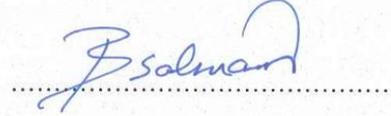Prof. Dr. Ayşenur UĞURLU
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ABSTRACT

ENHANCEMENT OF BIOGAS PRODUCTION FROM MICROALGAE BY ENZYMATIC PRETREATMENT

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Renewable energy sources have gained considerable attention due to increasing global energy demand and environmental concerns on fossil fuels. Anaerobic digestion is one of the renewable technologies that can produce gaseous energy from several types of substrates. Microalgal biomass is a good candidate for anaerobic digestion, however; rigid cell wall characteristics prevent accessibility of anaerobic bacteria to degrade microalgal biomass and limits methane production from microalgae. To enhance methane production from microalgae, application of pretreatment methods prior to anaerobic digestion provides rigid cell wall disruption and cell membrane solubilization. As a result, enhanced methane production potentials could be achieved by increasing the accessibility of microalgal biomass for hydrolytic bacteria.

Main objective of this thesis was to enhance methane production from microalgae biomass by enzymatic pretreatment prior to anaerobic digestion. Enzymatic pretreatment was carried out with two different microalgae (*Scenedesmus sp.* and

Porphydium cruentum) at different pretreatment conditions including different enzymes (cellulase, protease, viscozyme, and enzyme mix including protease and visczoyme), enzyme doses (0.1-0.3-0.5 mL/ g biomass), temperatures (25-55°C), and time (1-24 hours). In order to determine the effectiveness of the enzymatic pretreatment, organic matter solubilization and cell wall disruption after enzymatic pretreatment were evaluated and methane production potential of pretreated biomass were determined by Biochemical Potential Tests (BMP) at both mesophilic (35°C) and thermophilic (55°C) range.

The results showed that enzymatic pretreatment at 55 °C showed highest solubilization efficiencies and efficiency of enzyme used in pretreatment is directly related to characteristics of microalgae. In case of *Scenedesmus sp.,* cellulase (8.8-12.3% solubility increase), protease (14.7-16 % solubility increase), viscozyme (8.2-16% solubility increase), and enzyme mix (22.5-37.8% solubility increase), were effective for cell wall degradation and biomass solubilization. In case of *P. cruentum*, protease (14.9-32.3 % solubility increase), viscozyme (27-30.4 % solubility increase), and enzyme mix (22.3-30.53 % solubility increase) were effective to improve biomass solubilization. After enzymatic pretreatment, highest methane improvements were achieved for *Scenedesmus sp.* as 109% after enzyme mix pretreatment (0.5 mL) at mesophilic temperature and 71.4% after protease pretreatment (0.5 mL dose) at thermophilic temperature. For *P. cruentum,* methane improvements were achieved as 71.7 % after protease pretreatment (0.5 mL dose) at mesophilic temperature and 100% (0.5 mL dose) after protease pretreatment at thermophilic temperature. According to the results, thermophilic digestion provided higher methane yields for both microalgae and highest methane improvements were achieved with enzyme mix and protease pretreatment for both microalgae Kinetic model studies showed that modified Gompertz model were able to fit experimental values than first order kinetic model. Energy assessment results indicated the ratio of energy input to the energy output were less than 1 for both untreated microalgae. However, the ratios were reduced by application of enzymatic pretreatment for both microalgae.

Keywords: Microalgae, Enzymatic Pretreatment, Anaerobic Digestion, Biogas

ÖZET

ENZİMATİK ÖN ARITIM İLE MİKROALGLERDEN BİYOGAZ ÜRETİMİNİN ARTTIRILMASI

Ece KENDİR ÇAKMAK

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Yenilenebilir enerji kaynakları, küresel enerji ihtiyacının artması ve fosil yakıtlardan kaynaklı çevresel endişelerin artması sebebiyle kaydadeğer bir dikkat kazanmıştır. Anaerobik çürütme yenilenebilir enerji teknolojilerinden bir olup çeşitli biyokütlelerden gaz enerjisi üretmeye olanak sağlamaktadır. Mikroalgler, anaerobik çürütme için güzel bir adaydırlar, fakat sert hücre duvarı yapısına sahip mikroalglar anaerobik bakterinin mikroalg biyokütlesini parçalamasını engellemekte ve metan üretiminde limitleyici olmaktadır. Mikroalglerden metan üretimini geliştirmek için, ön arıtım yöntemlerinin anaerobik çürütme öncesinde uygulanması, sert hücre duvarı yapısının parçalanmasını ve biyokütle çözünürlüğünün artmasını sağlamaktadır. Sonuç olarak, iyileştirilmiş metan üretim potansiyelleri elde edilebilmektedir.

Bu tezin ana amacı, anaerobik çürütme öncesinde mikroalg biyokütlesine enzimatik ön arıtım uygulayarak metan üretiminin geliştirilmesidir. Enzimatik ön arıtım, iki farklı mikroalg türü (*Scenedesmus sp.* ve *Porphydium cruentum*) kullanılarak enzim (selülaz,

proteaz, karbohidraz karışımı, enzim karışımı) enzim dozu (0.1-0.5 mL/ g biyokütle), sıcaklık (25-55°C) ve zaman (1-24 saat) gibi farklı koşullarda gerçekleştirilmiştir. Enzimatik ön arıtımın etkisini belirleyebilmek için enzimatik ön arıtım sonrasında organik madde çözünürlüğü ve hücre duvarının parçalanması incelenmiştir ve ön arıtım uygulanmış biyokütlenin metan üretim potansiyelleri biyokimyasal metan potansiyel testleri ile hem mezofilik hem de termofilik sıcaklık aralığında incelenmiştir. *Scenedesmus sp.* için selülaz (8.8-12.3% çözünürlük artışı), proteaz (14.6-17% çözünürlük artışı), karbohidraz (8.2-16% çözünürlük artışı) ve enzim karışımı (22.5-37.8% çözünürlük artışı), hücre duvarının parçalanması ve biyokütlenin çözünürlüğü açısından etkili olmuştur. *P.cruentum* için ise, proteaz (14.9-32.3% çözünürlük artışı), karbohidraz karışımı (27-30.4% çözünürlük artışı) ve enzim karışımı (22.3-30.53% çözünürlük artışı) etkili olmuştur. Enzimatik ön arıtım sonrasında en yüksek metan gelişim potansiyelleri *Scenedesmus sp.* için mezofilik sıcaklıkta, enzim karışımı (0.5 mL doz) ile yapılan ön arıtım sonrasında 109 %, termofilik sıcaklıkta ise proteaz (0.5 mL doz) ön arıtımı sonrasında 71.4% artış göstermiştir. *P.cruentum* için ise mezofilik sıcaklıkta, proteaz (0.5 mL doz) ile yapılan ön arıtım sonrasında 71.7 %, termofilik sıcaklıkta ise proteaz (0.5 mL doz) ön arıtımı sonrasında 100 % artış göstermiştir. Her iki mikroalg türü için de termofilik çürütmede daha yüksek metan üretim potansiyelleri elde edilmiştir ve en yüksek metan iyileştirme değerleri her iki mikroalg türü için de enzim karışımı ve proteaz ön arıtımı ile elde edilmiştir. Kinetik model sonuçlarına göre modifiye edilmiş Gompertz modeli birinci derece reaksiyon kinetik modeline göre deneysel datalar ile daha iyi uyuşmuştur. Enerji değerlendirmesi sonuçlarına göre, ham mikroalg için enerji oranı 1 den daha yüksek çıkmıştır. Fakat, bu oranlar enzimatik ön arıtım ile düşürülmüştür.

Anahtar Kelimeler: Mikroalg, Enzimatik Ön Arıtım, Anaerobik Çürütme, Biyogaz

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1. INTRODUCTION

Energy demand due to population increase and industrialization is a global problem that each country tends to focus on alternative energy production strategies to achieve their development goals. Instead of fossil fuels, use of renewable energy reduces concerns about environmental issues and energy security. Among renewable energy sources, according to future projections, the role of biomass energy has the great potential with an increase in market share. They are environmentally friendly with less CO_2 emissions than fossil fuels and they are various biomass sources that can be utilized for energy production. Moreover, they can be produced locally which means that there is no external dependence like in fossil fuel case. Therefore, countries focus on more bioenergy production, put budget for investments year by year and find alternative biomass sources for energy production. Microalgae are one of the promising biomass sources, which have a great potential for generation of bioenergy. Microalgae biomass has many advantages such as higher biomass production per unit area, higher photosynthetic efficiency, able to grow in wastewater and in non-arable lands. Microalgae biomass can be processed for biodiesel, bioethanol, biogas, and bioelectricity etc. production. Among these, anaerobic digestion is a simpler and environmentally friendly way to produce gaseous energy as methane. It has some advantages over other bioenergy pathways such as use of all portions of biomass (carbohydrates, proteins, and lipids). Furthermore, it does not require expensive extraction processes such as transesterification, and it is possible to integrate into several biorefinery or wastewater treatment concepts to produce energy.

There are some factors affecting biogas production from microalgae such as macromolecular composition, cell wall degradability and operational parameters such as pH, temperature, mixing, and reactor design etc. Among these, cell wall degradability is considered as one of the most important characteristics of microalgae that may influence biogas yields. Microalgae having rigid cell wall characteristics including cellulose, hemicellulose, or algeanan are very resistant to degradation during anaerobic digestion and it reduces the availability of organic matter in the cell for hydrolytic

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bacteria. So, before anaerobic digestion, cell disruption of microalgae by various pretreatment methods provides biomass solubilization and enhanced contact of biomass with anaerobic archaea and thus higher biogas yields. Among pretreatment methods, enzymatic pretreatment is considered as an effective and low energy demand process. High selectivity of enzymes, mild environmental conditions, and no inhibitory products are some of the advantages of enzymatic pretreatment.

The literature studies that focus on enzymatic pretreatment and biogas production from microalgae is limited. Specifically, optimization of enzymatic pretreatment with investigation of pretreatment conditions for achieving maximum biomass solubilization has not studied well. So, further research is needed to understand the effect of different enzymatic pretreatment conditions on solubilization of microalgae biomass and biogas production improvement. Furthermore, different microalgae species should be also investigated for their methane production potentials and their responses to enzymatic pretreatment. Lastly, there are very few studies concerning thermophilic digestion of microalgae. These gaps should be fulfilled to have a better understanding of use of microalgae for biogas production and applicability of enzymatic pretreatment to microalgae for improved biogas production.

The thesis aimed to apply enzymatic pretreatment for investigation its effect on biomass solubilization and ultimately methane yields from microalgae. It was also aimed to quantify potential methane yields from untreated and enzymatically pretreated microalgal biomass at different anaerobic digestion temperatures (mesophilic and thermophilic) to observe effect of temperature on methane production potential from microalgal biomass.

In order to fulfill these aims, two different microalgae (*Scenedesmus sp.* and *Porphydium cruentum*) that have different cultivation conditions and cell characteristics (cell wall and composition) were selected in order to investigate the effect of microalgae characteristics on enzymatic pretreatment and methane yields. Enzymatic pretreatment was applied with enzymes including cellulase, protease, viscozyme, and enzyme mix

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(including viscozyme and protease), enzyme doses (0.1-0.5 mL/ g biomass), temperatures (25-55°C), and contact times (1-24 hours) in order to determine optimum conditions for maximum solubility of microalgae biomass after pretreatment. Biochemical Methane Potential tests (BMP) were applied under mesophilic and thermophilic temperatures to observe the effect of digestion temperature on methane yields from untreated and pretreated microalgal biomass. Kinetic models including first order kinetic model and modified Gompertz model were applied to better understand the behavior of anaerobic digestion of microalgae. Energy assessment was also carried out in order to evaluate the feasibility of biogas production of commercial scale from enzymatically pretreated microalgae.

The organization of this thesis is divided into five chapters. Chapter 2 gives a brief literature on microalgal biorefinery process including cultivation, harvesting, pretreatment and bioenergy production strategies from microalgae biomass. Special attention was given to advantages and common limitations of anaerobic digestion of microalgae biomass and possible solutions to enhance biogas production with a detailed discussion of pretreatment methods.

Chapter 3 includes material and methods followed throughout the study including cultivation conditions for microalgal biomass, harvesting, application and optimization of enzymatic pretreatment, the procedures followed through anaerobic digestion, kinetic models for methane production, statistical analysis and analytical methods.

Chapter 4 includes the evaluation and discussion of the results. In detail, the performance of the enzymatic pretreatment conditions was discussed in terms of change in solubilization and optimization studies were conducted to achieve optimum pretreatment conditions for maximized solubilization. Chapter 4 also focuses on methane production potential of untreated and pretreated microalgal biomass under both mesophilic and thermophilic conditions. Detailed energy assessment was conducted to visualize the feasibility of enzymatic pretreatment for biogas production from

microalgae. Finally, Chapter 5 focuses on conclusions derived from this study and gives a comment about future studies.

2. LITERATURE REVIEW

2.1. Background

The growing global demand on energy forces due to increase in human population and industrial activities forces more pressure on fossil energy. However, fossil energy sources cannot fulfill this demand since they are limited sources. Furthermore, fossil fuels lead to several types of environmental problems including air, soil and water pollution. At this point, as a sustainable alternative, use of renewable energy sources, have been studied and applied extensively to tackle the global energy demand. Renewable energy is more sustainable when compared to fossil fuels since the releases of greenhouse gases are lower or carbon neutral and their sources are inexhaustible [1]. For these reasons, renewable energy sources began to have more attention in the global energy production sector. Future projections showed that the share of renewable energies for electricity would increase 20% to 30% in 2040 with a rising rate of 2.8% per year, although the shares of coal and liquid fuel will decrease gradually [2].
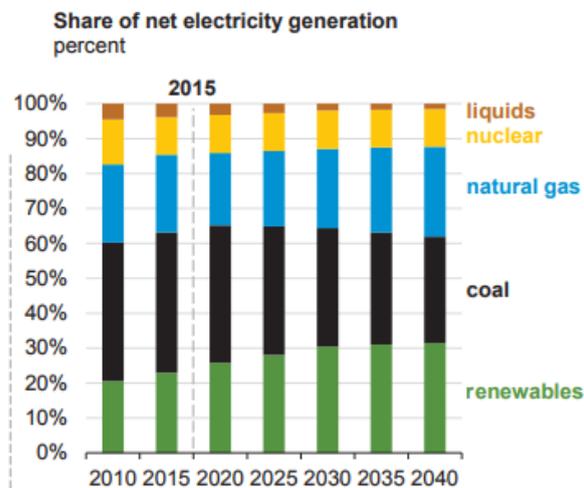
Figure 2. 1. Future projections for the contribution of energy sources for electricity generation [2]

Renewable energy sources are classified in terms of their sources as solar, wind, geothermal, or biomass. Among these, biomass energy has greater importance with a supply of 14% of global renewable energy (18%) (Figure 2.2). Biomass is defined as all organic material derived from plants as woody, herbaceous and aquatic plants and wastes such as manures [3]. Plants are able to produce biomass using CO_2, water, and sunlight via photosynthesis. During photosynthesis, sunlight energy is stored as the chemical energy in biomass and through combustion or decomposition, the chemical energy produced can be used as electricity, heat, fuel and chemical feedstock [3]. Bioenergy is a good alternative especially for countries which do not have enough fossil fuel sources.
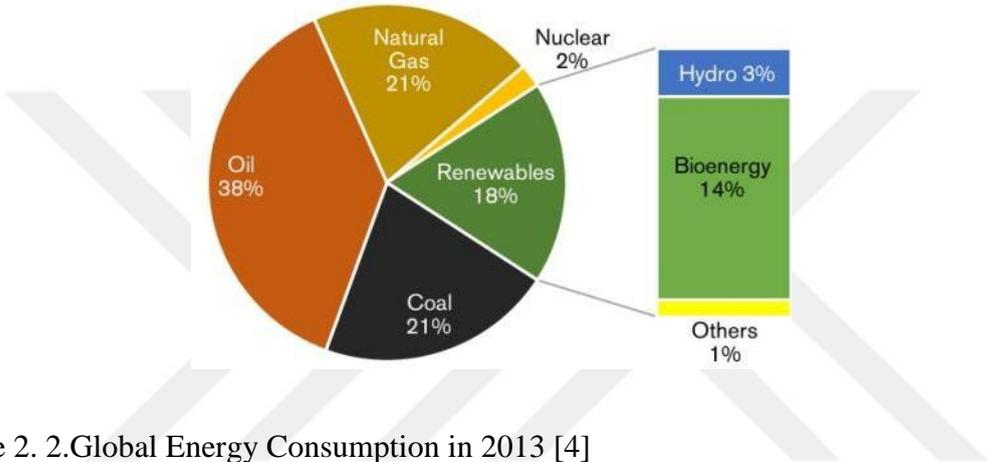
Figure 2. 2.Global Energy Consumption in 2013 [4]

Fuels derived from biomass directly and indirectly are called as 'biofuel'. Biofuels are classified into three categories that are namely, first generation, second generation, and third generation [5]. In the last years, 'fourth generation' is also used. First generation of biofuels is derived from energy crops such as sugar, starch, and vegetable oil. It includes ethanol production from wheat, barley, corn, potato, and sugarcane and biodiesel production from soybean, sunflower and animal fat. The main obstacle for this generation is requirement for arable land for growth [6]. Furthermore, the energy crops are also used for human nutrition leading to increase in market prices and threatening food security. Second generation of biofuels is defined as biofuels produced from non-food biomass such as agricultural, forestry and various types of wastes [7]. The main advantages of this type of biofuels are the use of non-food biomass and providing a sustainable alternative for waste management. Third generation of biofuels is the type of biofuel derived from algal biomass and fourth generation of biofuels is the use of genetically modified algae f or

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biofuel production. Algal biomass has higher extraction yield per unit cultivated area, high biomass production, high photosynthetic efficiency (3-8%) higher carbon dioxide sequestration potential than terrestrial crops [8,9]. Additionally, they can grow up anywhere (no need for arable land) including wastewaters [10,11]. These advantages make algal biofuel production more favorable than other biofuel generations.

The following sections gave solid information about algae characterization, biorefinery concept, and bioenergy production from especially from microalgae.

2.2. Characteristics of Algae

Algae consist of both unicellular (microalgae) and multicellular (macroalgae or seaweed) photoautotroph and live in freshwater and marine environment [12, 13]. They are plant like organisms that do photosynthesis for their growth using carbon dioxide, water and solar energy to produce oxygen and organic matter. Algae are responsible for 70% of oxygen produced on earth [14].

'Macroalgae 'are referred as large, multicellular organisms having a size from a few millimeters to meters that are grown in marine and freshwater environment [12]. Macroalgae have similar characteristics with plants, however; they do not have roots, stems, or leaves [15]. They can be classified into three groups according to pigmentation, which are namely brown seaweed (*Phaeophyceae*), red seaweed (*Rhodophyceae*) and green seaweed (*Chlorophyceae*).

'Microalgae' are referred as algae having a size of 5-50 µm, which are unicellular or filamentous [5, 16]. In terms of taxonomy, microalgae are classified into three categories: diatom (*Bacillariophyceae*), green algae (*Chlorophyceae*) and golden algae (*Chrysophyceae*) [12, 15]. Some types of cyanobacteria such as *Arthrospira platensis* and *Arthrospira maxima* are also considered as microalgae [12]. Microalgae are also classified according to carbon supply. Photosynthetic algae produce glucose and oxygen by using light and carbon dioxide. On the contrary, heterotrophic algae do not use sunlight or carbon

dioxide and needs organic carbon for their growth. Mixotrophic algae can use both carbon dioxide and organic carbon [14].

The composition of microalgae species depends on the type of strain. Table 2.1 shows macromolecular composition (protein, lipid, and carbohydrate) of some microalgae. Generally, selection of microalgae for industrial production or biofuel production is based on their composition. For instance, microalgae having high protein content mostly used to supply protein for human nutrition or animal feeding purposes. On the contrary, microalgae having high lipid content are used extensively for biodiesel production.

Table 2. 1.Composition of some microalgae species [17, 18]

Species	Protein (%)	Lipid (%)	Carbohydrate (%)
Euglena gracilis	39-61	14-20	14-18
Chlamydomonas reinhardtii	48	21	17
Chlorella pyrenoidosa	57	2	26
Chlorella vulgaris	51-58	14-22	12-17
Spirulina maxima	60-71	6-7	13-16
Spirulina plantesis	46-63	4-9	8-14
Scenedesmus obliquus	50-56	12-14	10-17
Scenedesmus quadricauda	47	1.9	-
Scenedesmus dimorphus	8-18	16-40	21-52
Spirogyra sp.	6-20	11-21	33-64
Dunaliella bioculta	49	8	4
Dunaliella salina	57	6	32
Prymnesium parvum	28-45	22-38	25-33
Tetracelmis maculata	52	3	15
Porphydrium cruentum	28-39	9-14	40-57
Synechoccus sp.	63	11	15
Anabena cylindrica	43-56	4-7	25-30
Chroococcus sp.	39-41	19-21	20-25

Apart from general characteristics of microalgae, environmental conditions during cultivation such as light, temperature, and available nutrients can change composition of microalgae [19]. Depending on the aim of microalgae production, some stress conditions may be applied. For instance, limitation of nutrients provides accumulation of lipids in microalgae. Moreover, lipid content of microalgae may be manipulated for production of biodiesel. So, environmental manipulations can be applied to achieve desired composition of microalgae.

1.3. Microalgal Biorefinery Concept

Biorefinery concept can be defined as sustainable biomass processing to produce various marketable productis and energy. Biorefinery concept combines processing of valuable products, fuel, and energy with a sustainable approach [20]. Microalgae are good candidates for biorefinery concept since many valuable products and biofuels can be produced through various processes by microalgae biomass (Figure 2.3).

Microalgal biorefinery concept includes both upstream and downstream processes. Upstream processes constitute cultivation of microalgae and efficiency is dependent on type of microalgae, cultivation (open/closed systems), CO_2, nutrient, and light supply [21]. Harvesting and cell disruption are commonly applied to microalgae to increase the efficiency of the downstream processes. Downstream processes include extraction and purification processes to get valuable products from microalgae. In the following sections, both upstream and downstream processes will be explained indetail.
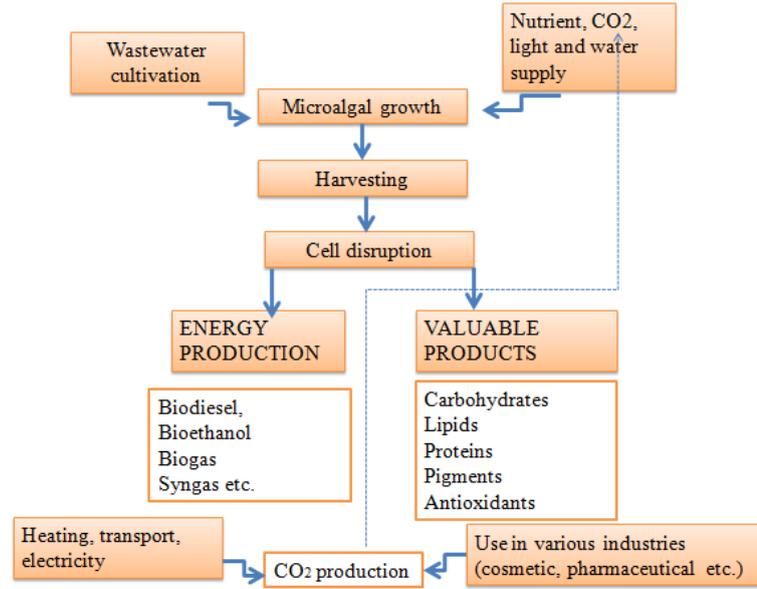
Figure 2. 3. Schematic view of microalgal biorefinery concept

2.3.1. Cultivation of Microalgae

Microalgae cultivation is mainly dependent on light, nutrients, temperature and CO_2 [22, 23]. Moreover, pH, dissolved oxygen, presence of zooplankton grazers and pathogens are other parameters that influence growth of microalgae [24]. For cultivation of microalgae, there is no need for arable land for growth; however, at least a pond or an engineered system is required depending on the aim of cultivation. Generally, cultivation systems for microalgae are open ponds, closed ponds, photobioreactors (PBRs), plastic bag, and well systems (Figure 2.4) Among these, open ponds and PBRs are regarded as the most well-known and cost-effective techniques [12,15].

Figure 2. 4. Different types of microalgae cultivation systems [25, 26, and 27]

Open ponds are open to the environment. Raceway ponds and high rate algal ponds (HRAPs) are some of the examples of open ponds used for cultivation [28]. The main advantages of open ponds are their cost effectiveness, easy maintenance and operation; however, contamination due to other species may become very quickly and controlling of culture is not possible [12]. Climatic conditions and nutrient limitation are also important for the effectiveness of cultivation. So, both climatic conditions and nutrients should be provided in open ponds to promote biomass growth [29]. In open ponds, mixing may be poor and sunlight may not be sufficient that may lead to limited biomass growth [30]. Paddle wheels, water jet or air pump be used to improve mixing in open ponds [12]. Biomass growth in open ponds is found to be between 10-25 g. m^2/day and it is comparatively lower than biomass growth in PBRs (20-100 g. m^2/ day) [28, 29]. Furthermore, use of open pond grown microalgae for production of commercial products may be limited due to contamination risks [29].

PBRs are closed reactor systems that have several configurations such as tubular, flat, or column. Tubular and flat PBRs have larger surface area and lower cost than column PBRs [12]. PBRs are suitable for both phototrophic and Mixotrophic cultivation. PBRs are more likely to be protected from external contamination, high mass transfer rate, possibility of optimization related to each specie and lower medium and nutrient loss. In addition, control of the process is easier than open ponds and area requirement is lower in PBRs. On the contrary, PBRs have high installation and maintenance costs, scale up is difficult and they are susceptible to fouling[30].

Although both PBR and open ponds has some general advantages and disadvantages, microalgae specie is the main parameter for the selection of cultivation systems. Autotrophic algae require light for growth, so; illuminated raceways or tubular PBRs can be preferred for cultivation. Furthermore, both raceways and PBR are suitable for Mixotrophic algae [12]. Apart from microalgae specie, cost is the most important parameter for microalgae cultivation. Since it may require fresh water and nutrients for biomass growth, the cost of cultivation may be limiting. To solve this problem, microalgal biorefinery concept can be combined with wastewater treatment to decrease cost of microalgal cultivation and provide environmental protection [23,31]. Moreover,

entegrating microalgae cultivation with wastewater treatment can provide microalgae as a co-substrate in anaerobic digestion [32]. Agricultural, domestic or industrial wastewaters are commonly used in microalgal cultivation [33]. Domestic wastewater contains human waste, organic waste, nutrients, chemicals, heavy metals, and microorganisms. Agricultural wastewater has higher amounts of organic nitrogen, phosphorus, and organic matter than municipal wastewater. Components of industrial wastewater depend on the industrial activity. Mainly, nitrogen and phosphorus are not commonly found in industrial wastewater, however; high metal tolerant microalgae cultivation can be proceeded with industrial wastewater [33]. Although use of wastewater for microalgae cultivation has several benefits, inhibition of microalgal growth due to other bacteria, fungi, and zooplankton found in wastewater is the major limitation. This problem can be eliminated by sterilization of wastewater, however; it is also costly and non-feasible in large-scale applications.

2.3.2. Harvesting of Microalgae

Harvesting is defined as separation of solid content from liquid media [12]. It is commonly applied to microalgae to increase biomass concentration to be used in further processes. Generally, initial microbial biomass concentration is 0.02-0.5% and after harvesting step, the biomass can be concentrated up to 15-20% [15]. The main limitation in harvesting is the high cost because of lower concentration of biomass and negative charge of microalgae. In addition, stability in suspension, size and shape of microalgal cells, and chemical contamination increases cost of the harvesting process [5]. Harvesting contributes to more than 20-30% of the total cost of the microalgal biorefinery systems. For high value products (PUFA, pigments, antioxidants etc.), such cost can be compensated; however, for energy production, this cost may be limiting[34].

Harvesting can be classified as primary harvesting (thickening) and secondary harvesting (dewatering). Primary harvesting is referred as thickening of algal slurry from 0.5% total solids to 6% total solids. Flocculation, sedimentation and floatation are some of the examples can be used in primary harvesting. Secondary harvesting is referred as thickening

of algal slurry to TS composition between 10%-20% and centrifugation and filtration are some of the examples of secondary harvesting.

Chemical Harvesting Methods

Chemical coagulation/flocculation is mainly applied to increase the particles size before additional harvesting step. It includes addition of coagulants including electrolytes and synthetic polymers for charge neutralization and flocculation of cells. Most effective coagulants are aluminum sulfate and ferric chloride, however; negative effects of these metal salts to downstream processes should be taken into account [35]. For instance, in anaerobic digestion, these chemicals can be inhibitory for anaerobic microflora [23]. As alternative to metal salts, polymers can be used as coagulants with high harvesting efficiency and low sludge volume [5]. Cationic starch and chitosan are some of the coagulants used in microalgae harvesting. Chemical methods are easier to apply, fast, and low energy processes. However, in general, the cost of chemical and toxicity are the disadvantages of this process [36].

Mechanical harvesting methods

Mechanical based methods apply physical forces to separate microalgal biomass from liquid media. Centrifugation is considered as the most effective harvesting method among other methods. In this method, centrifugal forces provide a differential separation of particles on fluid suspensions according to individual densities [22]. Nozzle type centrifuge and multi-chamber centrifuge are some of the examples. The selection is based on size of microalgae cells and harvesting efficiency [5]. Centrifugation is the most rapid and effective method, however; higher operational costs makes its application limited [22].

Gravity sedimentation is another mechanical based method that is based on sedimentation of microalgal cells and separation from liquid media. It is a cheap and simple method but it takes longer time to settle and it may lead to biomass loss. In addition, at the end, the concentration of biomass is lower than other methods [36].

Filtration is another mechanical process that microalgae cells pass through a porous filter media and collected on the surface of the filter. The harvesting efficiencies are very high (95%) for this process, however; flux of medium, presence of extra cellular polymers (EPS), cell size, microalgae surface charge, contact angle, biofouling and membrane type are main parameters that may hamper the efficiency of filtration [5]. Furthermore, clogging, pumping, and membrane change costs are other main limitations increasing operational costs[36].

Floatation process includes introducing of air bubbles to media, trapping microalgal cells and separation from liquid medium with 80-90% harvesting efficiency. It is a simple to operate, cheap, and time saving process. In addition, it is feasible for large-scale applications. However, higher power input and electrode scaling are needed to achieve high efficiencies [5]. As other mechanical methods, floatation also requires high capital and operational cost[23].

Electrical based harvesting is mainly based on electrophoresis of microalgae cells, which aims movement of negatively charged cells in an electric field. Major advantage of this process is that it does not require for additional chemical. However, high energy demand nd cost are the main limitations for electrical based harvesting [35]. Furthermore, process is directly influenced by pH, conductivity and chemical composition of water[23].

Biological harvesting methods

Biological harvesting methods are classified into auto flocculation and bio flocculation. In auto flocculation, consumption of dissolved CO_2 results in high pH levels and calcium phosphate precipitate charges positively. Algae support the precipitant and charge neutralization occur [35]. Bio-flocculation with auto-flocculating microalgae has several advantages such as no need for chemical flocculants, reuse of microalgal biomass and no disruption in downstream processing. Additionally, additional cost for cultivation is not required and contamination risk is reduced when compared to other types of bio-flocculators such as bacteria and diatoms [34]. However, it requires long time due to slow processing.

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On the other hand, bio flocculation can be described, as flocculation due to secreted biopolymers (extra cellular polymers). Microbial flocculation is another biological method that microorganisms such as microbes and planktivorous can flocculate microalgae culture [35]. Bio flocculation does not require additional costs such as organic/inorganic substrate [22]. However, bio-flocculation with bacteria needs additional substrate for energy source for bacteria and that may result in bacterial contamination [34].

2.3.3. Cell Disruption (Pretreatment) of Microalgae

Cell disruption of microalgae is essential for downstream processes for microalgae having rigid cell wall. Generally, microalgal cell wells are made of two components: fibrillary component and amorphous component. Fibrillary component forms skeleton of the wall which contains cellulose, hemicellulose, mannan and xylan. These compounds are hard to degrade when compared to other carbohydrate groups. Amorphous component of the cell wall forms matrix in which fibrillary component is embedded and it is composed of uronic acid, rhamnose, arabinose, fucose, xylose, mannose, galactose and glucose [37]. In addition to these components, an outer layer called trilaminar sheet (TLS) may be included. TLS is composed of lignin-like polymer called as algeanan (sporopollenin) which is resistant to degradation [37, 38, and 39].

Depending on the type and growth conditions of microalgae, the complexity of microalgal cell wall varies. Generally, Cyanobacteria have cell walls including peptidoglycan outer-membrane [40]. *Dunaliella salina* is lack of cell wall and *Euglena gracilis* has a cell wall composed of protein. *Nannochloropsis sp.* is a small marine algae having a cell size about 3μm has a cell wall with an outer layer having trilaminar structure [41]. This layer consists of algeanan and inner wall composed of mainly cellulose. *Botryococcus braunii* has biopolymers resistant to degradation [42]. *Porphydium cruentum* is red marine microalgae with a cell wall covered by mucilage including exopolysaccharides [43]. Cell wall of *Chlamydomonas reinhardtii* contains proteins but it does not contain cellulose or hemicelluloses. *Chlorella sp.* has also have a rigid cell wall which is composed of rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose [44].

Haematococcus pluvial is are commonly green oval motile cells under favorable conditions. However, under unfavorable conditions, the microalgae turn into red with a highly resistant and thick cell wall that contains TLS [41].

Pretreatment (cell wall disruption) methods are commonly applied in biorefinery processes including biofuel production and extraction of valuable products to reach desired components of the microalgae. These methods are classified as physical, chemical, and biological. In addition, two or more pretreatment methods can be applied in combination [45]. This section was discussed in detail in section 2.4.3. for their possible use in anaerobic digestion.

2.3.4. Valuable Products from Microalgae

After cultivation, harvesting and cell wall disruption steps, microalgal biomass can be processed for extraction of valuable products used in human nutrition, animal feed, aquaculture, and agriculture. Polyunsaturated fatty acids (PUFAs), proteins, antioxidants, lipids, polysaccharides, pigments including carotenoids, phycobilins and chlorophylls are common products extracted from microalgae and used in cosmetic, pharmaceutical, and food industry [16, 46]. For human nutrition, microalgal biomass is used extensively for production of powders, tablets, capsules and pastilles. Especially, *Spirulina (Arthrospira)* biomass is used as food products such as pasta and biscuits to support digestive tract [16]. The main limitation for valuable product extraction is the cost of the processes. For human nutrition, the production cost varies between 40 and 50 US $/kg. For animal feed the cost is approximately10 US $/kg. However, in pigment sector, the cost is much higher[46].

2.3.5. Use of Microalgae for Wastewater Treatment and CO_2 Mitigation

Microalgae are well known organisms that are capable of remediate environment by several ways. One of the most important capabilities of microalgae is to use in wastewater treatment. Microalgae require nutrients for protein, nucleic acid and phospholipids synthesis, so; nutrients can be removed easily with microalgae in wastewater. This natural process is called as 'phycoremediation'. Phycoremediation includes nutrient removal,

xenobiotic compounds removal with algae-based bio sorbents, wastewater treatment, CO_2 sequestration, toxic compound detection by algae-based biosensors [47].

The second major contribution of microalgae to environmental remediation is CO_2 mitigation. Using microalgae to make photosynthesis has significant potential to reduce atmospheric CO_2 since microalgae have higher CO_2 fixation rates when compared to terrestrial plants. Additionally, no disposal requirement is needed for trapped CO_2 [33]. It is reported that 100 tons of microalgae can capture 183 tons of CO_2 from the atmosphere which is an important capability of microalgae for mitigating greenhouse effects[24].

2.3.6. Biofuel Production from Microalgae

Biofuels are renewable fuels derived from natural sources. They are good alternatives to conventional fossil fuels due to being unlimited and environmentally friendly [12]. Bio hydrogen, hydrocarbons, bioethanol, biodiesel, biogas and bioelectricity are some of the biofuels derived from microalgae [48] (Figure 2.5).
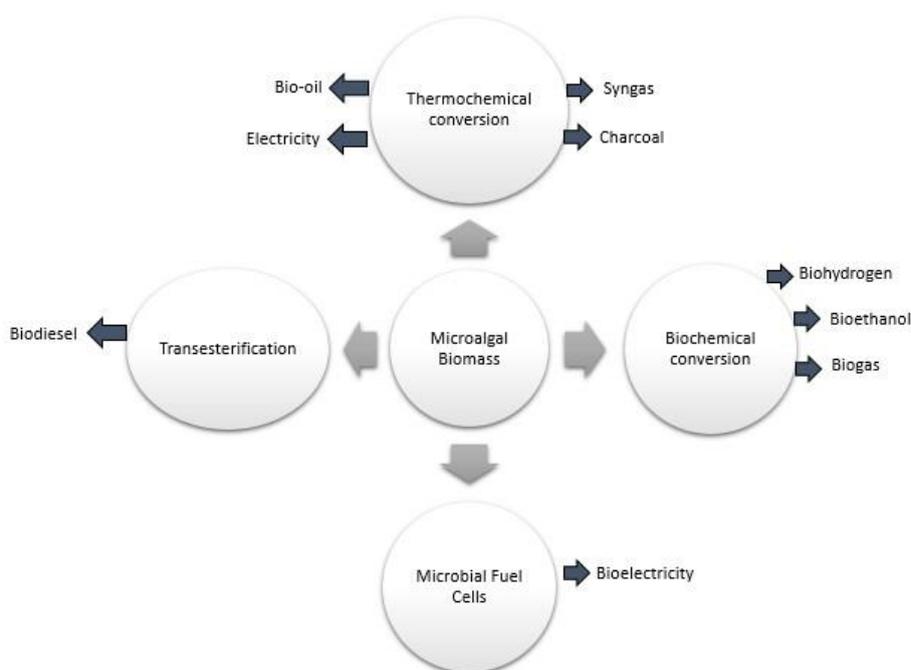
Figure 2. 5.Biofuel conversion processes from microalgae

Thermochemical conversion of microalgal biomass includes gasification, direct combustion, pyrolysis, and hydrothermal liquefaction (HTL). Direct combustion is referred as thermal conversion of biomass under air/oxygen environment. Gasification is defined as thermal conversion of biomass via partial oxidation under temperatures at 800-900°C [49]. Pyrolysis occurs at high temperatures ranging between 400-600°C without oxygen environment. The oil produced after pyrolysis is called as bio-oil. In this process, dried microalgae should be used for an effective process, however; drying biomass increases total cost. Hydrothermal liquefaction (HTL) process provides transformation of wet microalgal biomass to a liquid crude oil. The process temperatures are between 280 and 370°C with a pressure of 10-25 Mpa. HTL is able to convert proteins, carbohydrates, and lipids onto crudeoil.

Bio-diesel is composed of mono-alkyl esters and obtained by the reaction (transesterification) between triglycerides and alcohols [5]. Microalgae can accumulate lipids in large amounts (20-50% dry weight) under stress conditions such as nutrient deprivation or photo-oxidative stress [50]. Biodiesel production includes lipid extraction and transesterification processes after cultivation and harvesting steps. Generally, lipid extraction and transesterification are complex processes. Energy demand for harvesting (20-30% of the total cost) is quite high [51]. The cost of biodiesel production from microalgae is 3-4 US $/kg [46] are not able to compete with the cost of petroleum (0.3-0.6 US $/kg) yet.

Biochemical conversion of microalgal biomass consists of bioethanol, bio hydrogen, and biogas production from microalgae. Bioethanol formation has been extensively studied by several researchers. In bioethanol process, carbohydrate portion of microalgal biomass is converted into ethanol by biological means. Generally, bioethanol production includes pretreatment, enzymatic hydrolysis and fermentation. The main limitation is high cost of the process. Dark fermentation and phytofermentation are the alternative pathways used to produce bioethanol [52]. Bio hydrogen production is also can be considered to produce

energy from microalgal biomass. Hydrogen production from microalgae biomass can be processed with photolysis or dark fermentation. However, bio hydrogen production is quite expensive when compared to other biofuels.

Anaerobic digestion is considered as a straightforward method to produce energy from microalgae since it is simple and it does not need complex drying and oil extraction processes used commonly in other biofuel production processes [6, 51, 53]. Moreover, in biodiesel or bioethanol productions, only small portion of cell is used. On the other hand, anaerobic digestion uses all macromolecules to produce methane. There are three main ways to implement anaerobic digestion into microalgal biorefineries. First way is direct conversion of concentrated biomass into methane. This pathway can be integrated into wastewater treatment process to reduce cost of the process and treat wastewater with biological activities. Second way is the use of cell disrupted biomass after pretreatment for anaerobic digestion and final way is the use of biomass in anaerobic digestion after lipid extraction [51]. Moreover, waste microalgal biomass produced after other extraction and biofuel processes (bioethanol, pigment extraction, protein extraction etc.). Higher energy yield, no biomass drying requirement, possibility of co-generation, possibility of upgrading such as CO_2 biosequestration, co-digestion are the main advantages of anaerobic digestion over production processes [42]. As compared to valuable products, wastewater grown microalgae can be used safely in anaerobic digestion. Additionally, anaerobic digestate effluents can be recycled for microalgae growth or fertilization [54].

2.4. Anaerobic Digestion Process

Anaerobic digestion is a biological process that organic matter is converted into biogas through consecutive processes performed by microbial consortia in the absence of oxygen. Biogas consists of CH_4 (60-70%) and CO_2 (30-40%) and trace amounts of H_2S and H_2O. In nature, methane is produced in the digestive tract of ruminants, wet composting, rice fields and marshes [55]. The engineered systems aim to produce methane for energy purposes. Produced biogas can be used for combustion, transport and electricity purposes. The characteristics of biogas produced in anaerobic digesters are given in Table 2.2.

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Table 2. 2. Characteristics of biogas [55]

Characteristics	Biogas (60-70% CH_4, 30-40% CO_2 and other gases)
Energy	6.0 – 6.5 kWh /m^3
Fuel equivalent (as oil)	0.60 – 0.65 L oil/m^3 biogas
Normal density	1.2 kg/m^3
Molar Mass	16.043 kg/kmol

The formation of biogas was equated by Buswell [56] as stated in Equation 2.1. The composition of biogas depends on the content of long-chain hydrocarbons, carbon atoms, lignin content of biomass, digestion time, and liquid content in the reactor, temperature, andpressure.

$$C_c H_h O_o N_n S_s + \frac{1}{4}(4c - h - 2o + 3n + 2s)H_2O \rightarrow \frac{1}{8}(4c - h + 2o + 3n + 2s)CO_2 +$$

$$\frac{1}{8}(4c + h - 2o - 3n - 2s)CH_4 + nNH_3 + sH_2S$$
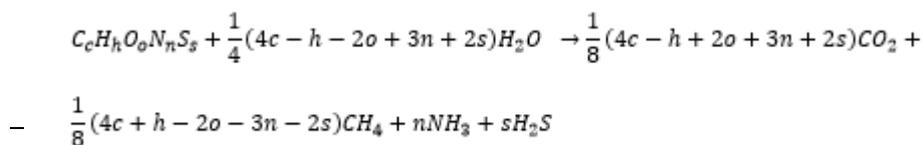
Equation 2.1

2.4.1. Stages of Anaerobic Digestion

Anaerobic digestion has four consecutive steps that are namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 2.6). All these steps are linked to each other, so; as one of the steps is inhibited, complete digestion process can collapse.

Hydrolysis

During hydrolysis phase, complex molecules (carbohydrates, proteins, and lipids) are hydrolyzed by facultative and obligatory hydrolytic bacteria and converted into simpler molecules as alcohol, amino acid, sugar and long chain fatty acid [31]. During hydrolysis, different types of bacteria are involved for hydrolysis of substrate by producing exo-cellular enzymes. In case of microalgae biomass, exo-cellular enzymes are produced to disrupt cell wall of microalgae. For hydrolysis of carbohydrate, cellulase, hemicellulase, xylanase, and amylase enzymes are produced by bacteria including *Clostiridium, Acetivibrio,* and *Staphylococcus*. For hydrolysis of proteins, protease enzyme is produced by bacteria such as *Clostiridium, Vibrio Peptococcus, Bacillus, Proteus,* and *Bacteriodies*. Furthermore, lipids are hydrolyzed by lipase and phospholipase enzymes produced by *Clostiridium, Micrococcus,* and*Staphylococcus* [31].

Time required for hydrolysis of complex molecules is variable due to the structure. For instance, a few hours are required for hydrolysis of carbohydrates. On the other hand, for proteins and lipids, the required time increases to a few days. For complex organics like lignin and lignocelluloses, rate of hydrolysis is very slow and complete degradation may not be achieved [55].

Acidogenesis

In **acidogenesis** phase, monomers produced after hydrolysis phase are converted into volatile fatty acids (VFAs) as butyric acid, acetic acid and propionic acid, acetate and alcohols. *Lactobacillus, Micrococcus, Pseudomonas, Desulfuromonas,* and *Streptococcus* are some of the bacteria responsible for acidogenesis phase [31]. Depending on the partial pressure of hydrogen, concentration of intermediate products varies. As partial pressure of hydrogen increases, some reduced compounds such as acetate are less produced [55].

Acetogenesis

In **acetogenesis** phase, acetogenic bacteria such as *Syntrophobacter* and *Syntrophomomas* transforms VFAs into acetate and hydrogen. Acetogenic bacteria are sensitive to partial hydrogen pressures. They are able to produce H_2 and CO_2 under low partial pressure of

hydrogen, and produce butyric, carbonic, propionic, and valeric acids and ethanol under higher partial pressures of hydrogen. However, methanogens are only able to use acetate, H_2 and CO_2 and they can live under high partial pressure of hydrogen. There is a symbiotic action between acetogens and methanogens, which is called as 'syntrophy'. Acetogenesis phase is important since approximately 70% of the methane is produced through acetate reduction [55, 57].

Methanogenesis

In **methanogenesis** phase, intermediate products from previous steps including acetic acid, H_2, and CO_2 are converted into CH_4 and CO_2 by acetoclastic methanogens such as *Methanotrix*, *Methanosarcina, Methanospirillum,* and *Methanosaeta*.They are unicellular and prokaryotic microorganisms [15] and they are responsible for 70% of the produced. CH_4. H_2 and CO_2 are also converted into CH_4 by hydrogenotrophic methanogens which are responsible for 30% of methane production[31].
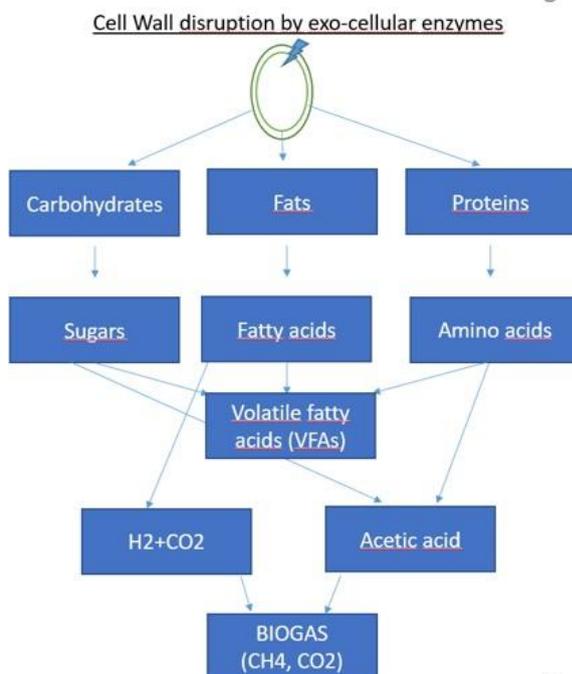
Figure 2. 6. Steps of anaerobic digestion process

2.4.2. Biogas Production Potential from Microalgae

The first attempt for anaerobic digestion of microalgae was done by Gouleke et al. [58] who investigated anaerobic digestion of *Chlorella vulgaris* and *Scenedesmus* biomass. After oil crisis in 1973, countries moved forward to their research to renewable energy production. Climate change and environmental pollution concerns also forced countries to take actions against use to fossil fuels. Use of microalgae for energy production has achieved great attention at the beginning of 2000s. The research related to use of microalgae in anaerobic digestion had become one of the popular research in bioenergy production and the number of research has been increasing dramatically year by year (Figure2.7).
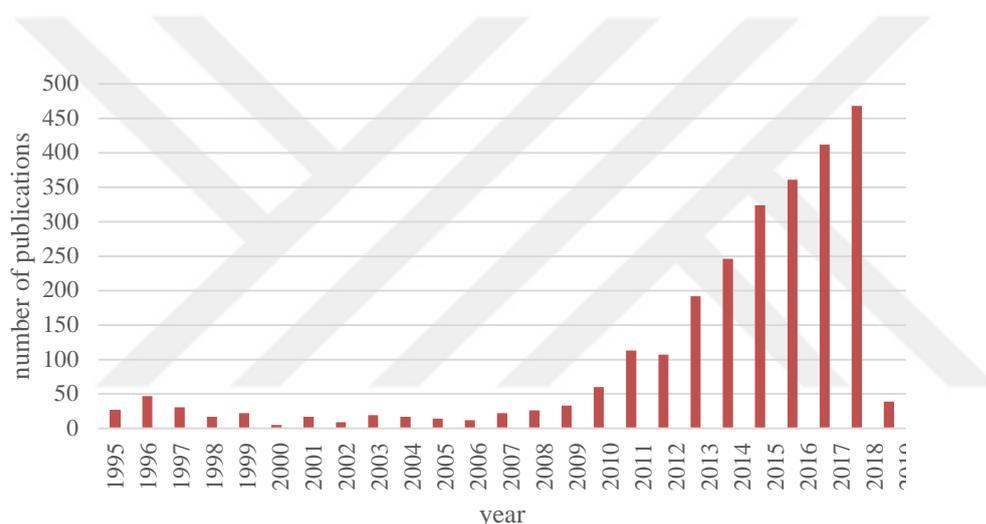
Figure 2. 7. Number of publications related to anaerobic digestion of microalgae biomass (taken from www.sciencedirect.com, accessed date: 07.11.2018)

Biogas production potentials from microalgae are various due to different characteristics of microalgae strain and operating conditions (See Table 2.3). Especially, rigid cell wall characteristics resulted in lower biogas yields (i.e. *Scenedesmus sp., Chlorella sp.*). Carbon to nitrogen ratio also important for biogas production rate. Low C/N ratios result in ammonia inhibition during anaerobic digestion [45]. Generally, biogas production potential from microalgae changes from 63-587 mL CH_4/ g VS of microalgae biomass. As these values are compared with other substrates used in anaerobic digestion such as chicken litter (126 mL biogas/g fresh matter), fat (826-1200 mL biogas/g fresh matter), food waste (110

mL biogas/g fresh matter), horse manure (56 mL biogas/ g fresh matter), maize silage (200-220mL biogas/ g fresh matter), municipal solid waste (101.5 mL biogas/ g fresh matter) and sewage sludge (47 mL biogas/ g fresh matter), it is clear that biogas production from microalgae has a great potential to producebiomethane.

As can be seen in Table 2.2, even for the same strain, different biogas yields have been obtained. These variations were mainly due to cultivation conditions (i.e. nitrogen depletion cultivation), C/N ratio or operational parameters including pH, temperature, hydraulic retention time (HRT), mixing, biomass concentration, organic loading and substrate to inoculumratio.

Table 2. 3.Biogas potential for some microalgae strains (mesophilic digestion, batch mode)

Microalgae strain	Biogas production	Reference
Arthrospira maxima	173 mL CH_4/ gVS	[59]
Arthrospira platensis	481 mL CH_4/ gVS	[60]
Botryococcus braunii	404 mL CH_4/ gVS	[61]
	343-370 mL CH_4/ gVS	[62]
Chlamydomonas reinhardtii	405-549mL CH_4/ gVS	[63]
	587 mL biogas /g VS	[60]
Chlorella sp.	262 mL CH_4/ gVS	[64]
	170 mL CH_4/ gVS	[58]
	310-360 mL CH_4/ gVS	[65]
	230 mL CH_4/ gVS	[66]
Chlorella kessleri	335mL CH_4/ gVS	[60]
Chlorella pyrenoidosa	264mL CH_4/ gVS	[23]
Chlorella sorokiniana	298mL CH_4/ gVS	[67]
	172-518 mL CH_4/ gVS	[68]
	212 mL CH_4/ gVS	[69]
Chlorella vulgaris	337mL CH_4/ gVS	[70]
	240 mL CH_4/ gVS	[71]
	245 mL CH_4/ gVS	[72]
	403 mL CH_4/ gVS	[73]
	286 mL CH_4/ gVS	[74]
	189 mL CH_4/ gVS	[69]
Dunaliella sp.	276 mL CH_4/ gVS	[75]
Dunaliella salina	323mL CH_4/ gVS	[60]
	63mL CH_4/ gVS	[76]
	440mL CH_4/ gVS	[77]
Dunaliella tertiolecta	24 mL CH_4/ gVS	[74]

Microalgae strain	Biogas production	Reference
Euglena gracilis	485 mL CH_4/ gVS	[60]
Isochrysis sp.	408 mL CH_4/ gVS	[62]
Isochrysis galbana	349 mL CH_4/ gVS	[75]
Micractinium sp.	209 mL CH_4/ gVS	[66]
Microcystis sp.	70–153 mL CH_4/ gVS	[78]
	302 mL CH_4/ gVS	[79]
	201 mL CH_4/ gVS	[80]
Nannochloropsis sp.	357 mLCH_4/g VS	[70]
	360 mL CH_4/ gVS	[53]
Nannochloropsis gaditana	228 mL CH_4/ gVS	[62]
	109 mL CH_4/ gVS	[81]
Nannochloropsis oculata	204 mL CH_4/ gVS	[82]
	231 mL CH_4/ gVS	[75]
Nannochloropsis salina sp.	557 mL CH_4/ gVS	[70]
	430mL CH_4/ gVS	[83]
Phaeodactylum tricornutum	337 mL$CH4$/g VS	[70]
	360 mL CH_4/ gVS	[84]
Scenedesmus sp.	37 dm^3/kgCOD	[31]
	320 mL CH_4/ gVS	[58]
	163 mL CH_4/ gVS	[85]
	170 mL CH_4/COD	[86]
	140.3 mL CH_4/ gVS	[87]
Scenedesmus obliquus	240 mL CH_4/ gVS	[84]
Scenedesmus dimorphus	397 mL CH_4/ gVS	[62]
Spirulina	190 mL CH_4/ gVS	[88]
Spirulina platensis	210 and 260 mL/gVS.	[89]

Microalgae strain	Biogas production	Reference
Spirulina maxima	320 mL CH_4/ gVS	[77]
Tetraselmis	420 mL CH_4/ gVS	[53]
Tetraselmis suecica	91-174 mL CH_4/ gVS	[90]
Thalassiosira weissflogii	265 mL CH_4/ gVS	[62]

Although, mesophilic digestion of microalgae is common for researchers, studies concerning thermophilic digestion of microalgae is scarce. Lavric et al. [91] showed that methane production of thermally pretreated microalgal biomass was elevated by 62% with a methane yield of 254.3 mLCH_4/g VS after thermophilic digestion. Anaerobic digestion of *Scenedesmus sp.* in CSTR (mesophilic and thermophilic) biodegradability was achieved as 30.4% after 50 days of SRT. However, mesophilic digestion in anaerobic membrane reactor also found to be affective. Gonzalez-Fernandez et al. [50] found out that thermophilic digestion of *Chlorella sorokiana* and *Scenedesmus sp.* yielded the methane production as 108.2 ± 1.9 mL CH_4/ g CODin which was higher than methane yield after mesophilic digestion (79.2 ± 3.1 mL CH4/ g CODin. Caporgno et al. [92] showed that *I. galbana* and *S. capricornutum* showed higher methane yields at mesophilic conditions. The anaerobic digestion of mixed indigenous microalgae showed methane production yield as178-207 mL CH_4/g VS after mesophilic digestion. Thermophilic digestion showed better methane production (390 mL CH_4/g VS); However, thermophilic condition had a lag period as 30 days [93]. The effect of pretreatment on thermophilic digestion of microalgae was also studied by the researchers, but there is still lack of knowledge. Thermal pretreatment of microalgae increased methane production by 21% in mesophilic digestion and by 6%in thermophilic digestion. Pretreatment of microalgae with anaerobic bacteria improved methane production by 13% in thermophilic digestion. No improvement was observed after mesophilic digestion. Combined pretreatment (thermal-biological) improved methane production by 12% in thermophilic digestiom and by 6% in mesophilic conditions (94).In another study, Magdalena et al . [95] found out that after thermophilic digestion of

protease pretreated *Chlorella sp.,* the methane yield was found to be as 235.6 mL CH_4/ gCOD. Apart from raw/pretreated microalgae biomass, lipid extracted microalgae was also used in thermophilic digestion. For instance, thermophilic digestion of algae biomass *(Ulva lactuca)* after oil extraction resulted in $342.59\, cm^3 CH_4/g\, VS$[96]. Another study showed that thermophilic digestion of lipid-extracted *Nannochloropsis gaditana* did not improved the biogas production when compared to mesophilic conditions, However; hydrolysis step was found to be enhanced [97].

2.4.3. Factors Affecting Anaerobic Digestion Performance

Due to being biological process, anaerobic digestion is sensitive to operational parameters such as pH, temperature, reactor configurations, and characteristics of substrate.

pH

Anaerobic microorganisms involved in anaerobic digestion have various optimum pH ranges. Methanogens are mostly susceptible to pH changes. pH between 6.5 and 7.2 was considered as the optimum range for this type of microorganisms [98]. As pH values are less than 6.5, organic acid production causes more decrease in pH and anaerobic digestion process fails. In addition, at high pH values, activity of anaerobic bacteria also is lost. So, in order to provide production of methane, the pH values should be kept at optimum ranges. The main reason for pH decrease in an anaerobic reactor is the overaccumulation of volatile fatty acids (VFAs) due to acidogenic activities. Generally, VFAs production can be compensated by alkalinity production by methanogenic bacteria. However, in an excessive accumulation, it may be inhibitory for methanogens. So, the ratio VFA to total alkalinity is an important indicator for pH changes in the reactor. VFA/TA ratio should be between 0.1-0.25 to provide system stability[55].

Temperature

Temperature had a direct effect on anaerobic digestion performance. Anaerobic digestion can be implemented in psychrophilic (4-25°C), mesophilic (30-40°C), thermophilic (50-60°C), or even in extremophilic (>60°C) range, however; the activity of microorganisms,

especially methanogens, varies depending on the temperature (Figure 2.8). In mesophilic range, the growth rate is moderate, endogenous death is low, and volatile fatty acids (VFAs) accumulation is lower. In thermophilic range, the growth rates of methanogens are higher, so; higher organic matter removal can be achieved. In addition, removal of pathogenic bacteria is provided during thermophilic anaerobic digestion [99]. However, thermophilic anaerobic digestion is more sensitive to environmental conditions and accumulation of free inhibits methanogens. Thermophilic anaerobic digestion has lower stability and longer time is needed for recovery. Furthermore, the cost of the process in thermophilic digestion is higher due to high energy requirement for heating [100]. The partial pressure of H_2 in the digester also effects metabolism kinetics in a way that at higher temperatures, endergonic reactions are more favorable than exergonic reactions (free energy is negative)[98].
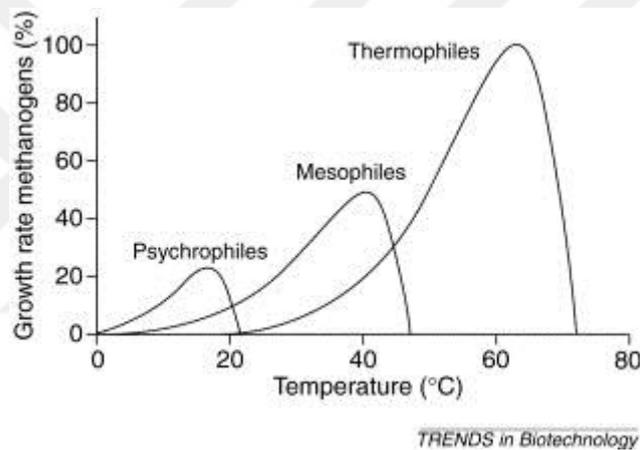
Figure 2. 8. Growth rate of methanogens at various temperatures [101]

Mixing

Mixing is important in terms of provide contact between anaerobic bacteria, its releasing enzymes and organic matter to be degraded. In addition, it provides complete utilization of biomass in the digester, prevents sedimentation and scum formation, prevents temperature and pH gradients in the digester, and provides biogas release from the liquid phase [102]. Mixing speed is also important since frequent mixing may disrupt biological processes. Mixing can be provided impellers and pumps or gas bubbling. Type and the intensity of mixing are chosen according to digester geometry and type and concentration of +substrate.

Organic Loading Rate (OLR) and Hydraulic Retention Time (HRT)

OLR and HLR are important parameters for efficient operation of anaerobic digestion. Organic loading rate is the amount or organic matter (volatile solids or COD) fed to the digester per day per unit digester volume. Hydraulic retention time indicstes total time liquid retaining in the digester. High OLRs reduce the volme of the digester and cost, correspondingly. However higher OLR results in disruption of bacterial population balance [24]. So, optimum operation conditions can be achieved at low OLR and high HRT, which provide methane production as maximum and constant. However, it is important to notice that, for each strain, the optimum OLR and HRT may change, so these parameters should be evaluated as strain specific. Generally, anaerobic digesters (complete mix reactors) can work under a HRT value of 15-30 days and an OLR value of 1-5 g COD/L.d for microalgae. In order to increase methane yield, pretreatment or different reactor configurations by increasing HRT can be applied [99].

Ammonia Inhibition

Ammonia is found to be toxic to anaerobic microflora after certain level [17]. Protein content of microalgae may result in high ammonia concentration during anaerobic digestion. The inhibitory concentrations of ammonia during anaerobic digestion are between 1.7 and 14 g/L [23]. As an example, *Spirulina maxima* has a high protein content (up to 60 %) and high concentration of ammonia is produced during anaerobic digestion. This results in volatile fatty acids accumulation due to ammonia toxicity on methanogens. Thermophilic conditions may also enhance inhibition effect of ammonia due to increasing free ammonia concentrations with increasing temperature.

To solve ammonia inhibition, use of acclimatized methanogens, and optimization C:N ratio by co-digestion are some of solutions [23]. Additionally, some ions such as Na^+, Ca^{+2} and Mg^{+2} lowers inhibition effects by increasing alkalinity [17].

Salt Inhibition

Salt inhibition is another problem faced during anaerobic digestion when saline microalgae is used as substrate. Freshwater algae may also contain high sodium concentrations (above 0.14 M) and it may inhibit anaerobic process [17]. Generally, at higher salinity concentrations (3500-5000 mg/L) anaerobic digestion is inhibited. However, at moderate concentrations (100-200 mg/L), it was observed that microbial activity could be improved. To use anaerobic inoculums having saline resistant microorganisms can be a solution to high salinity problem [42]. Moreover, washing substrate before feeding to the digester to remove salinity may be another solution to prevent salt inhibition.

Sulfur Inhibition

Sulfur can be present in waste/wastewaters as sulfate, sulfide, H_2S (dissolved in the liquid or gaseous form). Sulfate reducing bacteria (SRB) degrade organics by utilizing sulfate as electron acceptor and as H_2S is produced as the product. SRB compete against methanogens, acetogens, and fermentative bacteria for organic and inorganic substances including acetate, H_2, propionate, and butyrate [103]. Presence of H_2S above 200 mg/L may be inhibitory since H_2S in dissolved form can pass through cell membrane and form cross-links between polypeptide chains resulting in cell alteration. In addition, at lower pH values H_2S concentration increases, so, inhibition can be occurred at also lower pH values. Apart from its inhibition effect, higher H_2S content in biogas results in corrosion in concrete and steel material and cogeneration units. It produces sulfur dioxide emissions during combustion (energy production). Furthermore, it is known to be toxic for human health and bad odor [103]. In order to prevent H_2S inhibition and its concentration in biogas increasing pH, addition of iron salt, lowering organic loading rate, or a two-stage treatment system can be applied [55].

Other Inhibitors

Apart from ammonia, salt, and sulfur inhibition, other organic and inorganic compound may have inhibitory effects through anaerobic digestion. Heavy metals are toxic since thy

may disrupt enzymatic function and structure. Organic compounds can also inhibit anaerobic digestion by swelling and leakage in bacterial membrane and cell lysis.

Reactor Configuration

According to Shah et al. [1] anaerobic digesters were divided into three classes, which are namely, low rate digesters, high rate digesters, and tubular digesters. Plug flow and complete mix reactors are some of the examples of low rate digesters. Low rate digesters are divided into several configurations such as one/ two stage, dry/wet digestion or batch/ continuous digesters. One stage digestion provides whole anaerobic digestion process in one tank. On the contrary, two stage processes separate hydrolysis and acidogenesis (first tank) from acetogenesis and methanogenesis (second tank). One stage digesters are found to be easier to operate, lower cost, and less technical problem and mostly used in industrial scale [1]. Two stage process provider higher and more stable biogas production. On the other hand, in one stage digesters, the biogas production may be discontinuous and hydraulic retention time is higher than two-stage reactors. Digesters are also classified as dry matter content. Dry digesters work under 20-40% of dry matter and organic loading ranges are between 12-15 kg VS/ m3. d, on the contrary; wet digesters work under less than 20% dry matter and organic loading rate is less than 5 kg VS/ m^3d [1]. Batch vs. continuous digesters. In batch digesters, the substrate and feed are put into reactor and sealed and hold for a specific time. However, in continuous digesters the reactor is fed continuously. Plug flow, CSTR, UASB, and anaerobic filters are some of the continuously fed reactors. These type of reactors are commonly used for the industrial wastes[1].

High rate digesters works under 4-15% solids content and volatile solids reduction can reach up to 98%. Organic loading rate is approximately 10-15kg VS/ m^3d and HRT is 0.5-12 days. UASB, UAF and FBR are some of the examples for high rate digesters.
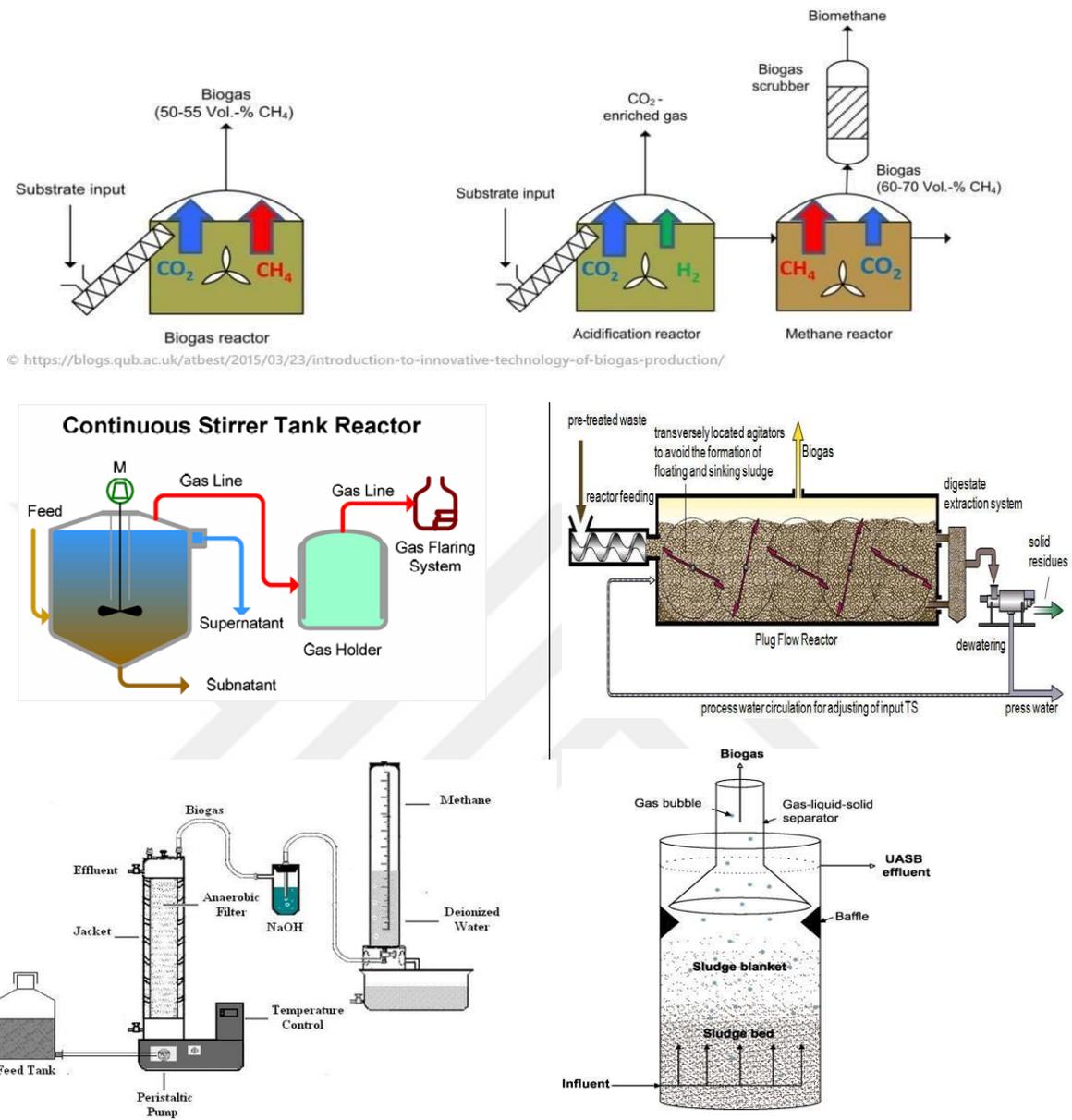
Figure 2.9. Some Types of Anaerobic Digesters

2.5. Characteristics of Microalgae Biomass

Microalgae biomass is a good candidate for substrate for biogas production. Due to being live organisms, the characteristics of microalgal biomass show variations due to type of microalgae, cultivation conditions, cell wall characteristics. In the following sections, these variations will be discussed in detail.

2.5.1. Macromolecular Composition ofMicroalgae

Various biogas production potentials have been stated in literature for each microalgae strain due to their macromolecular characteristics such as the content of protein, lipid and carbohydrate (See Table 2.2). Each macromolecule has different specific methane yield, so, the resulting methane yields are different. Specific methane yield for lipid $C_{57}H_{104}O_6$ is 1.014 CH_4 /gVS. Additionally, specific methane yield for protein $C_6H_{13.1}O_1N_{0.6}$ is 0.85 L CH_4/gVSand forcarbohydrates$(C_6H_{10}O5)n$ is0.415 CH_4/gVS[17].Although lipidshave higher methane yields than proteins and carbohydrates, high lipid content may lead to inhibition by production of high long chain fatty acids (LCFAs) or volatile fatty acids (VFAs) if alkalinity and buffering capacity is low. Additionally, microalgae with high lipid can be used for biodiesel production more effectively. So, it is suggested that less than 40% lipid content is favorable for anaerobic digestion to have the optimum energy [17, 51]. Additionally, substrates having complex compounds such as proteins, lipids and lignin, cellulose, and hemicellulose are limiting the rate of hydrolysis [38]. Microalgae does not contain lignin, however; they may contain cellulose and hemicellulose, which lowers their biodegradability. On the other hand, some compounds such as carbohydrates which are easily degradable are rate-limiting for acetogenesis and methanogenesis because of lower growth rates[38].

2.5.2. Carbon to Nitrogen Ratio

During anaerobic digestion, microorganisms are able to use carbon for energy production and use nitrogen for producing cell components. Carbon sources are consumed as 30 times higher than nitrogen sources, so carbon and nitrogen balance is an important parameter. For an effective digestion, optimum C:N ratios should be retained between 20:1 and 30:1 to provide higher biogas production [24]. Microalgae biomass has a C/N ratio of 4.16:1 to 7.82:1 [51]. Lower C:N ratios cause ammonia release which has inhibitory effects on methanogenic bacteria and volatile fatty acid accumulation [1]. To increase C:N ratio to desired values, co-digestion or manipulation in microalgae cultivation can be applied. Co-digestion is referred as anaerobic digestion of two substrates. It has several benefits such as improving reliability of feedstock, dilution of toxic substances, balance of nutrients,

synergistic effect on microorganisms, higher methane yield, and increase in the load of biodegradable organic matter [1]. Microalgal biomass can be digested with several wastes such as pig manure, cow manure, sewage sludge, paper waste or corn stalks [51]. In addition, changing macromolecular profile of microalgae during growth phase may increase C:N ratio. For instance, protein rich substrates can be converted into carbohydrate rich substrates by culturing microalgae in different amounts of phosphorus[31].

2.5.3. Biomass Productivity

Biomass productivity is the one of the major parameters for microalgae strain selection. Each strain has different cell characteristics and growth conditions that affect the productivity. For instance, *Spirulina* and *Dunaliella* can be cultivated with small amount of inoculum, however; alkaline/saline selective media which makes slower growth and lower biomass productivity [22] *Chlorella* and *Haematococcus* requires periodic startup of reactor, however; they do not require selective media for growth. On the other hand, *Nannochloropsis* and *Cyclotella* provide high biomass yields but they are not tolerant to contamination [22]. In addition, light intensity, nutrients and temperatureare important for biomass growth. Time takes for doubling biomass could change from a few days to longer periods depending on the environmental conditions. So, for the selection of microalgae strain, strains having higher biomass productivity has more advantageous over other strains since the amount of biomass produced should be high to feed the anaerobic reactors.

2.5.4. Cell Wall Degradability

Cell wall degradability is a significant parameter that directly affects the digestibility of biomass. Generally, microalgal biomass has cellulose and acetelysis resistant biopolymers such as algeanan and sporopollenin which results in high resistance to bacterial attack and influences yields of bacterial degradation [104]. Higher biogas production can be achieved with microalgae species not having cell wall. For the strains having rigid cell wall, use of pre-treatment methods provide disruption of cell wall before anaerobic digestion and increase biomass solubilization in the medium. By this way, hydrolytic bacteria can easily reach organic matter [51]. (See Section 2.3.3.)

Table 2.4. shows cell wall characteristics of different microalgae species. Some of them do not have cell wall (i.e. *Dunaliella sp.*); however, other may have rigid cell walls including several type of complex structures including biopolymers, cellulose, hemicelluloses, and trilaminar structures (TLS).

Table 2.4. Cell wall characteristics of some microalgae

Strain	Cell wallcharacteristics
Botryococcusbraunii	Biopolymers
Chlamydomonasreinhardtii	Lack of cellulose, includesglycoproteins andproteoglycans
Chlorellasp.	Harsh polysaccharide-based cell wall
Dunaliellasp.	Wall-less cells
Euglenagracilis	Protein rich
Haematococcuspluviali	TLS rich cell wall under unfavorable conditions
Nannochloropsis sp.	Sporopollenin polymers
Scenedesmussp.	Rigid cell wall including cellulose, hemicellulose,sporopollenin
Spirulina	Peptidoglycan outermembrane
Porphyridiumcruentum	Cell wall composed of sulfonated exopolysaccharides

2.5.5. Use of Pretreatment Methods to Enhance Biogas Production from Microalgae

Cell wall disruption (pretreatment) of microalgae biomass has been extensively used in microalgae biorefinery concept including, extraction of valuable biomass, bioethanol production, biodiesel production, and biogas production. Especially in biogas production, it is commonly used to provide cell wall disruption and increase biomass solubilization. By this way, biogas production can be enhanced. Pretreatment methods are classified as

physical, thermal, biological, and chemical depending on the way to use cell disruption and they will be discussed comprehensively in the following sections.

Physical Pretreatment

Physical treatment depends on application of physical forces such as solid-shear, liquid-shear and wave energy to break down and reduce size and crystalline cellulosic structure of cell wall of microalgae [105]. After cell wall disruption, increase in specific surface area is observed [13,24]. Types of physical pretreatment are chipping, shredding, grinding, homogenization, ultrasonication, microwave and electrolysis [8].

Chipping, grinding or milling (bead beating) methods are used to handle compact biomass. There are several types of milling including ball milling, disc milling, knife milling, and hammer milling [13]. The mechanisms involve mechanical compaction and shear stress [105]. Although, these methods are efficient, provides high mass loading and commercially available. They are considered as cost effective when substrate concentration is high (100-200 g/l) [8, 105].

High speed/pressure homogenization is another method used for cell disruption of microalgae. This process involves stirring with high rpm (high speed homogenization) or high pressure forces (high pressure homogenization) to provide hydrodynamic cavitation and shear stress at solid-liquid interphase. The advantages of this process are its effectiveness and simplicity. However, it requires high dry cell weight composition and it is an energy consuming process. Furthermore, protein denaturation may occur during homongenization [105]

Ultrasound pretreatment is a commonly applied method at industrial scale for various types of biomass. It supplies cavitation and micro turbulence in the microalgae cells [106]. During ultrasound pretreatment, heat and pressure increases that enhances organic matter solubilization. Main operational parameters of ultrasound pretreatment are power input, time, and temperature. Ultrasound pretreatment is commercially available process with

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high cell wall disruption efficiency, however; it has high energy demand which makes energy balance as negative for pretreatment. Use of high concentration of microalgal biomass reduces energy costs. Furthermore, combination of ultrasound pretreatment can improve the cell disruption and reduce energy demand during pretreatment [105].

Microwave pretreatment is applied by short waves of electromagnetic energy (microwaves), to disrupt microalgal cell wall by increasing molecular energy and heat. As in ultrasound treatment, water content of harvested biomass is an important factor that affects energy balance. To reach energy efficient process high solids concentration is needed [13].

Freezing is another method that provides ice-crystals formation and disruption of microalgal cell under low temperatures. Electrolysis pretreatment is a new method applied to microalgal biomass. It provides production of free radicals that can disrupt microalgal cell wall and reduce crystallinity of biomass. Free radicals production during pretreatment may result in inhibition during anaerobic digestion [45]. UV assisted TiO2 oxidation produces hydroxyl radicals (OH) that can disrupt microalgal cell wall. In this method, cost and energy demands are higher. Osmotic shock is an energy friendly process based on introducing salt to the solution to provide osmotic pressure. This process is simpler and scalable, however; the recovery for downstream processes should be taken into account [41].

In general, physical methods are very effective in terms of cell disruption; however, in terms of energy demand and maintenance costs, they are not considered as feasible. In order to tackle energy costs, use of biomass with high solid content or combine physical methods with another pretreatment method decrease the cost of the process.

Thermal Pretreatment

It stands for inserting heat to provide hydrolysis of cell wall and solubilization of organic matter. As pressure is applied in addition to heat, the method is called as hydrothermal

pretreatment [13]. Thermal pretreatment is generally applied at temperatures less than 100°C. Hydrothermal pretreatment is applied for temperatures above 100°C and with pressure. Temperature, pressure, and contact times are one of the important parameters for this method. Thermal pretreatment is one of efficient process for microalgae, however, it has high energy demand, and it may produce recalcitrant compounds lowering degradability in anaerobic digestion [13].

Chemical Pretreatment

It is applied by alkaline, acidic, or oxidative chemicals by disrupting and solubilizing substances of microalgal cell wall. Calcium, potassium or sodium hydroxide are some of the chemicals that are used for mitigate pretreatment at pH 9-12. Hydrochloric and sulfuric acid are commonly used for acidic pretreatment at low pH [45]. Oxidative chemicals such as chelating agents, solvents, ozone, hydroxyl radicals are also used during chemical pretreatment. Chemical pretreatment does not require energy, however; the cost of chemical, toxicity of chemicals, corrosion, contamination and inhibitory compounds are the main concerns for this pretreatment [40].

Biological Pretreatment

Biological pretreatment is the use of specific enzymes to disrupt microalgal cell wall in mild environmental conditions. Enzymes are able to hydrolyze bonds in the cell wall and release of intercellular organic matter. Cellulase, neutrase and pectinase are some of the examples used in biological pretreatment [5]. Furthermore, glycosidases, glucanases, peptidases, lipases, proteases, esterases, B-glucanase, xylanase are also used in biological pretreatment [105].

Biological pretreatment can be applied by commercial enzymes or on-site produced enzymes (enzymes produced by microorganisms). Pretreatment with commercial enzymes provides less energy demand, high selectivity of enzymatic reactions, no inhibition products, and mild pH and temperature conditions [(5)(105)(107). So, it can be concluded that biological pretreatment is a low energy demand and more environmentally friendly

process. The main limitation is being costly [5] and complexity of formulating enzyme mix to have optimum hydrolysis efficiency after pretreatment [107]. To reduce costs and difficulties related to externally added enzyme, on-site enzyme production by microorganisms (bacteria, fungi etc.) can be provided or immobilization of enzymes onto recyclable nanoparticles [31, 45].

2.4.5. Comparison of Pretreatment Methods Used for Biogas Production from Microalgae

Applicability of pretreatment to microalgae biomass is dependent on microalgae characteristics including cell wall structure and macromolecular composition, and energy/cost considerations. Most of the pretreatment methods are able to increase biomass solubilization by disrupting cell wall, however; cost and energy requirements are found to be dominant for selection of pretreatment method to apply in large scale systems. Furthermore, it can be said that, the selection of pretreatment system depends on several parameters in terms of biogas production from microalgal biomass. Table 2.5 shows various applications of pretreatment methods for biogas improvement from microalgae.

Table 2. 5. Research related to pretreatment of microalgal biomass and effect of biogas yields

Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
			Physical pretreatment				
Spirulina maxima	**Ultrasound**	Semi continuous	0.19 m^3 CH$_4$/ kg VS	0.17 m^3 CH4/ kg VS	0%	75%	[88]
Nannochloropsis salina	**Ultrasound**	Batch	347 mL STP/g VS	247mL STP/g VS	-		[38]
Nannochloropsis salina	**Microwave**	Batch	347 mL STP/g VS	487 mL STP/g VS	40%		[38]
Nannochloropsis salina	**French-Press**	Batch	347 mL STP/g VS	460 mL STP/g VS	33%		[38]
Chlamydomonas, Scenedesmus, and Nannochloropsis	**Ultrasound**	Batch	272 ± 1 mL CH$_4$/ g VS	301- 31 mL CH4/ g VS	12-14%		[106]
Acutodesmus obliquus, Oocystis sp.Phormidium and Nitzschia sp.	**Ultrasound**	Batch	198 ± 9 mL CH$_4$/ g VS	209-223 mL CH4/ g VS	6-13%		[106]
Microspora	**Ultrasound**	Batch	255 ± 2 mL CH$_4$/ g VS	301-314	18-24%		[106]

Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
Physical pretreatment							
Acutodesmus obliquus, Oocystis sp.Phormidium and Nitzschia sp.	**Ultrasound**	Batch	198 ± 9 mL CH$_4$/ g VS	209-223 mL CH4/ g VS	6-13%		[106]
Microspora	**Ultrasound**	Batch	255 ± 2 mL CH$_4$/ g VS	301-314	18-24%		[106]
Scenedesmus	**Ultrasound**	Batch	81.8 mL CH$_4$/ g COD	73-153.5 mL CH4/ g COD	-15% -87%		[6]
Hydrodictyon reticulatum	**Ultrasound**	Batch	170 mL/g-VS	313–384 mL/g-VS	84%-125%		[108]
Scenedesmus obliquus and Chlorella vulgaris	**Microwave**	Batch	172.48 mL biogas /g VS	194.06-307.11 mL biogas /g VS	13-84%		[48]
Chlorella sp. and Scenedesmus sp.	**Ultrasound**	Batch	336 mL CH4 /g COD	356-385 mL CH4 /g COD	6-14.5%		[9]
Scenedesmus obliquus	**Ultrasound**	Batch	265 mL biogas /VS	266-307 mL biogas /VS	2-27%	63-73%	[104]
Chlorella sorokiniana	**Ultrasound**	Batch	297 mL biogas /g VS	259-375 mL biogas /g VS	2-53%	62-65%	[104]
Arthrospira maxima	**Ultrasound**	Batch	185 mL biogas /g VS	180-206 mL biogas /VS	16-38%		[104]

Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
			Physical pretreatment				
Stigeoclonium sp., Monorraphidium sp., and diatoms Nitzschia sp. d Amphora sp., Chlorophyta	**Ultrasound**	Batch	105.6 mL CH_4/gVS	113.7 ml CH4/g VS	7.6%		[109]
Acutodesmus obliquus	**Ultrasound**	Batch	115.6 Nm3/ tCOD	173.6 Nm3/ tCOD	50%		[110]
Phaeodactylum tricornutum	**Ultrasound**	Batch	258 mL CH_4/gVS	284-287 mL CH_4/gVS	10-11%		[92]
Mixed microalgae culture	**Ultrasound /Electrolysis**	Batch	138 mL CH_4/g VS	66-294 mL CH_4/g VS	0-117%		[111]
Stigeoclonium sp., Monorraphidium sp., and diatoms Nitzschia sp. d Amphora sp., Chlorophyta	**Microwave**	Batch	105.6 ml CH_4/gVS	127.7 ml CH4/g VS	21%		[109]

Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
			Physical pretreatment				
Monorraphidium sp., Stigeoclonium sp., Scenedesmus sp. and Nitzschia	**Microwave**	Continuous (HRT: 15-20 days)	0.09 L CH$_4$/gVS	0.14 L CH$_4$/gVS	66%	68.5-69.3%	[112]
Isochrysis galbana	**Beading**	Batch	22.0 ± 1.0 mL biogas	12.7 ± 0.6 mL biogas	-	79%	
Acutodesmus obliquus	**Milling**	Batch	115.6 Nm3/ tCOD	197.89 Nm3/ tCOD	72%		[110]
			Chemical pretreatment				
Chlorella sp.	**Alkali**	Batch	≈120-140 mL/CH4 g COD	≈110-160 mL/CH4 g COD	17%	-	[114]
Scenedesmus sp.	**Alkali**	Batch	-≈150-160 mL/CH4 g COD	≈130-170 mL/CH4 g COD	17-20%	-	[114]
Isochrysis galbana	**Acid**	Batch	22.0 ± 1.0 mL biogas	26.0 ± 1.0 mL CH4 /g VS	18%	63.1%	[116]
Chlorella vulgaris	**Acid**	Batch	138.9 mL CH4 /g COD	221.8-228.8 mL CH4 /g COD	59-64%	66.3-70.6%	[113]

Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
			Chemical pretreatment				
Chlorella sp and Monorraphidium sp.	**Acid**	Batch	78 mL CH_4 /g VS	89.05- 142.5 mL CH_4 /g VS	14-82%		[117]
Tetraselmis striata	**Acid**	Batch	0.53 g CH4 /COD	0.46-0.82 g CH_4/g COD	0- 55%		[118]
Chlorella vulgaris	**Alkali**	Batch	138.9 mL CH4 /g COD	237.9 - 240.6 mL CH4 /g COD	71-73%	68.8- 70.1%	[113]
Spirulina maxima	**Acid-Alkali**	Batch		0-0.24 m^3 CH4/ kg VS	10-26%	53-76%	[88]
Chlorella sp. and Scenedesmus sp	**Alkali**	Batch	336 mL CH4 /g COD	213-363 mL CH4 /g COD	8%		[9]
Chlorella sp and Monorraphidium sp.	**Alkali**	Batch	78 mL CH_4 /g VS	80.78-145.10 mL CH_4 /g VS	3-86%		[117]
Mixed microalgae	**Oxidative pretreatment**	Batch	247.3-260 mL CH_4/g VS	268.7-432.7 mL CH_4/g VS	6-66%		[119]

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Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
Thermal /Hydrothermal pretreatment							
Monorraphidium sp. and Stigeoclonium sp.	**Hydrothermal**	Batch/Continuous	120mL CH_4/g VS	140-170 mL CH_4/g VS	17-40%		[120]
(Pediastrum sp., Micractinium sp. and Scenedesmus sp	**Hydrothermal**	Batch	103 mL CH_4/ g VS	136 mL CH_4/ g VS	32%		[121]
Chlorella vulgaris	**Hydrothermal**	Batch	156.4 mL CH4 /g COD	223-257 mL CH4 /g COD	42.5-64.3%	65.6-66.7%	[40]
Arthrospira maxima	**Hydrothermal**	Batch	185 mL biogas /g VS	138-235 mL biogas /g VS	(-5)-62%	-	[104]
Chlorella sorokiniana	**Hydrothermal**	Batch	297 mL biogas /g VS	298-461 mL biogas /g VS	9-69%	57-69%	[104]
Scenedesmus obliquus	**Hydrothermal**	Batch	265 mL biogas /VS	313-548 mL biogas /VS	18-107%	60-70%	[104]
Scenedesmus sp	**Hydrothermal**	Batch	0.15-0.22 L CH_4/gVS	$\approx$ 0.18-0.38 L CH_4/gVS	58-81%		[122]
Microspora	**Hydrothermal**	Batch	255 $\pm$ 2 mL CH_4/ g VS	359-413	41-62%		[106]

Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
Thermal /Hydrothermal pretreatment							
Acutodesmus obliquus, Oocystis sp.Phormidium and Nitzschia sp	**Hydrothermal**	Batch	198 ± 9 mL CH₄/ g VS	219-307 mL CH4/ g VS	11-55%		[106]
Chlamydomonas, Scenedesmus, and Nannochloropsis	**Hydrothermal**	Batch	272 ± 1 mL CH₄/ g VS	323-398 mL CH4/ g VS	19-46%		[106]
Spriulina maxima	**Thermal**	Batch		0.18-0.2 m³ CH4/ kg VS	0-10%	70-73%	[88]
Nannochloropsis salina	**Thermal**	Batch	347 mL STP/g VS	549 mL STP /g VS	58.2%		[38]
Scenedesmus sp	**Thermal**	Batch,	81.8 mL CH₄/ g COD	89.3-128.7 mL CH₄/ g COD	9.1-36.4%	64%	[6]
Chlorella sp. and Scenedesmus sp	**Thermal**	Batch	336 mL CH4 /g COD	351-405 mL CH4 /g COD	4.5-20.5%		[9]
Chlorella vulgaris	**Thermal**	Batch	180.3-267.7 mL/CH4 g COD	29.8-92.7%	67.3-68.3%		[113]

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Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
			Thermal /Hydrothermal pretreatment				
Nannochloropsis salina	**Thermal**	Batch	≈0.2 m³ CH₄ /kg VS	0.51-0.57 m³ CH₄ /kg VS	150%		[38]
Nannochloropsis salina	**Thermal**	Semi continuous OLR: 1.96 kg VS					[38]
		HLR: 119.84 days	0.3-0.5 m3/ m3.d	1.00 m3/ m3.d	100-233%		
Scenedesmus obliquus	**Thermal**	Batch	265 mL biogas /VS	270-381 mL biogas /VS	10-44%	60-68%	[104]
Chlorella sorokiniana	**Thermal**	Batch	297 mL biogas /g VS	310 -393 mL biogas /g VS	9-44%	55-69%	[104]
Arthrospira maxima	**Thermal**	Batch	185 mL biogas /g VS	127-250 mL biogas /g VS	(-12)-72%		[104]
Scenedesmus dimorphus	**Thermal**	Batch	101 mL CH₄/ g VS	130-137 mL CH₄/ g VS	60%		[123]
Nannochloropsis oculata	**Thermal**	Batch	0.279 L biogas/ g VS	0.283-0.436 L biogas/ g VS	0-56%		[124]
Stigeoclonium sp.,	**Thermal**	Batch	105.6 ml CH₄/gVS	134.9-181.3 mL CH4/g VS	28-72%		[109]

Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
			Biological pretreatment				
Chlamydomonas, Scenedesmus, and Nannochloropsis	**Microaerobical** 55 °C 12-24 h	Batch	272 ± 1 mL CH$_4$/ g VS	252-262 mL CH4/ g VS	0%		[106]
Acutodesmus obliquus, Oocystis sp.Phormidium and Nitzschia sp	**Microaerobical**	Batch	198 ± 9 mL CH$_4$/ g VS	171-193 mL CH4/ g VS	0%		[106]
Microspora	**Microaerobical**	Batch	255 ± 2 mL CH$_4$/ g VS	266	4-5%		[106]
Rhizoclonium	**Enzyme addition**	Batch	-	115-145 mL CH4/g TS	8-31%		[115]
Scenedesmus obliquus	**Commercial enzyme**	Batch	265 mL biogas /VS	1425-1699 ml biogas /gVS	273-485%	58-63%	[104]
Chlorella sorokiniana	**Commercial enzyme**	Batch	297 mL biogas /g VS	868-1292ml biogas /gVS	227%-387%	46-60%	[104]
Arthrospira maxima	**Commercial enzyme**	Batch	185 mL biogas /g VS	1461-1996	630-898%	-	[104]
Chlamydomonas reinhardtii	**Commercial enzyme**	Batch	263.1 mL CH$_4$/ g COD	255.7-311 mL CH$_4$/ g COD	0-16%	72%	[125]

Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
			Biological pretreatment				
Chlorella vulgaris	**Commercial enzyme**	Batch	190mL CH$_4$/ g COD	270-299 mL CH$_4$/ g COD	42-58%	72%	[125]
Chlorella vulgaris	**Commercial enzyme**	Batch	160.4 mL CH4/g COD	223.8 -255.6mL CH4/g COD	39-59%		[126]
Chlorella vulgaris	**Commercial enzyme**	Semi continuous	50 mL CH4/g COD	128.4± 15.3 mL CH4/g COD	156.8%		[87]
Chlorella vulgaris	**Commercial enzyme**	Batch	142 mL CH4/g COD	≈170- 250 mL CH4/g COD	20-77%		[87]
Scenedesmus sp.	**Commercial enzyme**	Batch	141.6 mL CH4/g COD	≈150-200 mL CH4/g COD	6-41%		[87]
Chlorella vulgaris	**Commercial enzyme**	Continuous	56 mL biogas/ day	86.6- 200.8 mL CH$_4$/ day	53-300%		[127]
Microalgal biomass	**Commercial enzyme**	Batch	188.6 mL CH$_4$/g VS	217 mL CH$_4$/g VS	15.4%		[39]
Acutodesmus obliquus	**Commercial enzyme**	Batch	115.6 Nm3/ tCOD	150.7Nm3/ tCOD	30.3%		[110]

Strain	Pretreatment Type	Reactor type	Biogas yield (before pretreatment)	Biogas yield (after pretreatment)	Biogas improvement (%)	Methane content	Ref.
Biological pretreatment							
Acutodesmus obliquus	**Commercial enzyme +Milling**	Batch	115.6 Nm3/ tCOD	189.2 Nm3/ tCOD	64%		[110]
Botryococcus braunii	*Anthracophyllum discolor* (in-situ enzyme)	Batch	27.8 ml CH4/VSS.d	27.5-32.8 ml CH4/VSS.d	17.9%	70%	[128]
Nannochloropsis gaditana	*Raoultella ornithinolytica* (in-situ enzyme)	Batch	109.37 CH4/VSS	262.84 -282.92 ml CH4/VSS	140.32 %-158.68%	-	[81]
Chlorella sp	*Bacillus licheniformis* (in-situ enzyme)	Batch	338.7 mL CH4/g VS	415.6 mL CH4/g VS	8.3 -22.7 %		[129]
Mixed culture of green microalgae	**Commercial enzyme/ *Trametes versicolor* (in-situ enzyme)**	Batch	83 ml CH4/ g VS	100-144 ml CH4/ g VS	20.5-73.5%	68%	[130]

Previous studies showed that ultrasound pretreatment at between 60-100 MJ/kgTS with a contact time of not more than 60 min resulted in up to 10-30 % biogas improvement depending on the microalgae strain [45]. However, for some cases, biogas production was lowered. In terms of energy balance, Ein/Eout were higher, which means, these pretreatments may not be feasible for large scale applications. As ultrasound pretreatment, microwave pretreatment showed similar concerns in terms of energy requirements [45]. Thermal pretreatment was applied commonly to microalgal biomass for temperatures more than 100℃ and biogas improvements were achieved as 20-50% (Figure 2.10). Net energy gain was also found to be negative for thermal pretreatment especially for high temperatures. Additionally, after high temperature pretreatment, cooling down of the biomass to mesophilic or thermophilic temperatures for anaerobic digestion also consumes energy and time. Regarding chemical pretreatment, pH, temperature and acid/alkali concentrations, the effectiveness of pretreatment varies (20-80% improvement in biogas production) (Figure 2.10). The most important advantage of the chemical pretreatment is its low energy demand. However, toxicity of the chemicals, corrosive effects, production inhibitory compounds, and cost of chemicals makes chemical pretreatment unsustainable. Biological pretreatment also showed better biogas yield improvements between 10-50%.(Figure 2.11).
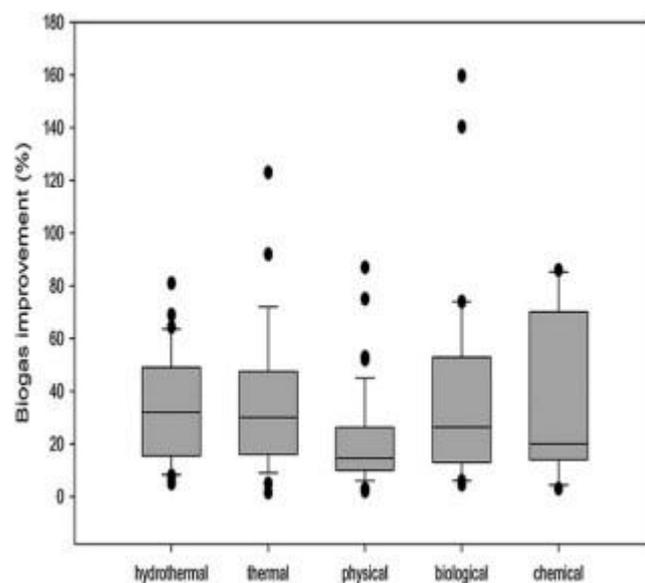
Figure 2. 10. Biogas improvement potential of various pretreatment methods [45]

Some research compared different pretreatment methods for specific strains. Schwede et al. [38] showed that thermal pretreatment was the most effective for improvement of methane production from *Nannochloropsis salina* when compared to ultrasonic, microwave, and French press. Alzete et al. [106] also found out that thermal pretreatment was the most effective process. Gonzalez-Fernandez et al. [6] also concluded that thermal and ultrasound pretreatment methods provided similar improvements in methane production from *Scenedesmus sp.*, however; energy consumption in thermal pretreatment is lower. Passos et al. [48] also suggested that thermal pretreatment had a better energy ratio than hydrothermal, microwave, and ultrasound pretreatments.

Ehimen et al. [115] stated that enzymatic pretreatment was more effective than mechanical pretreatment for *Rhizoclonium sp.* Furthermore, similar conclusion was stated by Ometto et al. [104] and enzymatic pretreatment were found to be more advantageous for *Scenedesmus obliquus, Arthrospira maxima, and Chlorella vulgaris.* Gruber-Brunhumer et al. [110] found out that mechanical pretreatment and freezing were most effective pretreatment when compared to ultrasonication, enzymatic pretreatment and milling for *Acutodesmus obliquus.*

These comparison studies showed that the feasiblity of pretreatment method is primarily dependent on type of microalgae. However, enzymatic and thermal pretreatment methods were stated as more effective especially in case of energy isssues. Thermal pretreatment at high temperatures may lead to high energy consumption, so; its feasibility may be challenging. On the other hand, the requirement for enzymatic pretreatment was low due to mild environmental conditions. Additionally, after thermal pretreatment some recalcitrant compounds that cannot be degraded by hydrolytic bacteria may be produced. On the contrast, no inhibitory compounds are produced during biological pretreatment (Figure 2.11).
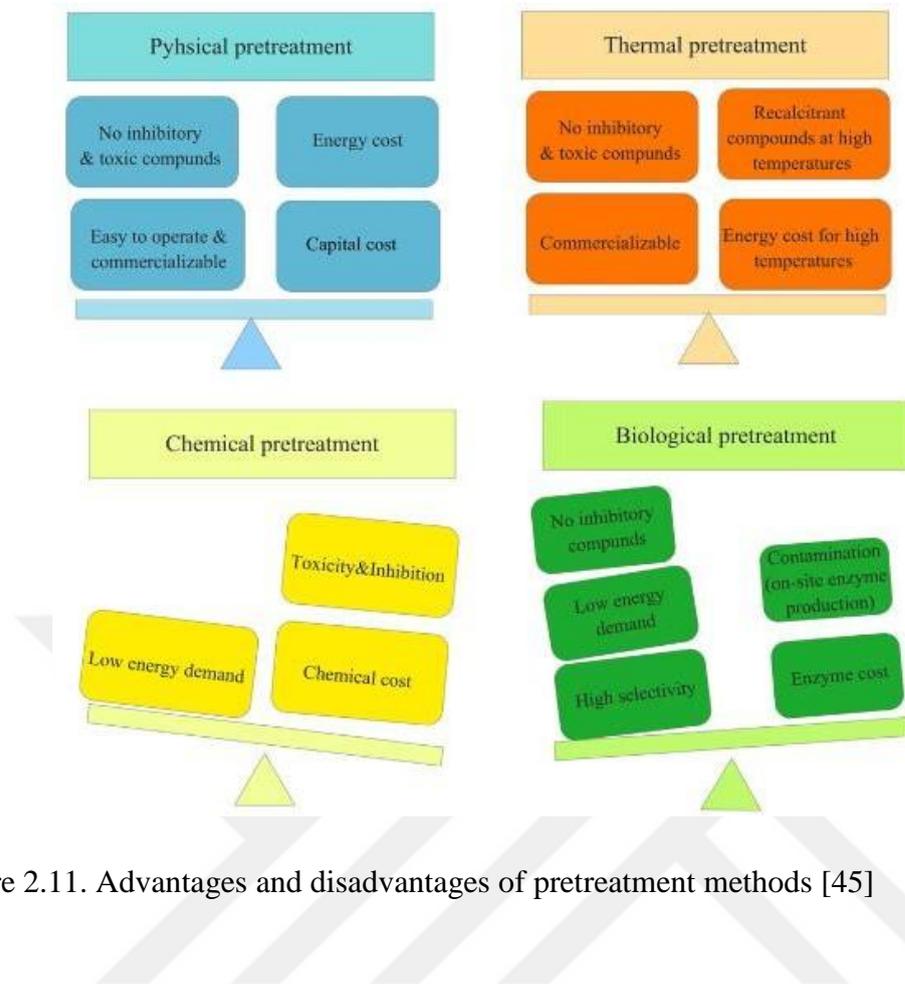
Figure 2.11. Advantages and disadvantages of pretreatment methods [45]

2.6. Evaluation of Previous Studies

Anaerobic digestion of microalgal biomass is found to be promising to tackle with increasing global energy demand. However, it has some limitations resulting in lower methane yields. To improve of methane yields from microalgae, one of the main problems, which is cell wall degradability, should be solved to achieve higher methane yields. Application of pretreatment before anaerobic digestion is the best alternative for cell wall disruption and enhanced biomass solubilization. Among pretreatment methods, biological pretreatment can be considered as promising due to low energy demand and higher selectivity of enzymes. Additionally, it does not produce inhibitory compounds that may lead in disruption of anaerobic digestion. So, the research should further focus on biological pretreatment with different pretreatment conditions and with different type of microalgae. In addition, most of the studies were conducted at mesophilic temperature after application of pretreatment. So, there is lack knowledge on the effect of digestion temperature on pretreated microalgae biomass.

In the light of previous research, this study aimed to give a deep understanding on application of enzymatic pretreatment and its effect on biomass solubilization and methane yields from microalgae. The effects of enzymatic pretreatment on methane production were investigated by comparing methane production from pretreated microalgal biomass with raw biomass. Two microalgae having different cell wall characteristics were chosen to evaluate the possible differences due to type of microalgae. It was also aimed to quantify potential methane yields from raw and pretreated microalgal biomass at different anaerobic digestion temperatures (mesophilic and thermophilic) to observe effect of temperature on methane production potential from microalgal biomass.

3. MATERIALS ANDMETHODS

3.1. Strain Selection and Characterization

In this study, two different microalgae specie were selected which are namely, *Scenedesmus sp.* and *Porphyridium cruentum. Scenedesmus sp.* is well-studied microalgae in terms of pretreatment and biogas production due to its rigid cell wall nature. Interior cell wall consists of cellulose covered by hemicelluloses and exterior layer consists of trilaminar structure including glycoproteins, glucosamine containing biopolymers, and algeanan (acetelysis resistant biopolymers, ARB) structure. The breakdown of the cell wall structure of this microalgae is the major concern for biogas applications since rigidity of cell wall decreases the accessibility of hydrolytic bacteria to organic matter during anaerobic digestion. So, this microalgae was selected to investigate the effect of enzymatic pretreatment. *Porphyridium cruentum* is red marine microalgae generally used due to its potential for polysaccharides (xylose, glucose, and galactose). Additionally, fatty acids, proteins, pigment and other chemical compounds are also produced from this strain. *Porphyridium sp.* cells are encapsulated by polysaccharide complex containing sulfate (EPS). Glucose, rhamnose, fucose, arabinose, xylose, mannose, orthomethyl sugar and acidic residues of glucuronic acid and galacturonic acid are the principal sugars present in the EPS. In addition, it includes proteins bounded to EPS. As they grow, this cell wall may dissolve in the medium resulting in viscosity increase. [131] [133]. This strain has not been studied for pretreatment and biogas production and there is not any research present in the literature. So this microalgae was selected to fulfill the gap in literature.

Scenedesmus sp. was obtained from Hacettepe University Department of Environmental Engineering. *Porphyridium cruentum* was purchased from Algae Supply Co (USA) (Figure 3.1). Substrate characterization of biomass is important since solids content, macromolecular composition and C: N ratio etc. affect the anaerobic digestion process. Prior to enzymatic pretreatment and BMP tests, characterization experiments were conducted for selected microalgae strains and the results were given in Table 3.1. As can be seen from the table, total solid content of both microalgae were 5 g/L.Volatile

solids (VS) content differs for both strains. *P. cruentum* had a lower VS content due to presence of minerals, especially salt, in the growth medium. sCOD/COD ratios were determined as 3% and 5% for *Scenedesmus sp.* and *P. cruentum,* respectively indicating low degradability of biomass. C: N ratios were found to be less than 10 indicating high N content of these microalgae.
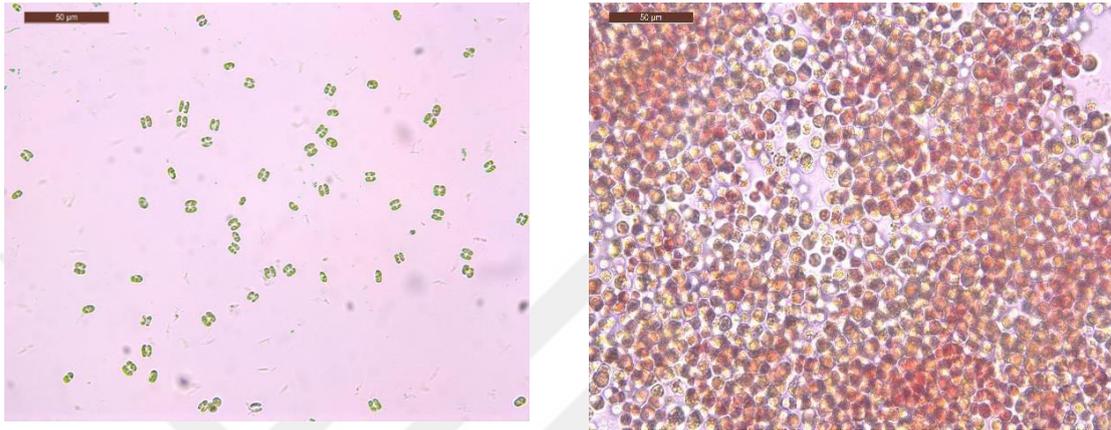
Figure 3. 1. Selected strains (Left: *Scenedesmus sp*., Right: *Porphyridium cruentum*)

Table 3. 1. Characteristics of microalgae used in this study

	Scenedesmus sp.	*P.cruentum*
Chemical oxygen demand(COD)	4822 mg/L	3414 mg/L
sCOD /COD (%)	3.0	5.0
Total solids (g/L)	5	5
Volatile solids (g/L)	4.2	3.8
Volatile solids (%) in total solids	84	76
Ash (%)	16	24
C:N ratio	6.53	8.88
CHSN	$C_{38.9}N_{5.95}H_{6.13}S_{0.44}$	$C_{7.02}N_{0.79}H_{3.19}S_{1.94}$

3.2. Microalgae Cultivation and Harvesting

For cultivation of microalgae, appropriate medium was chosen according to literature studies. *Scenedesmus sp.* was fed with Bold's Basal Medium (BBM) and *P. cruentum* was fed with F/2 medium which is a saline water medium. The medium was autoclaved at 120°C for 20 minutes and kept in the refrigerator until use. The ingredients of medium were given in the following tables (Table 3.2 and Table 3.3)

Table 3. 2. Preparation of 1L Bold's Basal Medium (BBM) [133]

Amount added	Chemical	g/ 400 mL H₂O	Amount added	Chemical	g/ 100 mL H₂O
		pH=7.0			
935 mL	Distilled water		1 mL	Na₂EDTA	5.0
10 mL	NaNO₃	10.0	1 mL	KOH	3.1
10 mL	CaCl₂.2H₂O	1.0	1 mL	FeSO₄	0.498 (in 0.1% H₂SO₄)
10 mL	MgSO₄.7H₂O	3.0	1 mL	H₃BO₃	1.142
10 mL	K₂HPO₄	3.0	1 mL	ZnSO₄.7H₂O	0.882
10 mL	KH₂PO₄	7.0	1 mL	MnCl₂.4H₂O	0.144
10 mL	NaCl	1.0	1 mL	MoO₃	0.071
			1 mL	CuSO₄.5H₂O	0.157
			1 mL	Co(NO₃)₂.6H₂	0.049

Table 3. 3. Preparation of 1L F/2 [133]

Amount added	Chemical
	pH=7.0
998 mL	Artificial Sea water (NaCl: 24.6 g/l; KCl: 0.67 g/L; $CaCl_2.2H_2O$: 1.36 g/L; $MgSO_4.7H_2O$:6.29 g/L; $MgCl.6H_2O$: 4.66 g/L; $NaHCO_3$:0.18 g/L)
0.075 g	KNO_3
$NaH_2PO_4.2H_2O$	$CaCl_2.2H_2O$
1 mL	Trace element stock solution
1 mL	Vitamin stock solution
Trace element stock solution (in 1000 mL distilled water	
Na_2EDTA	4.36 g
$FeCl_3. 4H_2O$	3.15 g
$CuSO_4.5H_2O$	0.01g
$ZnSO_4. 7H_2O$	0.022 g
$CoCl_2. 6H_2O$	0.01g
$MnCl_2. 4H_2O$	0.18 g
$Na_2MoO_4.2H_2O$	0.006 g
Vitamin stock solution (in 1000 mL distilled water	
B_{12} vitamin	0.0005 g
Thyamine HCl	0.1 g
Biotin	0.0005 g

Cultivation of microalgae was conducted in 5 L plastic bottles (Figure 3.1). Prior to use, they are washed with acid cleaning solution to remove unwanted materials. In each bottle, an air bubbling system was provided with an air pump and air stone. Sunlight was the main light source for photosynthesis and no additional artificial light was given to the bottles. Temperature was kept as the room temperature (20-25°C) and harvested after 9-10 days of cultivation (as the dark green color/ dark red color was observed). 10% of the biomass was kept for next cultivation cycle.

Figure 3. 2.Cultivation of microalgae in 5 L plastic bottles

To harvest microalgae biomass, firstly, the biomass was kept for one day without mixing for gravity settling. After then, supernatant was removed and remaining biomass was centrifuged (NÜVE NF 200) at 4100 rpm for 3-4 minutes. The concentrated biomass was stored for further experiments.

3.4. Experimental Setup

After cultivation and harvesting process, microalgal biomass was subjected to enzymatic pretreatment at different pretreatment conditions to investigate cell wall disruption and biomass solubilization potential. Then, Biochemical Potential (BMP)

tests were applied to estimate methane production of selected microalgae and to observe effect of enzymatic pretreatment on methane production on these microalgae biomass.

3.4.1. Enzymatic Pretreatment of MicroalgaeBiomass

To investigate effect of enzymatic pretreatment on cell wall disruption and biomass solubilization both microalgae strains were subjected to enzymatic pretreatment at different pretreatment conditions including enzyme type, enzyme dose, temperature, and time. In this study, enzymes were chosen as cellulase, protease, viscozyme, and enzyme mix (viscozyme+protease) and purchased from Sigma Aldrich. Cellulase (synonym Celluclast® 1.5L) is produced from *Trichoderma reesei* with an enzyme activity of > 700 EGU/G.Cellulase catalyzes the breakdown of cellulase into glucose, cellobiose and other higher glucose polymers. Protease is produced from *Bacillus licheniformis* with an enzymatic activity of 7-15 units/mg protein. Protease provides break down of proteins by hydrolyzing peptide bonds. Viscozyme is a carboydrase mix complex (arabinase, cellulase, beta-glucanase, hemicellulose, and xylanase) derived from *Aspergillus aculeatus* with an activity of >100 FBU/g. These enzymes have different activities under different pH and temperatures. Optimum temperature and pH values were investigated by the enzyme suppliers and they are given in Table3.4.

Table 3. 4. Optimum temperature and pH conditions for selected enzymes given by the supplier

Enzyme name	Optimum temperature	Optimum pH	Reference
Cellulase	50-60°C	4.5-6.0	[134]
Protease	50°C	8-10	[135]
Viscozyme	25-55°C	3.3-5.5	[136]

In this study, pretreatment temperatures were chosen as 25°C, 40°C, and 55°C. These temperature ranges were chosen to investigate the effect of temperature on biomass solubilization. It is known that at higher temperatures above 50°C, the activity of enzyme decreases and above 70°C, they are denaturated. pH was adjusted to optimum

pH ranges prior to pretreatment for each enzyme according to data sheet given by the suppliers (See Table 3.4). In terms of enzyme mix, pH was adjusted to between 8.0 and 8.5 at first half of the pretreatment and after then, pH was increased to pH 4.0-4.5 to provide both activities of protease and viscozyme. pH measurements were conducted by Mettler Toledo S700 Seven Excellence.

After pH and temperature adjustment, 100 mL of microalgae biomass (*Scenedesmus sp., P. cruentum)* as 5 g TS/ L were put into Erlenmayer flasks and subjected to enzymatic pretreatment by adding enzymes under the pretreatment conditions above. Enzyme doses were chosen as 0.1, 0.3 and 0.5 mL/g biomass. Each experiment was done in triplicate. The experiments were conducted in an incubator (NÜVE ES120) with continuous shaking at 100 rpm to provide contact of enzymes with the biomass. Experiments were carried out for 24 hours and at some time intervals (1 hour, 3 hours, 6 hours, 9 hours and 24 hours); 10 mL of pretreated samples were taken from the flasks.

Table 3. 5. Pretreatment conditions applied to microalgae biomass

Type of Enzyme	Cellulase	Protease	Viscozyme	Enzyme Mix
Enzyme dose	0.1-0.3-0.5 ml/g biomass	0.1-0.3-0.5 ml/g biomass	0.1-0.3-0.5 ml/g biomass	0.1-0.3-0.5 ml/g biomass
Temperature	25°C -40°C - 55°C	25°C -40°C - 55°C	25°C -40°C - 55°C	25°C -40°C - 55°C
Time	1-24 hours	1-24 hours	1-24 hours	1-9 hours

The pretreated samples were collected to investigate the effect of pretreatment on biomass solubilization. To separate liquid media from microalgae biomass, the samples were centrifuged at 4000 rpm for 5 minutes. After then, supernatant was filtered through 0.45 μm filter. Finally, the remaining supernatant were further analyzed to determine

solubilization increase in terms of COD, carbohydrates, and proteins. Solubility increase (%) in terms of COD can be found from the equation below:

$$Solubility\ increase\ (\%) = \frac{sCOD_{final} - sCOD_{initial}}{COD}$$

Equation 3.1.

3.4.2. Biochemical Methane Potential Test (BMP)

Biochemical Methane Potential Test (BMP) test is an easy to apply procedure that gives methane production potential of a specific substrate in a batch reactor. In this study, BMP protocol was performed as stated by Angelidaki et al. [137]. Microalgae biomass to inoculum ratio were retained as (S: I ratio) 1, which means for 1 g VS of microalgae biomass, 1 g VS of anaerobic inoculum was added. Inoculum was provided from an anaerobic digester unit of the municipal wastewater treatment plant in Ankara. Inoculum was fed with nutrients with and they were acclimatized to mesophilic and thermophilic conditions before BMP tests. BMP tests were performed in a closed bottle with a volume of 100 mL (Figure 3.3). The required amount of samples and inoculum were poured into the bottles and head space volume of 40 mL were left for biogas collection. pH was adjusted to 7.0-7.5 and sodium carbonate was added to provide alkalinity. pH measurements were conducted by Mettler Toledo S700 Seven Excellence. The bottles were flushed with N_2 for 1 minute for degassing O_2 and immediately capped with butyl rubber stoppers. After then, they were sealed with plastic crimp to prevent gas leakage. The bottles were placed into the incubator (NÜVE ES 120) and temperature was adjusted to $35\pm2°C/55\pm2°C$ for mesophilic digestion and thermophilic digestion, respectively. Continuous mixing was applied at 80 rpm to increase contact of microorganisms and substrate and to provide homogenous medium. The background methane production due to inoculum itself was performed by blank assays and they were subtracted from the sample assays. The sample assays and blank assays were performed in duplicate.
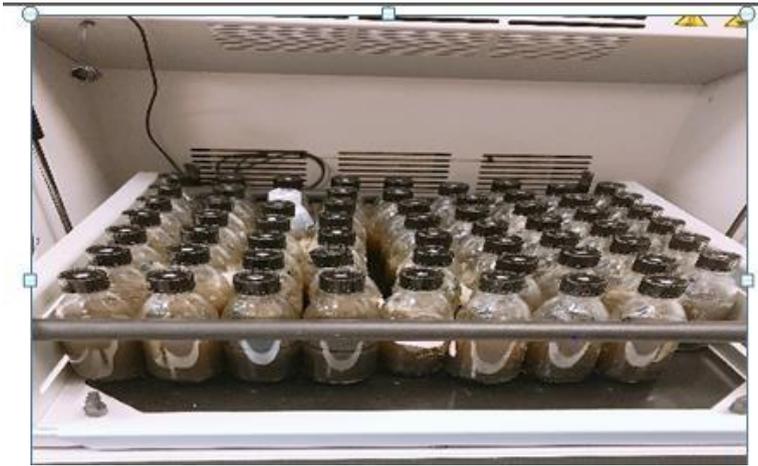
Figure 3. 3. Experimental setup for BMP tests

Biogas production was measured periodically using a digital manometer (Werth) (Figure 3.2) and the measured pressure values were converted into volume in normal conditions by the ideal gas law followingequation:

$$Vo = V_1 * P_1 T_o / P_o T$$

Equation 3.2

Where:

Vo: Volume of biogas (mL) at STP

V_1: Volume of measured biogas (mL)

Po: Measured pressure by the manometer (bar)

P_1: Ambient pressure at STP (1.013 bar)

To: Ambient temperature at STP (0°C, 273 K)

T_1: Ambient temperature in the BMP bottle

In order to determine CH_4 content of the biogas, CO_2 measurement device (Dansensor) was used (Figure 3.4). The device determined the CO_2 content of the biogas as (%) and assuming that content of other trace gases is negliable, the recorded values were subtracted from 100% to find CH_4 content as (%).

Figure 3. 4. Digital manometer and CO_2 analyzer

3.5. Data Evaluation

Data obtained from experimental setup were analyzed with different approaches. Data evaluation part includes optimization of enzymatic pretreatment, kinetics of methane production, and energy assessment of methane production from selected microalgae.

3.5.1. Optimization of Enzymatic Pretreatment

For optimization of enzymatic pretreatment, Response Surface Methodology (RSM) was applied in order to determine the effects of pretreatment conditions (enzyme dose, temperature, and time) on solubilization of microalgal biomass (*Scenedesmus sp.* and *P. cruentum*). Box-Benkhen design was introduced with three levels and three factors for each type of enzyme. Each type of enzyme was analyzed independently since the effect of each enzyme on the biomass was different from each other. The independent variables were chosen as enzyme dose, temperature, and time and the levels of these variables were chosen according to preliminary experiments given in the previous section. In case of time intervals, the range chosen between 3-9 hours. The independent variables and the levels were given in Table 3.6. Box-Benkhen design was simulated in Design Expert 10.0 with 27 runs.

Table 3. 6. Independent variables and levels of enzymatic pretreatment

Independent variables		Coded and actual levels		
		-1	0	1
Enzyme dose (mL)	A	0.1	0.3	0.5
Temperature (°C)	B	25	40	55
Time (hours)	C	3	6	9

3.5.2. Kinetics of Anaerobic Digestion

Biogas production curves are generally sigmodial which follow a lag period, a rapid gas production and a final plateau. This curve is similar to the typical bacterial growth curve. So, kinetic models are being used by several researchers to predict biogas production potential assuming that biogas production rate in the batch mode is consistent with specific growth rate of methanogens. Additionally, it provides prediction of lag period and maximum biogas production rate.

There are some kinetic models used for biogas production prediction namely first order kinetic model, Monod kinetics, Levi-Minzi, Gompertz model, modified Gompertz model, Cone model, and logistic model [138]. In this study, first order kinetic model and modified Gompertz model were applied to predict biogas production potentials. Modified Gompertz model was firstly developed by Zweietering et al. [139] to predict bacterial growth. Assuming that bacterial growth rate is coherent with biogas production, modified Gompertz model can be used for predicting biogas production potential. Modified Gompertz model has been used by several researchers so far [140, 141, and 142]. First order rate kinetics are the simplest models in which time is variable. It has been also studied extensively by the researchers for prediction of biogas production [138, 143].

First order kinetics:

First order kinetic model can be described as follows [141]:

$$y(t) = ym[1\text{-exp}(\text{-k*t})]$$ Equation 3.5

Where:

y (t): Cumulative methane production (mL/ gVS) at any time, t

y (m): Methane yield potential (mL/gVS)

k: First order rate constant (1/d)

t: Time at which M is calculated (days)

In this equation, the unknown parameters ym and were estimated by using non-linear regression with MATLAB. R^2 values and Mean Square Error (MSE) were also found to analyze the fitness of the model.

Modified Gompertz Model:

Modified Gompertz model can be also used to predict biogas potential yield as following equation[141]:

$$y(t) = ym * exp * \left[- \exp\left[\frac{Ue * e}{ym}\{\lambda - t\} + 1\right]\right]$$ Equation 3.4.

Where:

y (t): Cumulative methane production (mL/ gVS) at any time, t

ym: Methane yield potential (mL/gVS)

Use: Maximum methane production rate (mL/g VS. d)

λ: Duration of lag phase

t: Time at which M is calculated (days)

In this equation, the unknown parameters P, Rm, and λ can be estimated by non-linear regression with MATLAB.R^2 values and Mean Square Error (MSE) were also found to analyze the fitness of the model.

3.5.3 Energy Assessment

Energy assessment of pretreatment of biomass is essential in order to investigate as if application of pretreatment is feasible or not in terms of energy gain. Net energy gain (ΔE) is defined as the difference between energy gained from anaerobic digestion as methane/biogas and energy consumed for heating during pretreatment. Energy ratio is an another indicator which shows the ratio output energy to input energy.

$$\Delta E = Eoutput - E\ input \qquad\qquad \text{Equation3.5.a}$$

$$Energy\ ratio = Eoutput/Einput \qquad \text{Equation3.5.b}$$

According to Cordova et al. [144], total energy required is dependent on heat required for pretreatment of biomass:

$$Einput = \frac{\rho.V.\gamma.(Tp - Ta)(1 - \phi)}{VS} \qquad \text{Equation 3.6}$$

Where:

ρ: density (g/ml)

V: volume of the pretreated biomass (ml)

γ: specific heat (kJ/kg°C)

Tp: pretreatment temperature (°C)

Ta: ambient temperature (°C)

ϕ: heat recovery efficiency

VS: volatile solids content of biomass (g)

And the output energy can be calculated as:

$$Eoutput = \frac{\Delta P, CH4. LHV}{10^6}.$$

Where:

ΔP: methane production rate (m^3 CH$_4$/ m^3 reactor. day)

LHV: lower heating value of methane (kJ/m^3)

The assumed parameters for energy calculations were given in Table 3.7.

Table 3. 7. Parameters used for energy assessment [120]

Parameter	Unit	Value
ρ	g/mL	1
γ	$4.18*10^{-3}$	kJ/°C
Tp	°C	55
Ta	°C	25
ϕ	%	85
θ	kJ/m^3	1800
LHV	kJ/m^3	35800

3.6. Analytical Methods

3.6.1. Total Solids (TS) and Volatile Solids (VS) Determination

To determine microalgal biomass concentration after harvesting, TS and VS measurements were taken. TS and VS analysis were conducted according to [145]. First, a porcelain dish was dried at 550°C at least 1 hour. Then, it was put in a desiccator to cool down. After cooling, the weight of the porcelain disk was measured with a balance. After, 50 mL sample was put on the porcelain disk and dried at 105°C at least one hour. Then, it was put in the desiccator again to cool down. TS content of microalgal biomass can be calculated asfollows:

$$TS\left(\frac{mg}{L}\right) = \frac{\left((Wdryresidue + Wdish) - (Wdish)\right) * 1000}{SampleVolume\ (mL)}$$

Equation 3.3

In order to measure VS content, residue from TS experiment was ignited to 550°C in muffle furnace for 15-20 minutes. Then, it was put in the desiccator again to cool down and after cooling, the weight of porcelain disk and dried residue of sample was measured. As in TS experiment, the cycle continued after weight change is less than 4% of previous weight. VS content of microalgal biomass can be calculated as follows:

$$VS\left(\frac{mg}{L}\right) = \frac{\left((W_{dryresidue} + W_{dishafterign.}) - (W_{dryresidue} + W_{dishbeforeign.})\right) * 1000}{SampleVolume\ (mL)}$$

Equation 3.4

3.6.2. Soluble COD Measurements

Soluble COD measurements were conducted during pretreatment of biomass to observe the effect of biomass solubilization. The experiments were conducted with Hach Lange LCK 514 test kits (150-2000 mg/L) (Figure 3.5)

Figure 3. 5. COD test kits used in this study

Briefly, 2 mL of 0.45 μm filtered sample were put into test tubes. Dilutions were made if necessary. The tubes were shaked 3-4 times and put into the digester at 148°C for 2 hours. Then, the tubes were retained to cool down and the measurements were conducted by spectrophotometer (Figure3.6).

Figure 3. 6. COD digester and spectrophotometer used for COD analysis

3.6.3. Soluble Protein Measurements

In this part of the measurements, Lowry's method was followed [146]. In this method, four reagents (Reagent A, Reagent B, Reagent C, and Folin Reagent) were prepared. Reagent A was prepared by mixing %2 Na_2CO_3 with 1L 0.1 N NaOH solution. Reagent B was prepared by mixing 0.5 % $CuSO_4$ and %1 $Na_2Tartrat._2(H_2O)$ in 1 L distilled water. 50 mL of Reagent A and 1 mL Reagent B were mixed to prepare Reagent C. Reagent C was prepared one day before the experiment. Folin Reagent was prepared adding 2 mL Folin and Ciocalteu's Phenol Reagent (2N) and 2 mL distilled water. Folin Reagent was prepared just before the experiment.

Briefly, as the protocol stated, 3 mL Reagent C solution was pour into 10 mL test tubes and after then, 0.6 mL of standard BSA solutions (0-600 mg/L) were poured to the tubes. Test tubes were capped and vortexed for one minute. Then, they were incubated for 10 minutes at room temperature in dark. After incubation, 0.3 mL of Folin Reagent was added to each tube and vortexed for one minute. Then, they were incubated again at least 30 min. at room temperature in dark. Finally, before spectrophotometric analysis, they are vortexed shortly and transferred to cuvettes. Spectrophotometer (Beckman DU530) was turned at least 30 min before the analysis on to warm up and stabilize. As it was stabilized, the blank solution (distilled water) and samples were analyzed in spectrophotometer at 750 nm. For calibration, Albumin bovine serum, Cohn fraction V was used as the standard and seven different concentrations from 0 mg/L to 600 mg/L were used. To achieve this, stock solution of 600 mg/L was prepared and other concentrations were prepared by diluting stock solution in different ratios. Absorbance values for each standard solution was recorded and a calibration curve was drawn using absorbance values. After then, absorbance values of the samples were converted into concentration values(mg/L).

3.6.4. Soluble Carbohydrates (Sugar)Measurements

For determination of soluble sugars, the method established by Debuis et al. [147] was followed. Briefly, 2 mL of sample was pipetted into a tube, and then 0.05 mL of 80% phenol (by weight) was added. After, 5 mL of concentrated sulphuric acid was poured to the tubes. The tubes were waited for 10 minutes for cooling down and after then

mixed and waited for additional 30 minutes at room temperature. The absorbance values of the samples were measured at 490 nm. Blanks were prepared with distilled water as the sample. Standard solutions were prepared with glucose at different concentrations and the same procedure was followed in order to construct a standard curve.

3.6.5. Optical Microscopy

The treated and untreated samples were observed under optical microscopy (Leica DM 5000 B) equipped with digital camera to investigate the effect of enzymatic pretreatment on microalgae cells (Figure3.7).

Figure 3.7. Optical microscope used for visualizing microalgae cells

3.6.6. Summary of Materials and Methods

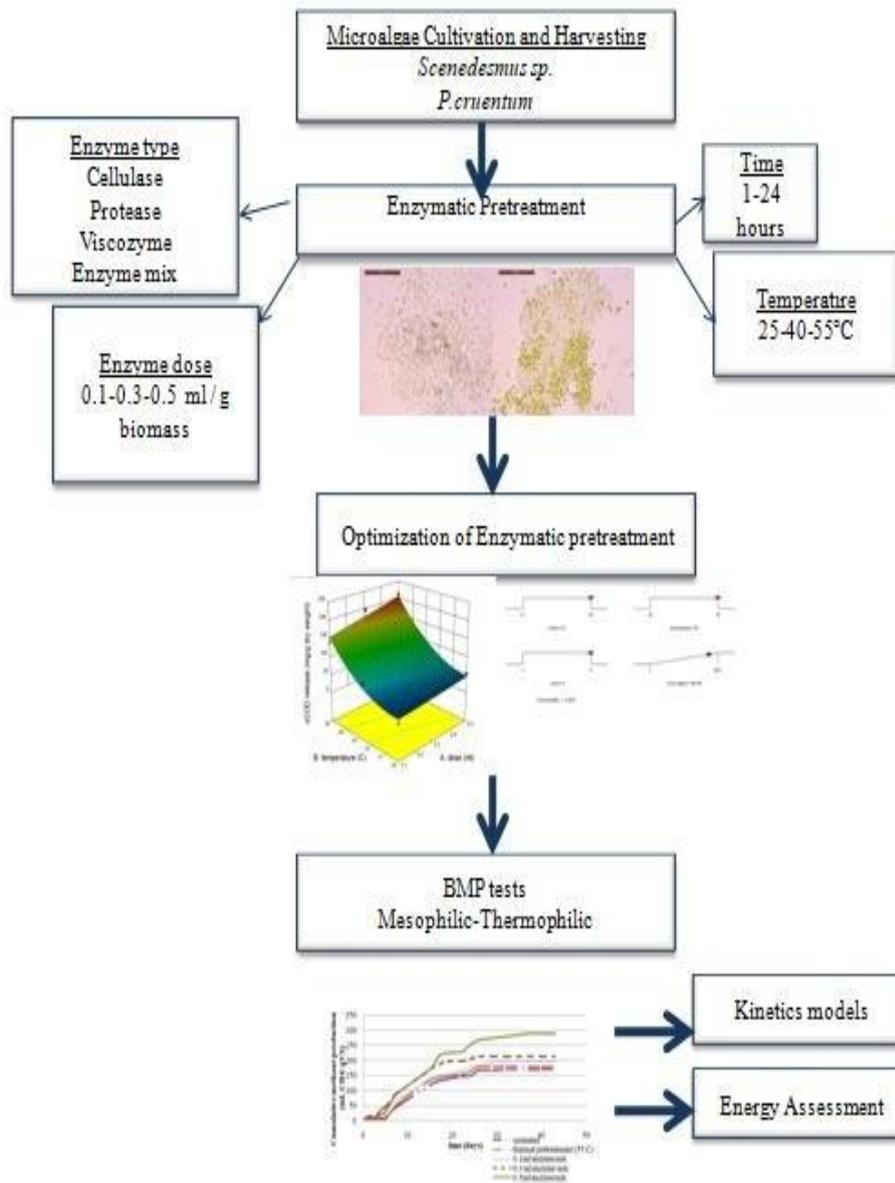The following figure summarizes the material and methods followed thought the study.

Figure 3.8. Flowchart of the methodology followed throughout the study

4. RESULTS AND DISCUSSION

This study aimed to investigate methane production yields from two different microalgae strains (*Scenedesmus sp.* and *Porphyridium cruentum*) and application of enzymatic pretreatment to improve methane production from these microalgae. The effects of enzymatic pretreatment conditions (enzyme type, enzyme dose, time, and temperature) on solubilization of biomass and the effect of enzymatic pretreatment on methane production were investigated by comparing biomass solubilization and methane production from pretreated microalgal biomass with untreated microalgal biomass. It was also aimed to quantify methane yield potentials from untreated and pretreated microalgal biomass at different anaerobic digestion temperatures (mesophilic and thermophilic) to observe effect of temperature on methane production potential from microalgal biomass. This study also aimed to determine feasibility of the enzymatic pretreatment in terms of energy considerations.

In this part of the thesis, the data obtained during experimental setup were given and evaluated in detail. Section 4.1. focuses on effect of enzymatic pretreatment on methane production from *Scenedesmus sp.* Same outline was followed for enzymatic pretreatment and BMP tests of *P. cruentum* biomass in Section 4.2. In Section 4.3, the comprehensive comparison and discussion of the results obtained from two selected microalgae were given.

4.1. Effect of Enzymatic Pretreatment on Biogas Production from *Scenedesmus sp.*

Scenedesmus sp. has a rigid multilayer cell wall including cellulose and hemicelluloses in the interior layer and exterior layer consists of trilaminar structure including glycoproteins, glucosamine containing biopolymers, and algeanan (acetolysis resistant biopolymers, ARB) structure [148]. The rigidity of cell wall decreases the accessibility of hydrolytic bacteria to organic matter during anaerobic digestion. So, breakdown of the cell wall of this microalgae is the major concern for biogas applications.

In order to overcome this problem, several pretreatment conditions can be applied to increase hydrolysis of the cell. In this study, *Scenedesmus sp.* biomass was subjected to enzymatic pretreatment with different enzymes including cellulase, viscozyme, protease and enzyme mix (protease and viscozyme). The effects of enzymatic pretreatment were investigated for cell wall disruption and organic matter solubilization by using different enzyme doses (0.1, 0.3, and 0, 5 mL/ g biomass) at different temperatures (25°C, 45°C, and 55°C) and at different application periods. Thermal pretreatment was also applied at 55°C to investigate the effect of temperature on *Scenedesmus sp.* solubilization. After enzymatic pretreatment, biomethane potentials of pretreated *Scenedesmus sp.* biomass under mesophilic (35°C) and thermophilic (55°C) conditions were tested in comparison with raw biomass. Two kinetic models (First order kinetic model and Modified Gompertz Model) were applied to biomethane potential test results to determine kinetic parameters of anaerobic digestion. Energy assessment of the pretreatment was applied in order to discuss the feasibility of the enzymatic pretreatment and methane production from *Scenedesmus sp.* The results obtained in the scope of this study and comprehensive data evaluations are given in the following sections in detail.

4.1.1. Thermal Pretreatment of *Scenedesmus sp.*

Thermal pretreatment at 55°C was applied to *Scenedesmus sp.* biomass without enzyme addition to observe the temperature effect on biomass solubility itself. Thermal pretreatment increased sCOD content of the medium as 59 mg sCOD/g dry weight in 9 hours. In 24 hours, sCOD release was reached to 65.6 mg sCOD/g dry weight (increase in solubility as 6.8%). This result showed that temperature itself had an effect on solubilization of *Scenedesmus sp.* biomass. Another studies also indicated increase in solubilization after thermal pretreatment between 55-60°C [6, 48]. However, they were not as effective as high temperature pretreatment. At high temperatures above 70-80°C, cell wall disruption of microalgae could be observed. At 55°C, microalgae are not able to grow and cell structure collapses for some type of microalgae. In case of *Scenedesmus sp.*, such collapse was not observed due to multilayer cell wall characteristics and cells were intact. However; solubilization of biomass to some extent was observed. This can be attributed to exopolymer release from cell wall after thermal pretreatment[6].

4.1.2. Enzymatic Pretreatment of *Scenedesmus sp.* by Cellulase

Cell wall of *Scenedesmus sp.* includes cellulosic structure and application of cellulase pretreatment was expected to disrupt cell wall of these microalgae to some extent. Results of cellulase pretreatment are given in Figure 4.1 and 4.2. Highest increase in COD solubilization with cellulase pretreatment was observed at 55°C as 138.8 mg sCOD/g dry weight (solubility increase as 12.3%)in 9 hours with cellulase dose of 0.5 mL. In 3 hours, the solubilization was also high as 118.8 mg sCOD/g dry weight (solubility increase as 11.53%) which showed that between 3-9 hours the rate of sCOD release was lower than at first 3 hours. With 0.3 mL cellulase pretreatment, sCOD release was found as 122.6 mg sCOD/dry weight (solubility increase as 11.5%) in 9 hours. The lowest cellulase dose pretreatment resulted in (0.1 mL cellulase) as COD releases 84.8 mg sCOD/ g dry weight (solubility increase as 8.8%)in 9 hours. These results showed that there was a positive correlation between enzyme dose and solubilization of *Scenedesmus sp.* biomass. After 9 hours, all samples reached a state that sCOD did not increase when contact time was further increased. These results showed that 9-hour pretreatment was enough to reach to maximum sCOD release. Furthermore, for the highest cellulase dose (0.5 mL cellulase), after 3 hours, most of the enzymes were used up. Between 6 and 9 hours, the rate of sCOD release was lower than the rate of sCOD release at first 3 hours. Lower cellulase doses required at least 6 hours contact time to reach maximum solubilization and after which sCOD releases were insignificant.

Cellulase pretreatment at 40°C resulted in improvement of biomass solubilization, however; the efficiencies were lower than the cellulase pretreatment at 55°C (Figure 4.1- Figure 4.2) since the enzymatic activity is higher at 55°C. The increase in sCOD release with pretreatment at the lowest dose (0.1 mL) was found as 33.4 mg sCOD/ g dry weight (solubility increase as 3.5%) in 24 hours. Highest sCOD release was observed for 0.3 and 0.5 mL cellulase dose in 6 hours as 42.4-76.4 mg/g dry weight (solubility increase as 4.4-7.9%), respectively. After 9 hours, sCOD release was not significant.

Cellulase pretreatment at 25°C resulted in lowest solubilization efficiency. Lowest dose (0.1 mL cellulase) pretreatment at 25°C resulted in sCOD release, which was negliable (less than 2%). 0.3 mL cellulase dose had a solubilization increase of 40 mg sCOD/g dry weight (solubility increase as 4.1%) in 9 hours and it did not change after then. Highest sCOD release was observed with 0.5 mL cellulase pretreatment. This pretreatment yielded 64.8 mg sCOD/g dry weight increase (solubility increase as 6.7%) in 9 hours (Figure4.2).

These results showed that temperature had a positive effect on enzymatic pretreatment and biomass solubilization. This can be attributed to release of cell wall exopolymer by temperature. Solubilization increase after thermal pretreatment can be the evidence for that. In addition, cellulase activity at 55°C were higher than lower temperatures. It was clear that there was a synergistic relationship between high temperature and cellulase activity on biomass solubilization. At lower temperatures, the sCOD release was observed after 3 hours, on the other hand, at high temperature (55°C), the sCOD release was observed even after 1 hour. Therefore, high temperature increased the rate of solubilization by the increased cellulase activity and decreased contact time required. Apart from effect of temperature, the enzyme dose was also found to be significant for COD solubilization.

When the amount of carbohydrate and protein solubilized from cell at various temperatures by cellulase pretreatment were compared, it was observed that higher carbohydrate release was observed under all pretreatment conditions applied. This can be attributed to degradation of cellulosic cell wall components via cellulase into the solution as soluble sugar. Protein release was also observed and this can be an indication of destruction of cell wall and percolation of intracellular protein into to the solution. It was also concluded that low cellulase doses resulted in lower release in carbohydrate content (Figure4.3)
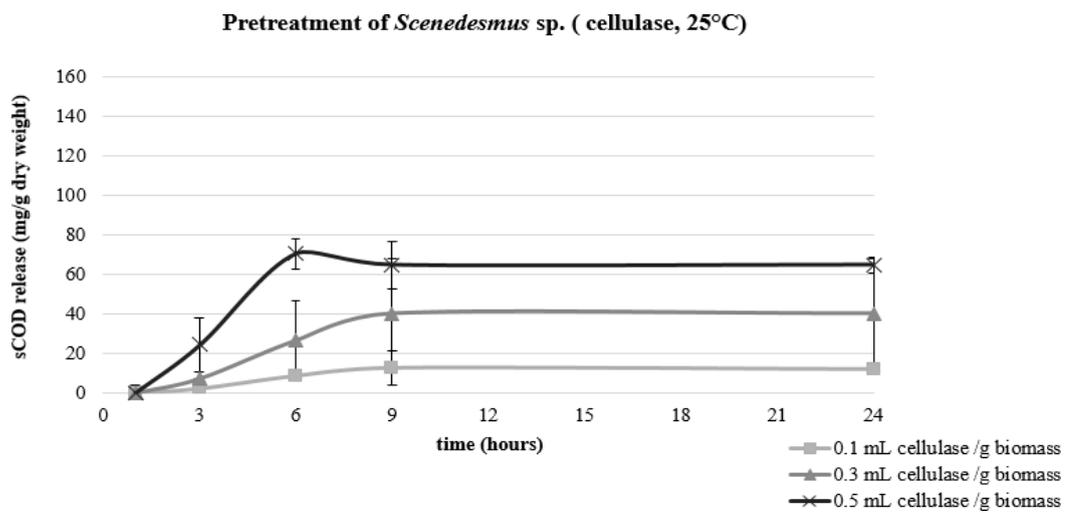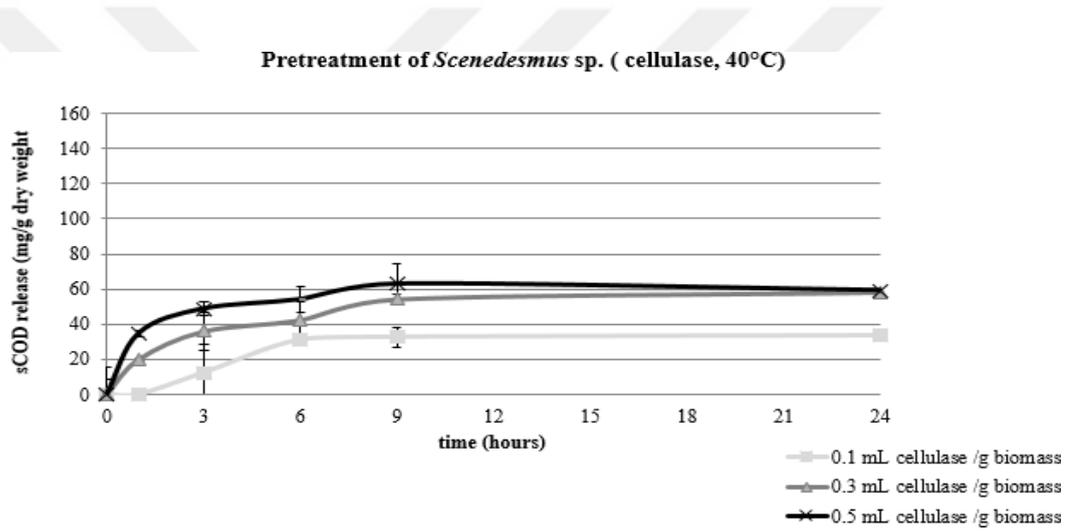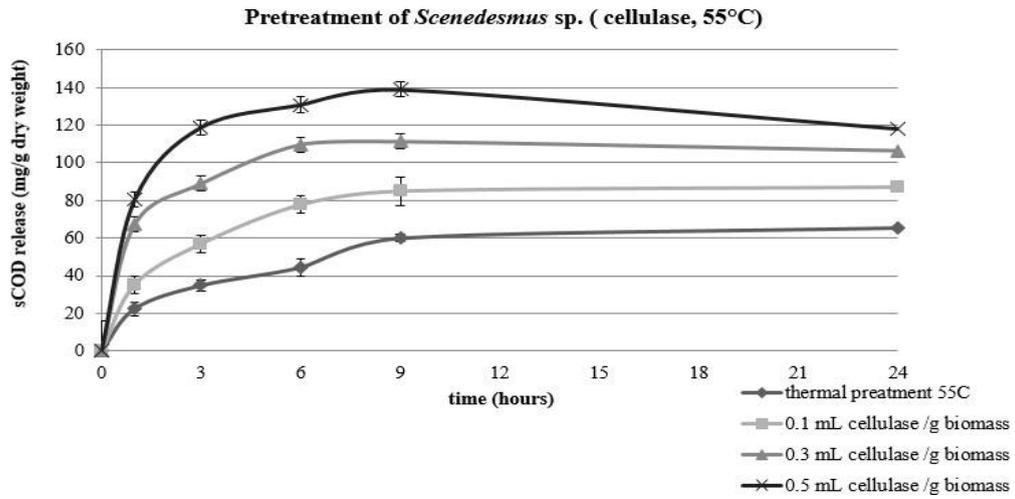
Pretreatment of *Scenedesmus* sp. (cellulase, 55°C)

- thermal preatment 55C
- 0.1 mL cellulase /g biomass
- 0.3 mL cellulase /g biomass
- 0.5 mL cellulase /g biomass

Pretreatment of *Scenedesmus* sp. (cellulase, 40°C)

- 0.1 mL cellulase /g biomass
- 0.3 mL cellulase /g biomass
- 0.5 mL cellulase /g biomass

Pretreatment of *Scenedesmus* sp. (cellulase, 25°C)

- 0.1 mL cellulase /g biomass
- 0.3 mL cellulase /g biomass
- 0.5 mL cellulase /g biomass

Figure 4.1. The effect of enzyme dose on soluble COD release by cellulase pretreatment at different temperatures (*Scenedesmus sp.*)

Pretreatment of *Scenedesmus* sp. (0.5 mL cellulase)

Pretreatment of *Scenedesmus* sp. (0.3 mL cellulase)

Pretreatment of *Scenedesmus* sp. (0.1 mL cellulase)
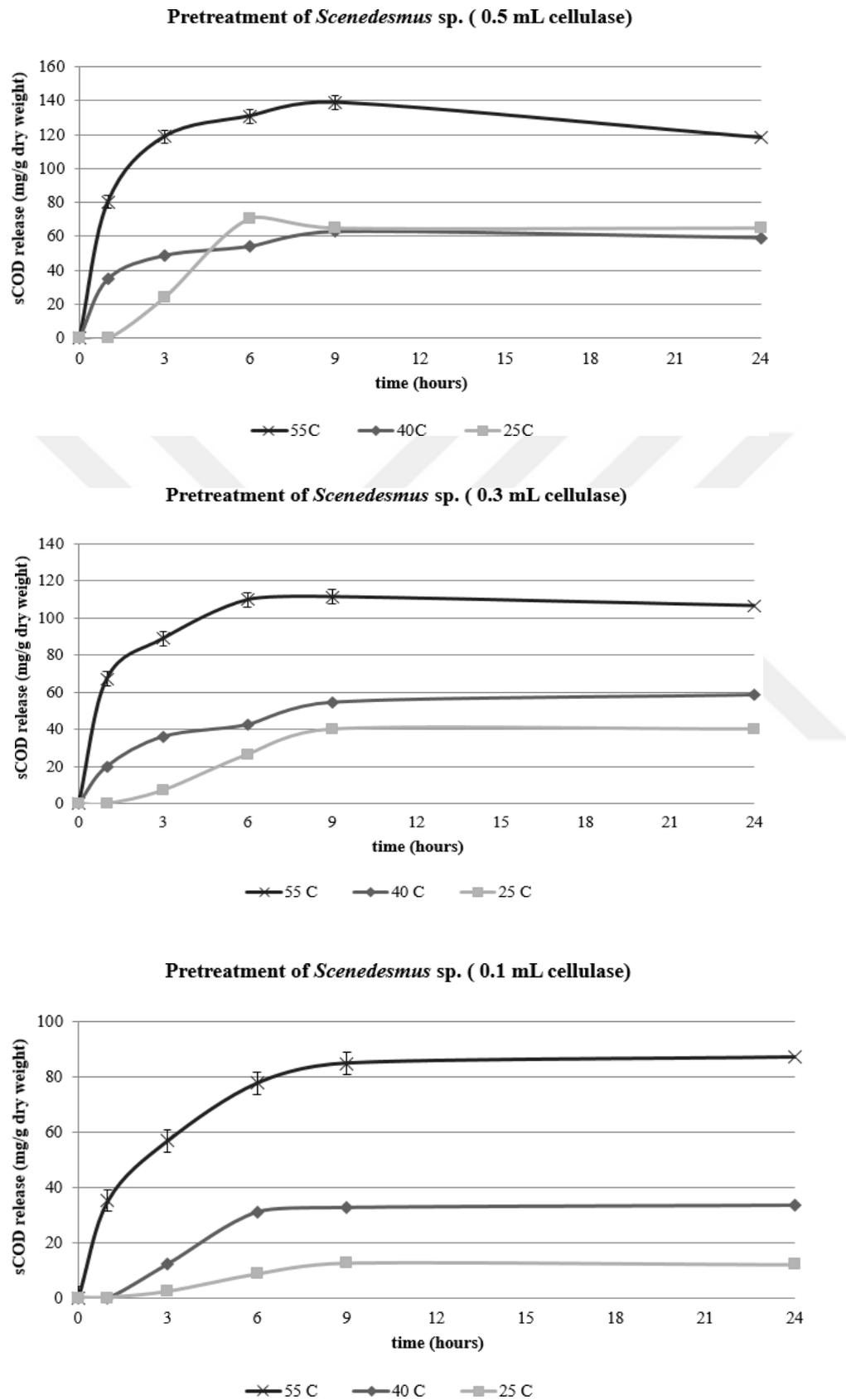
Figure 4.2. The effect of temperature on soluble COD release by cellulase pretreatment at different enzyme doses (*Scenedesmus sp*.)
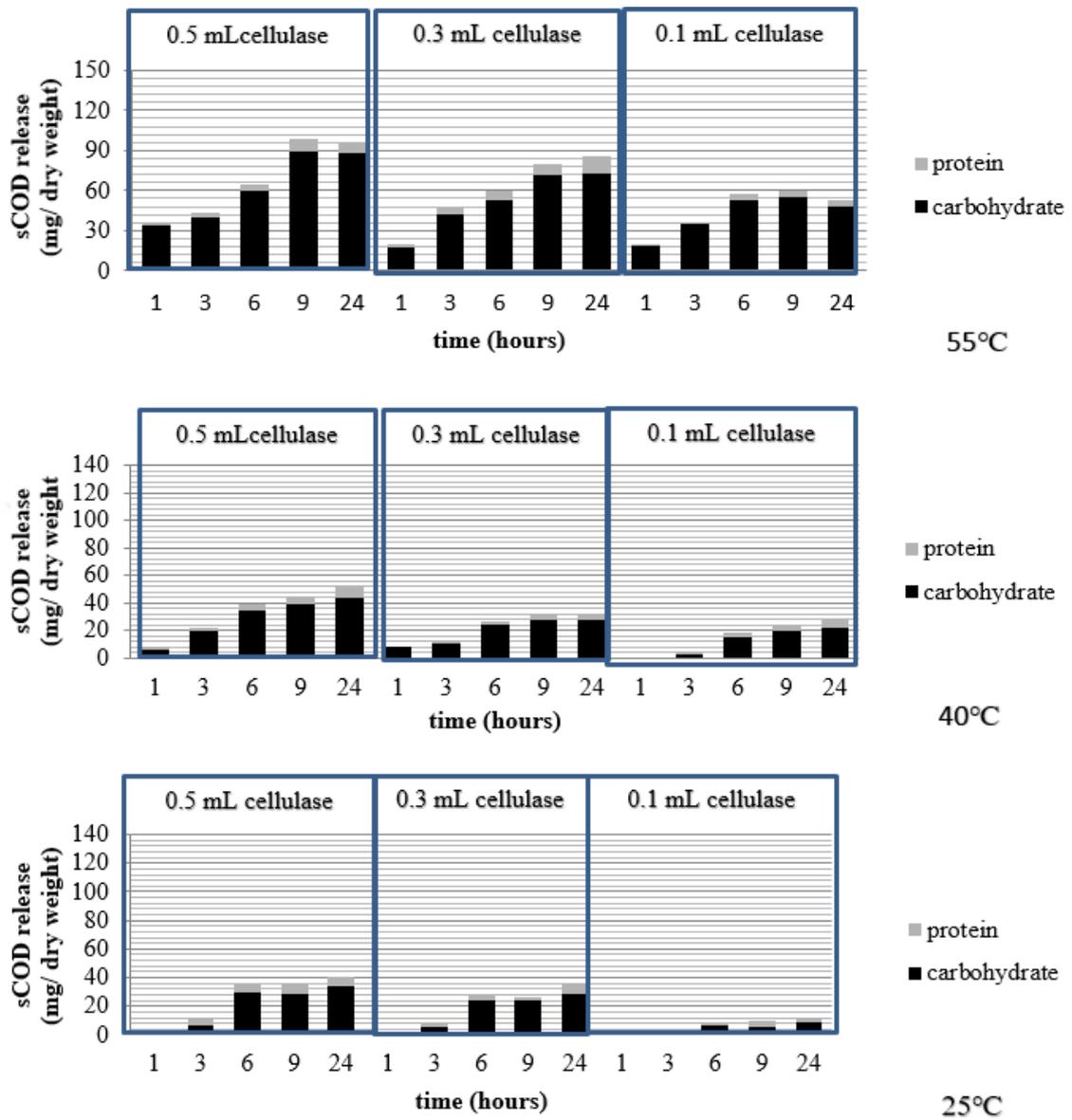
Figure 4.3. Change in soluble carbohydrate and protein content after cellulase pretreatment of *Scenedesmus sp*. at various temperatures

4.1.3. Enzymatic Pretreatment of *Scenedesmus sp.* by Protease

Cell wall of *Scenedesmus sp.* contains glycoproteins, so; application of protease pretreatment is expected to provide exterior cell wall disruption. Moreover, protein content of intracellular microalgae can also be converted into monomers by application of protease pretreatment.

In this study, highest solubilization increase was observed after protease pretreatment at 55 °C. Optimum activity provided by the enzyme manufacturer is 50°C, so highest solubilization can be attributed to the higher activity of proteases. When compared to thermal pretreated biomass at 55°C, there was a positive effect on protease pretreatment on sCOD release from *Scenedesmus sp*. Lowest dose (0.1 mL protease) provided sCOD release with increasing time and highest sCOD release was achieved as 141.6 mg sCOD/g dry weight (solubility increase as 14.7%) in 24 hours. Pretreatment with 0.3 mL protease had a sCOD release in 9 hours of 139.6 mg sCOD/g dry weight (solubility increase as 14.5%) and in 24 hours it was 147.2 mg sCOD/g dry weight (solubility increase as 15.2%). 0.5 mL protease pretreatment had the highest sCOD release after 24 hours as 154.8 mg sCOD/g dry weight (solubility increase as 16%). Moreover, in 9 hours, solubility increase reached to 15.4%, which meant that increasing contact time did not increase solubilization significantly (Figure 4.4-4.5). The similar trend was also observed for 0.3 mL protease pretreatment. These results are very important in terms of selecting contact time, which affects the cost of pretreatment process significantly.

Protease pretreatment at 40°C improved solubility of *Scenedesmus sp.* biomass. The lowest dose (0.1 mL protease) provided highest solubilization in 9 hours with an increase of 63 mg sCOD/g dry weight (solubility increase as 6.5%). Pretreatmentwith 0.3 mL protease had sCOD release in 9 hours as 64 mg sCOD/g dry weight (solubility increase as 6.6%) and 0.5 mL protease pretreatment had the highest solubilization increase among other doses as 67.1 mg sCOD/g dry weight (solubility increase as 7%) in 9 hour pretreatment. As the values were compared, it was noticed that maximum

increase was close to each other for all doses. However, at first 6 hours, the sCOD release rates were higher for the highest protease dose. Furthermore, it is important to mention that pretreatment efficiencies at 40°C were lower than pretreatment efficiencies at 55°C (Figure 4.4-4.5).

Protease pretreatment at 25°C showed also lower sCOD release efficiencies when compared to pretreatment at 55°C. Highest sCOD increase was observed as 53.2mgsCOD/g dry weight (solubility increase as 5.6%) in 9 hour pretreatment with 0.5 mL protease. For other enzyme doses, solubility increase was achieved after 9 hours as between 4.7-4.8%. After 9 hours, the sCOD release did not change, which means that time did not have a significant effect after 9 hours (Figure 4.4-4.5).

These results showed that protease pretreatment at 55°C showed the highest sCOD release for all doses. Cell wall of *Scenedesmus sp.* includes glycoproteins, so; with the increased activity of protease at 55°C, higher solubilization increase was achieved. At this part of the study, it was also observed that the protease doses yielded similar sCOD releases after 9 hours for low temperature pretreatment. At 55°C protease pretreatment, it was achieved after 9 hours for 0.3-0.5 mL protease dose. These results showed that lower protease doses also showed same sCOD releases as pretreatment was applied for longer periods. However, heating requirement will be higher if longer periods are used.
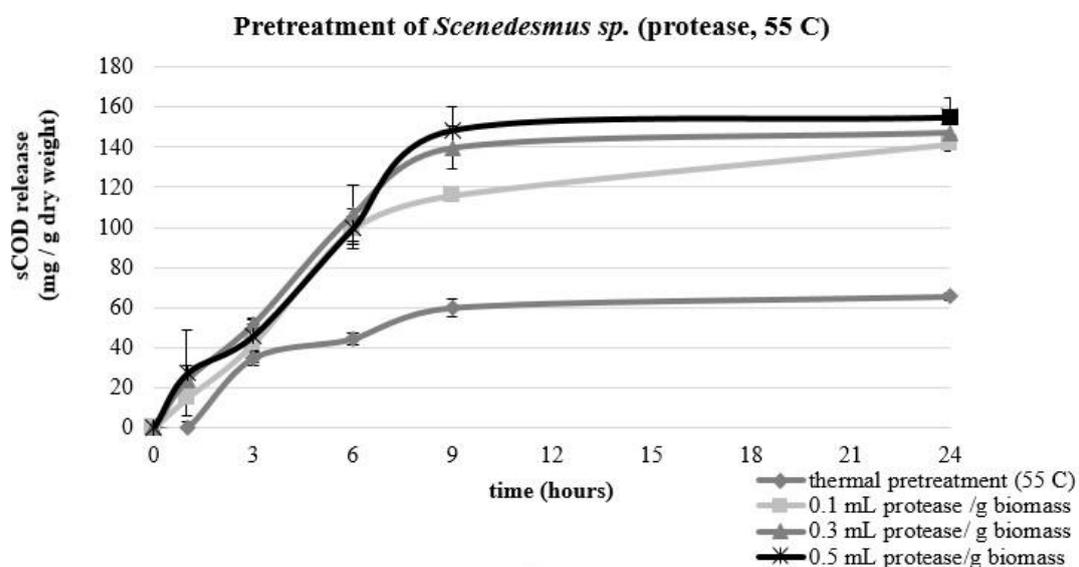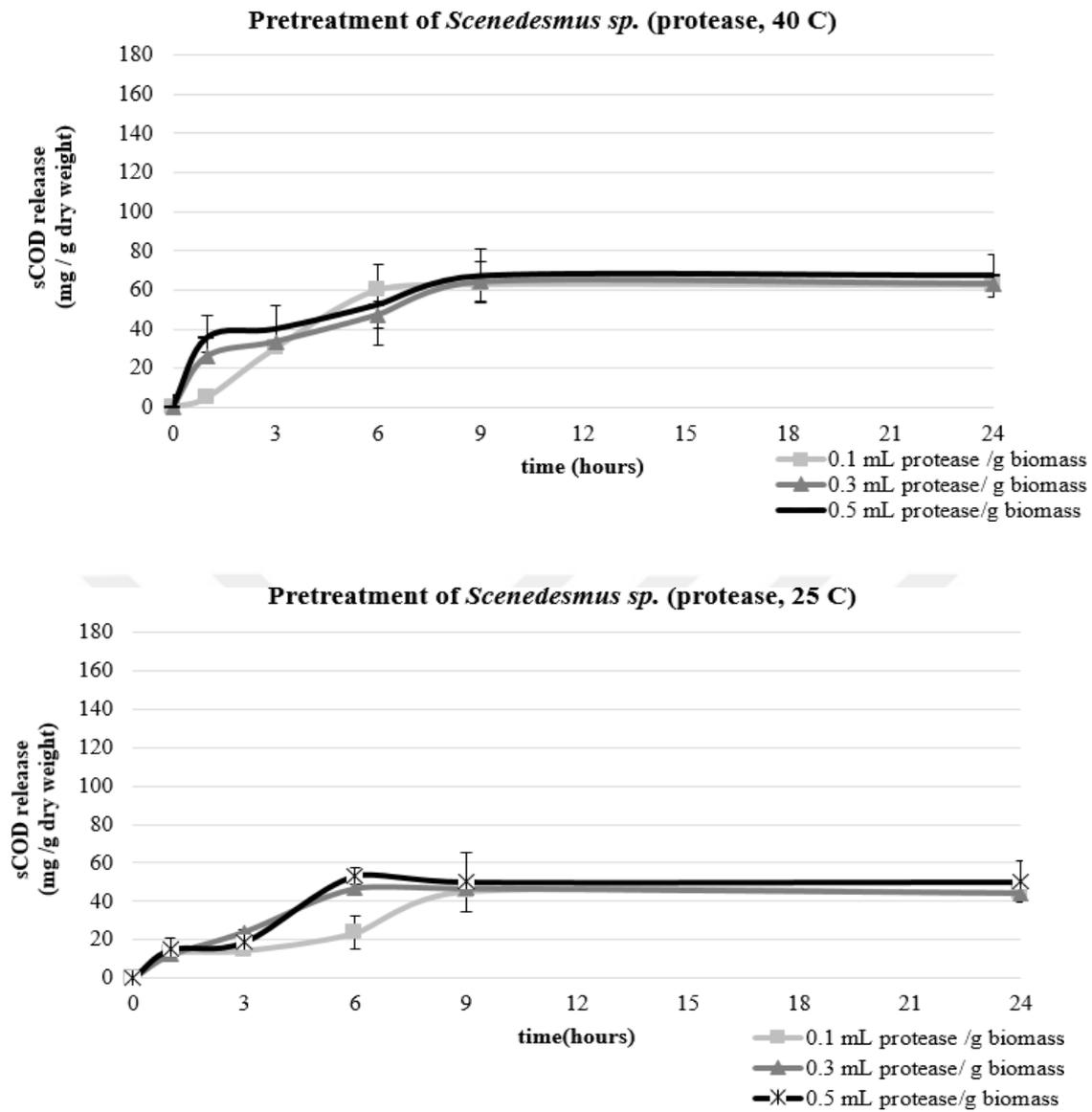
Figure 4.4. The effect of enzyme dose on soluble COD release by protease pretreatment at different temperatures (*Scenedesmus sp.*)
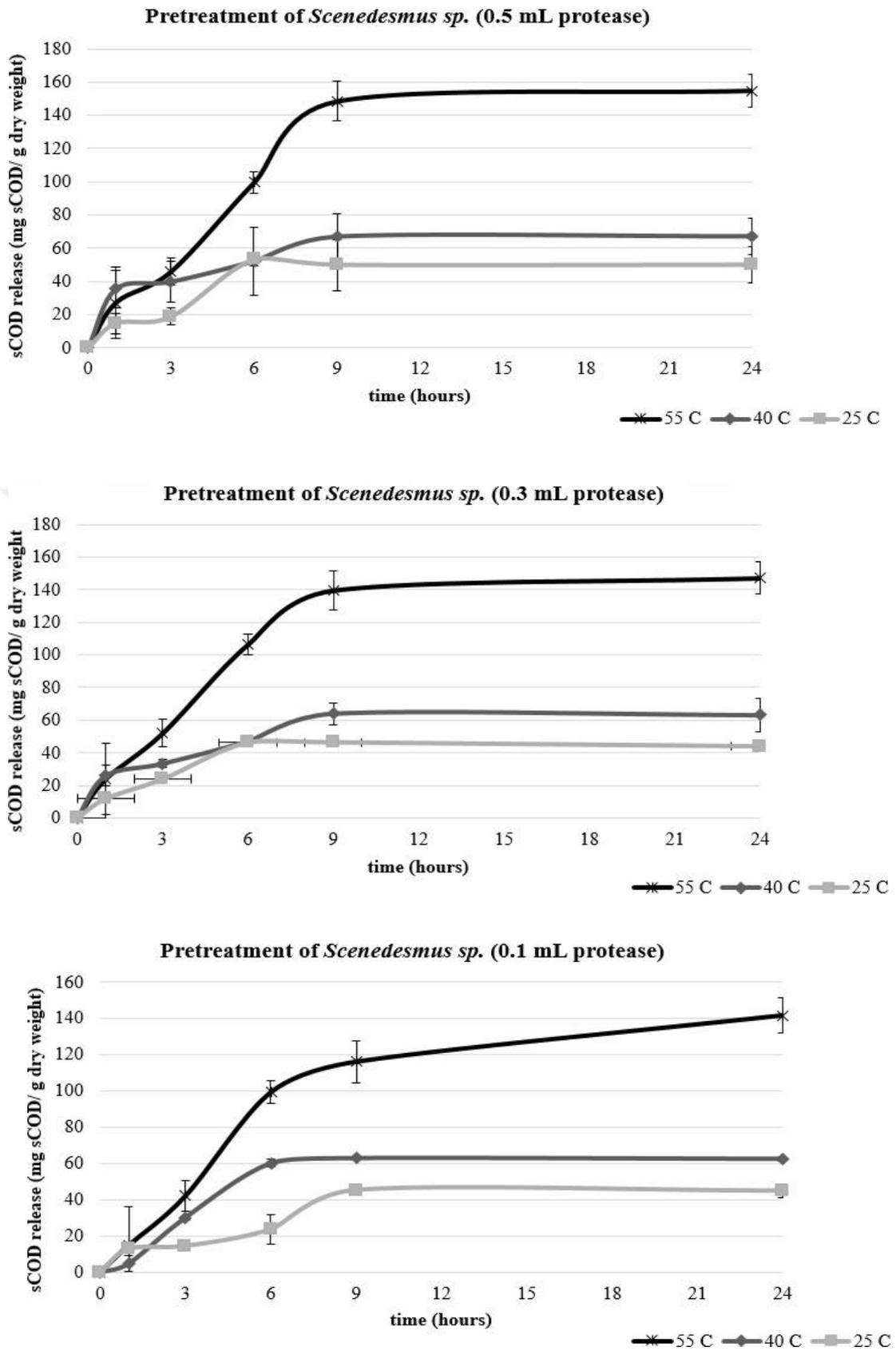
Figure 4.5. The effect of temperature on soluble COD release by protease pretreatment at different enzyme doses (*Scenedesmus sp.*)

Soluble carbohydrate and protein concentrations increased after protease pretreatment for all doses. This can be the indication of cell wall and cell components solubilization. It was also observed that protein solubilization protease pretreatment at 55°C yielded higher protein solubilization than protease pretreatment at lower temperatures due to high activity of protease at 55°C.
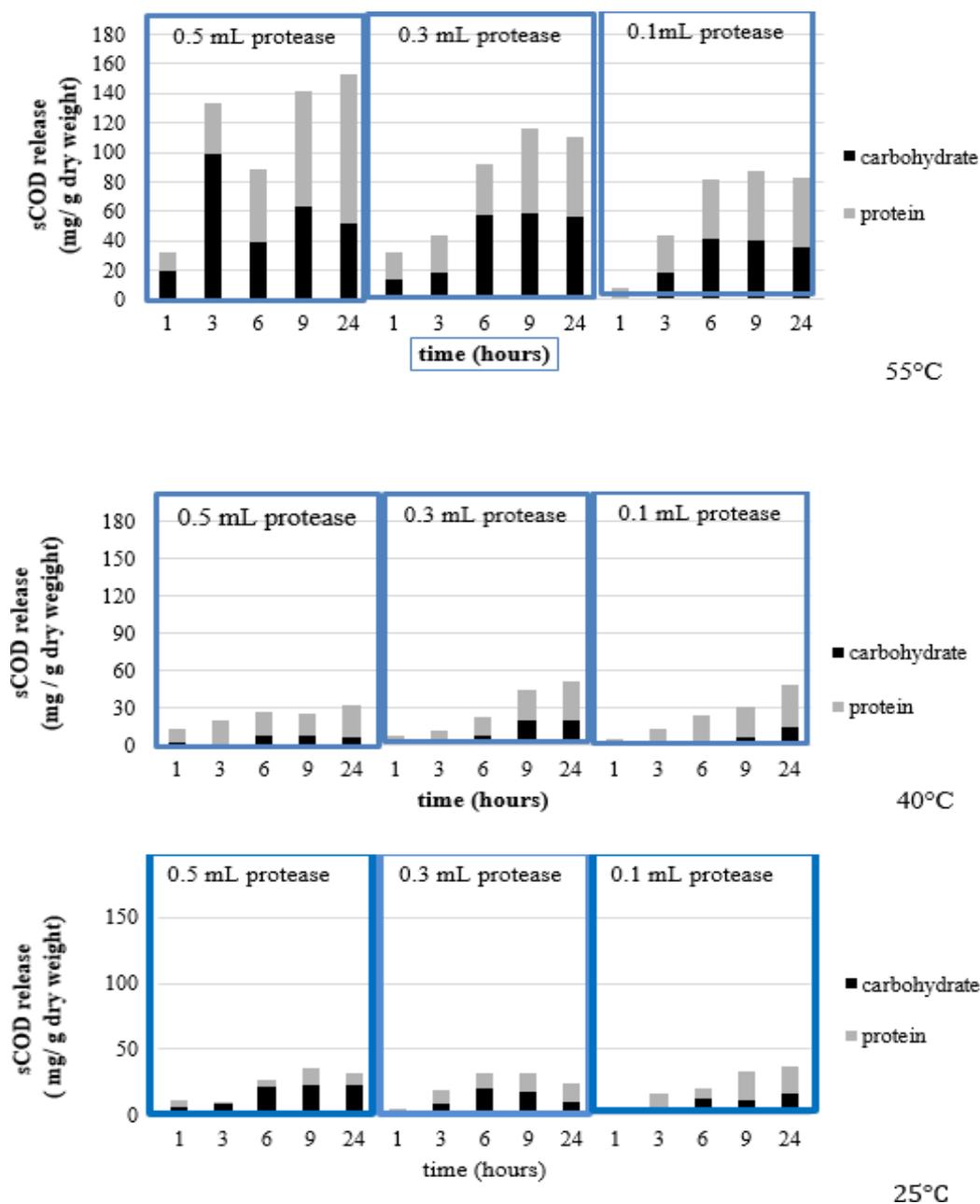
Figure 4.6.Change in soluble carbohydrate and protein content after protease pretreatment of *Scenedesmus sp*. at various temperatures

4.1.4. Enzymatic Pretreatment of *Scenedesmus sp.* by Viscozyme

Viscozyme enzyme is a mix of carbohydrases including cellulase, hemicellulases, arabinase and xylanase which can be suitable for cell wall disruption of *Scenedesmus sp.* In this study, pretreatment by viscozyme affected COD solubilization depending on enzyme dose, application temperature, and contact time (Figure 4.7-4.8). Highest sCOD release was observed at 55°C in 6 hours with 0.5 mL viscozyme pretreatment as 152.8 mg sCOD/g dry weight and solubility increase was achieved as 16%. The sCOD release with 0.1 mL viscozyme pretreatment in 24 hours increased solubility by 8.2% (84.8 mg sCOD/g dry weight). With 0.3 mL viscozyme, pretreatment, highest solubility was achieved in 3 hours by 9.7% (94 mg sCOD/g dry weight increase) (Figure 4.7-4.8). At 55°C, it was observed that at highest viscozyme dose (0.5 mL) resulted in maximum sCOD release at lower contact time of pretreatment. Viscozyme pretreatment at 40°C showed lower solubilization than pretreatment at 55°C as other enzymes (cellulase and protease). Highest sCOD release at 40°C was observed with 0.5 mL viscozyme pretreatment in 6 hours as 99.5 mg sCOD/g dry weight (solubility increase as 10.3%). On the other hand, for 0.3mL viscozyme pretreatment, the highest sCOD release was found to be 92.7 mg sCOD/g dry weight (solubility increase as 10.2%) after 24 hours. Additionally, for 0.1 mL pretreatment, the solubility increase was found to be between 3.8-3.9% in 24 hours. Viscozyme pretreatment at 25°C showed poor solubilization than pretreatments at higher temperatures (40°C and 55°C). Total solubility increases were observed as 1 %, 6.3%, and 6 % after 24-hour pretreatment for 0.1, 0.3, and 0.5 mL viscozyme pretreatment, respectively.

In general, it was observed that as pretreatment temperature decreased, for the same dose, the time reach to maximum sCOD release increased. Moreover, it was also observed that lower temperatures yielded lower sCOD release. This can be attributed to the effect of temperature on cell wall exopolymers and higher enzyme activity at that temperature. In terms of enzyme dose, it was concluded that, at 55°C, increasing enzyme dose resulted in higher sCOD release, however, at lower temperatures the differences between enzyme doses was relatively insignificant. Figure 4.9 showed that viscozyme pretreatment increased solubilization of carbohydrates rather than proteins at high temperature pretreatment. Because viscozyme is a carbohydrases mixture and it increased the degradation of cell wall containing carbohydrates like cellulose,
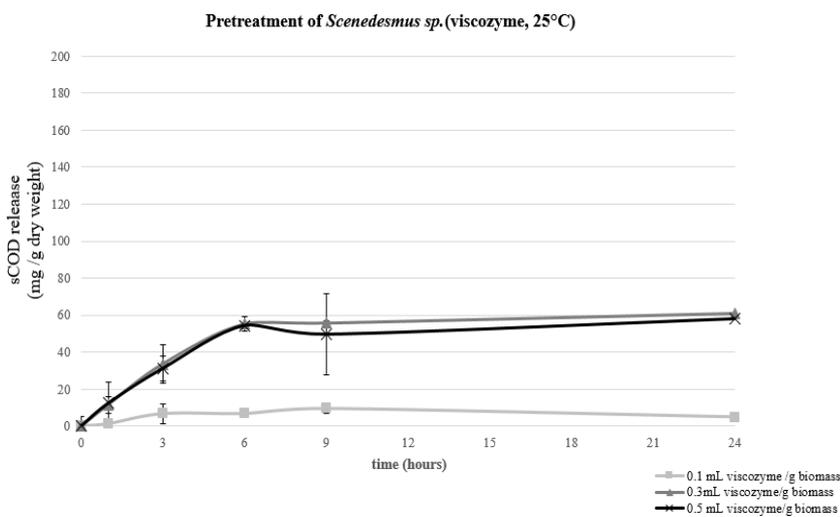
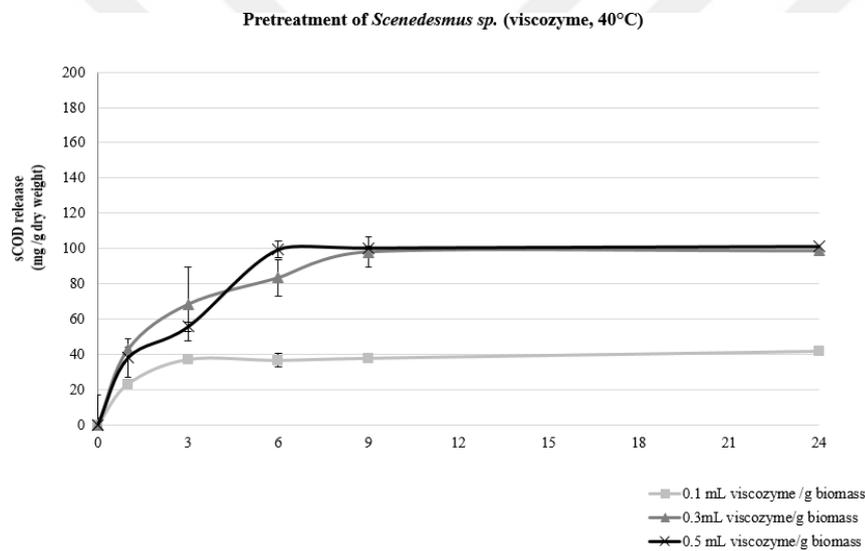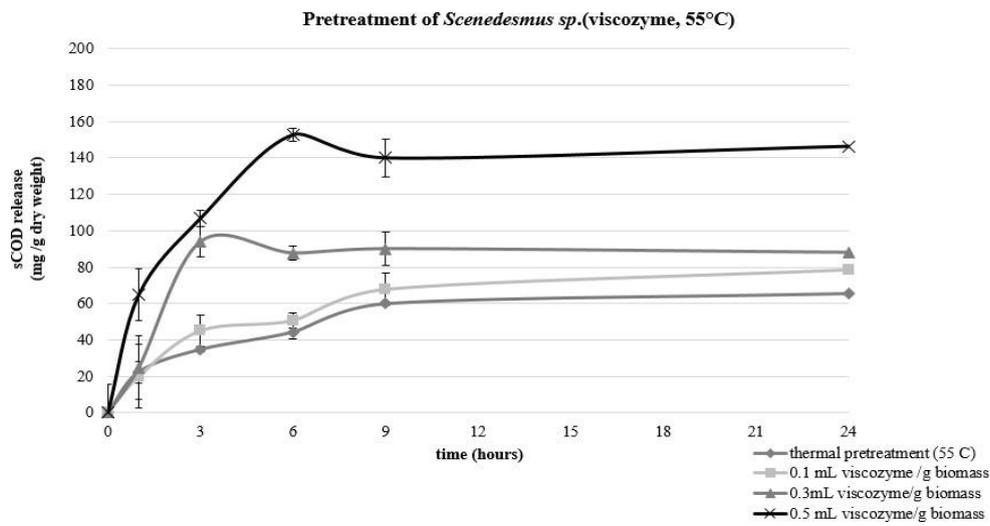hemicellulose and other carbohydrates.

Figure 4.7. The effect of enzyme dose on soluble COD release by viscozyme pretreatment at different temperatures (*Scenedesmus sp.*)
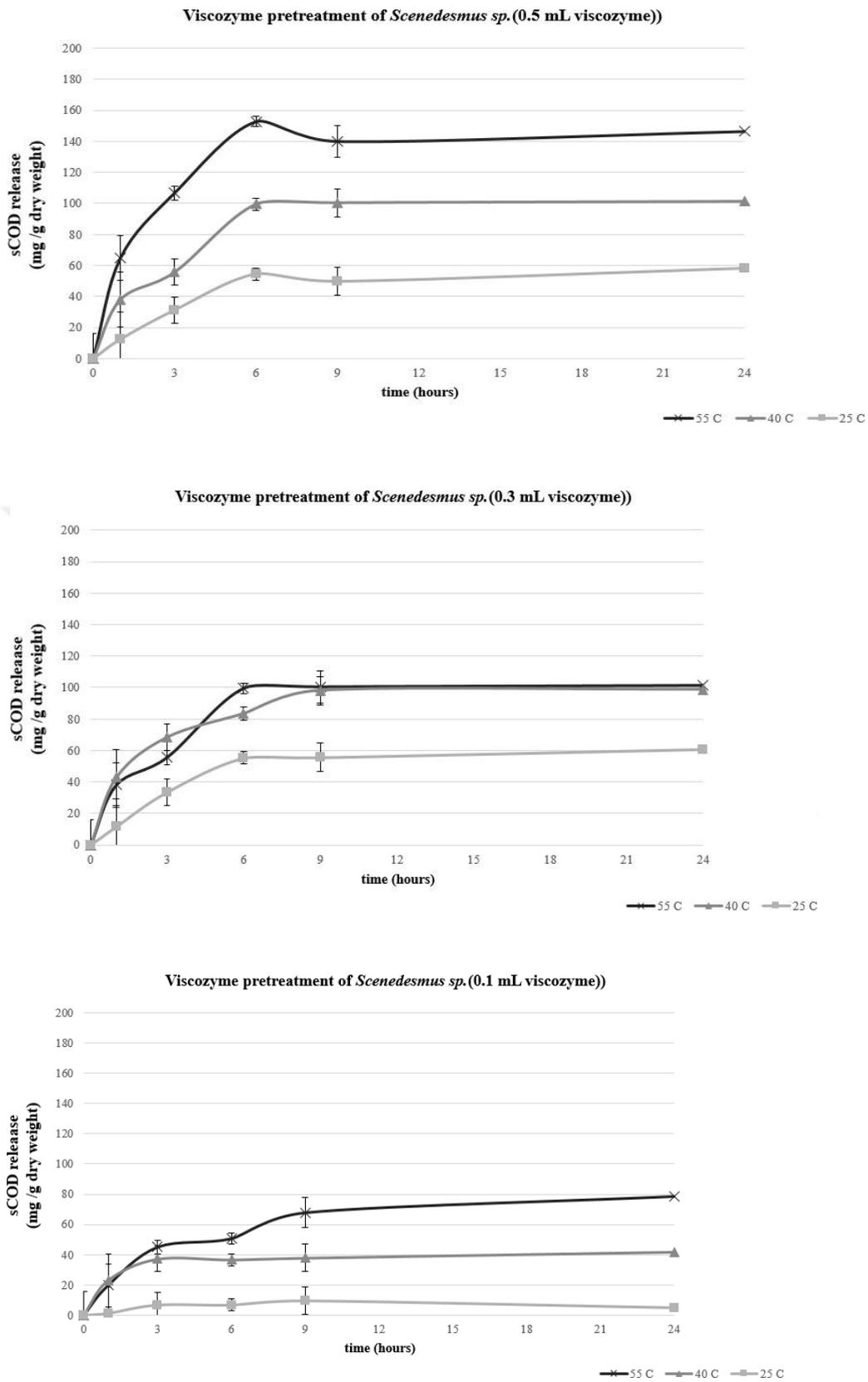
Figure 4.8. The effect of temperature on soluble COD release by viscozyme pretreatment at different enzyme doses (*Scenedesmussp.)*
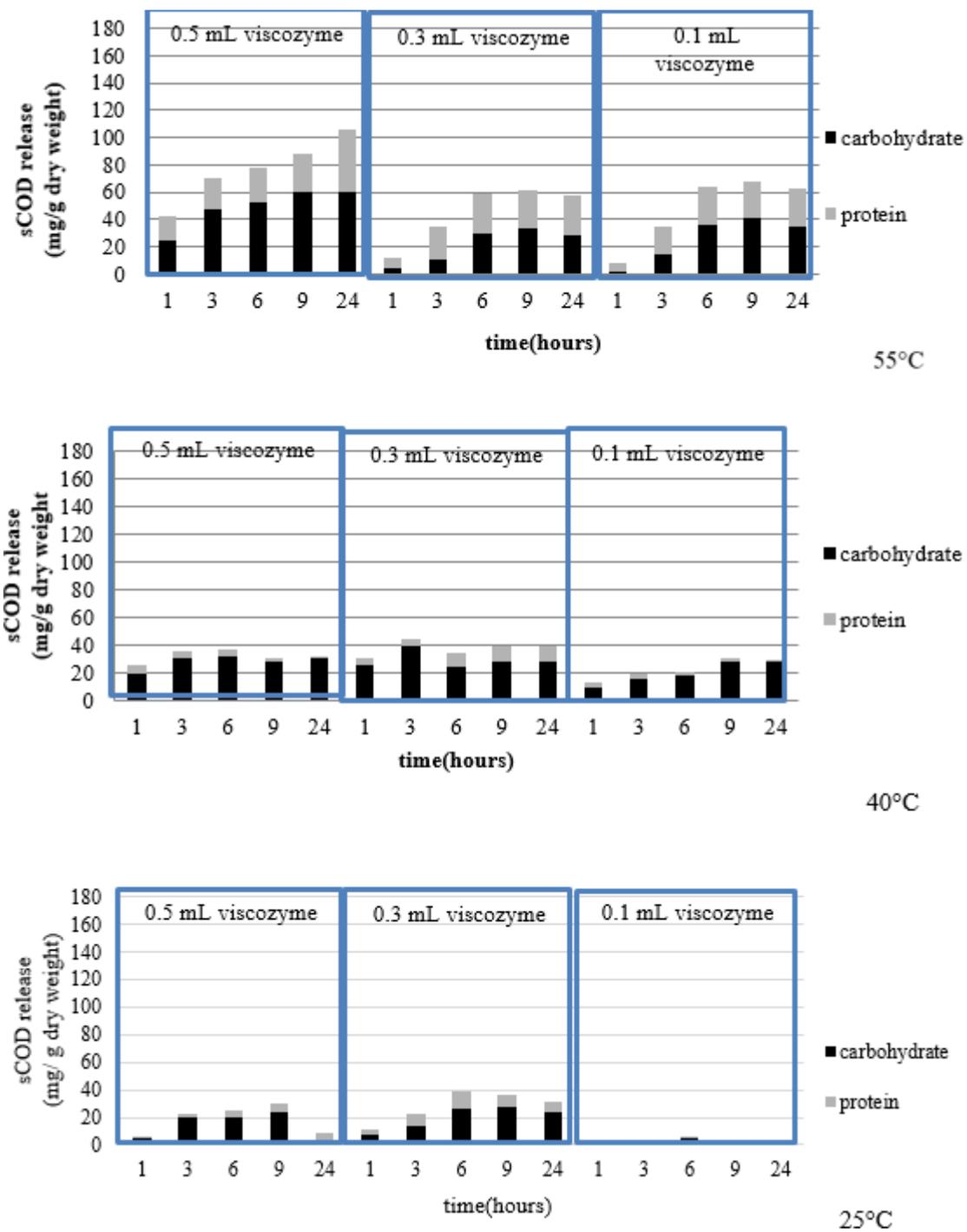
Figure 4.9. Change in soluble carbohydrate and protein content after viscozyme pretreatment of *Scenedesmus sp.* at various temperatures

4.1.5. Enzymatic Pretreatment of *Scenedesmus sp.* by Enzyme Mix

Use of enzyme mix provides multi disruption of complex cell wall structure and cell membrane of microalgae. In this study, viscozyme was combined with protease enzyme which were expected to increase cell wall degradation and organic matter solubilization. After pretreatment studies with single enzymes, enzyme mix pretreatment was also applied with viscozyme and protease mix at 55°C for 9 hours. The enzyme mix pretreatment was not applied due to lower solubilization results obtained from previous single enzyme pretreatment. At lower temperatures (40°C and 25°C). In addition, pretreatment was applied for 9 hours since for all single enzyme pretreatment, it was observed that after 9 hours the rate of sCOD release was not significant. It is seen from Figure 4.10, sCOD release was observed until 9 hours and the rate of sCOD release decreased as contact time increased. After 6 hours, most of the sCOD released for all doses. For the highest enzyme mix dose (0.5 mL), sCOD release was achieved as 268.8 mg sCOD/g dry weight (solubility increase as 37.8%) in 9 hours. For 0.3 mL enzyme mix pretreatment, highest solubility increase was observed as 27.5% in 9 hours (210.4 mg sCOD/g dry weight) and for 0.1 mL enzyme mix pretreatment, the solubility increase was reached to 22.5% in 9 hours (Figure 4.10). Carbohydrate and protein solubilization after enzyme mix pretreatment resulted in better carbohydrate and protein solubilization than the other enzymes applied. At lower doses, higher carbohydrate solubilization efficiencies were obtained. On the contrary, at the highest enzyme dose applied, protein solubilization was higher than carbohydrate solubilization from the cell (Figure4.11).

Enzyme mix pretreatment resulted in highest sCOD release among other enzymes. This can be explained by synergistic effect of enzymes. Viscozyme and protease together provided better disruption of both exterior and interior cell wall components and more biomass solubilization was observed. Therefore, use of mixed enzymes was more effective than single enzyme pretreatment.
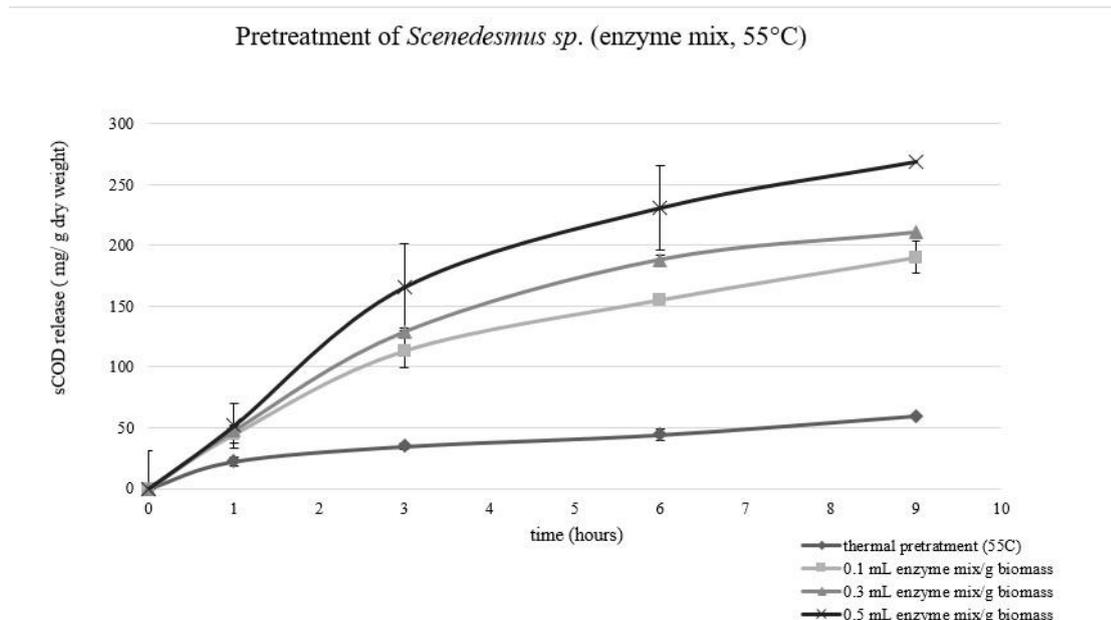
Figure 4.10. The effect of enzyme dose on soluble COD release by viscozyme pretreatment at 55°C (*Scenedesmus sp.*)
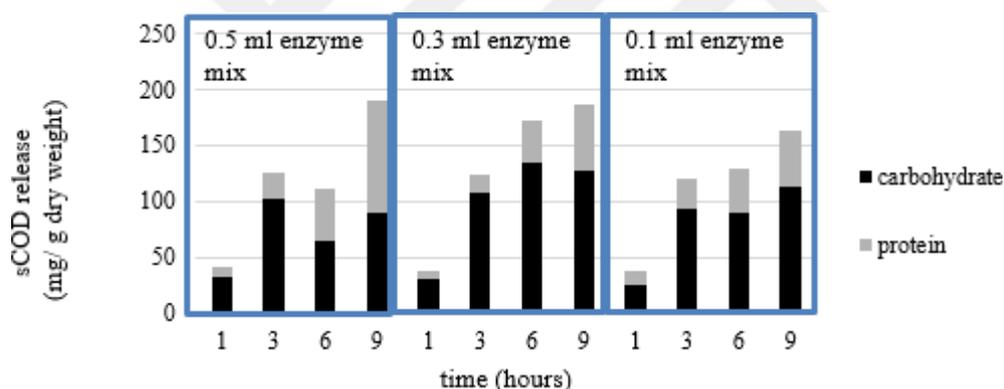

Figure 4.11. Change in carbohydrate and protein content after enzyme mix pretreatment of *Scenedesmus sp.* at 55°C

4.1.6. **Optimization Studies for Enzymatic Pretreatment of *Scenedesmus sp.***

Response Surface Methodology (RSM) is a combination of mathematical and statistical methods that aims to establish a model for a response that is affected by several variables and aims to optimize the response to achieve desired goal. In case of pretreatment of microalgae biomass, it provides to examine the effects of pretreatment conditions and the interaction between these conditions on biomass solubilization. In addition, it is able to fit a model to explain the relationship between pretreatment

conditions and sCOD release after enzymatic pretreatment. In this study, RSM was employed in order to determine the effects of pretreatment conditions (enzyme dose, temperature, and time) on solubilization of *Scenedesmus sp.* biomass. The ultimate aim was to optimize response to achieve the desired goal which was 'maximization of sCOD release'. To analyze experimental data obtained from enzymatic pretreatment studies, Box-Benkhen design was introduced with three levels and three factors for each type of enzyme. Each type of enzyme was analyzed independently since the effect of each enzyme on the *Scenedesmus sp.* biomass was different from each other. The independent variables were chosen as enzyme dose, temperature, and contact time. Therefore, the interactions between these parameters and sCOD release could be determined. The levels of these variables were chosen according to preliminary experiments given in the previous section. Time intervals were chosen between 3-9 hours instead of 1-24 hours according to experimental results to be able to run RSM.

RSM studies showed that cellulase pretreatment of *Scenedesmus sp.* had various responses on sCOD release with pretreatment conditions. Figure 4.12 represents the interaction of time and cellulase dose on sCOD release from *Scenedesmus sp*. As can be seen from the figure, cellulase dose had more significant effect on sCOD release from *Scenedesmus sp.* than time. As the dose increased, release of sCOD also increased. As the effect of time was investigated, it was observed that as time increased, sCOD release was enhanced, however; cellulase dose was more effective. Figure 4.13 shows that pretreatment temperature was significant on sCOD release and the time was found to be less significant than temperature. Figure 4.14 indicates that as temperature increased from 25°C to 55°C, more sCOD was released. In addition, increased cellulase dose also increased sCOD release, however; the increase trend was smoother than temperature increase.
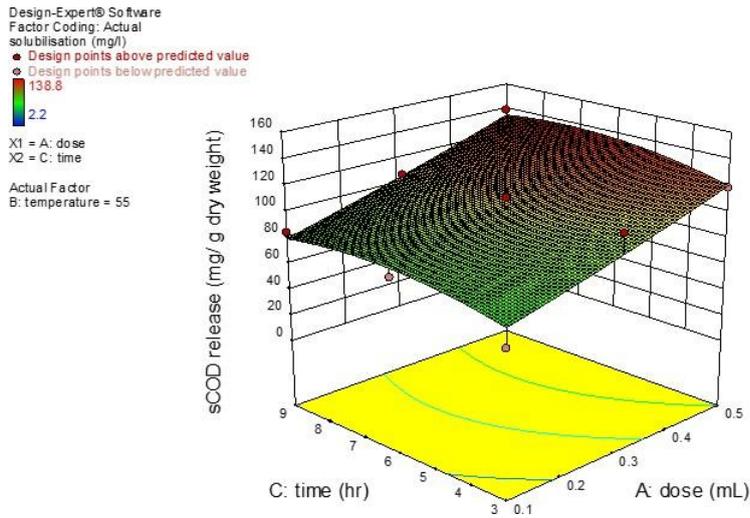
Figure 4.12. The interaction between time and enzyme dose on sCOD release from Scenedesmus sp. throughout cellulase pretreatment
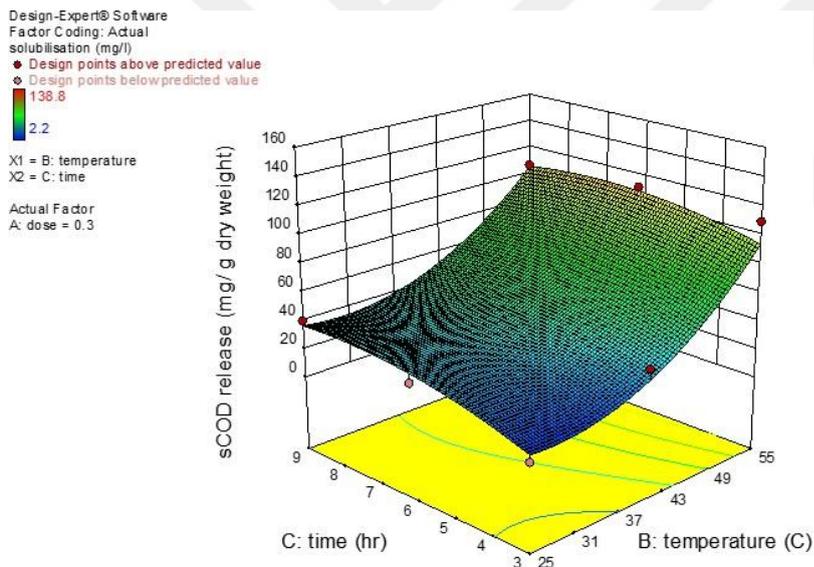

Figure 4.13. The interaction between time and temperature on sCOD release from *Scenedesmus sp.* throughout cellulase pretreatment
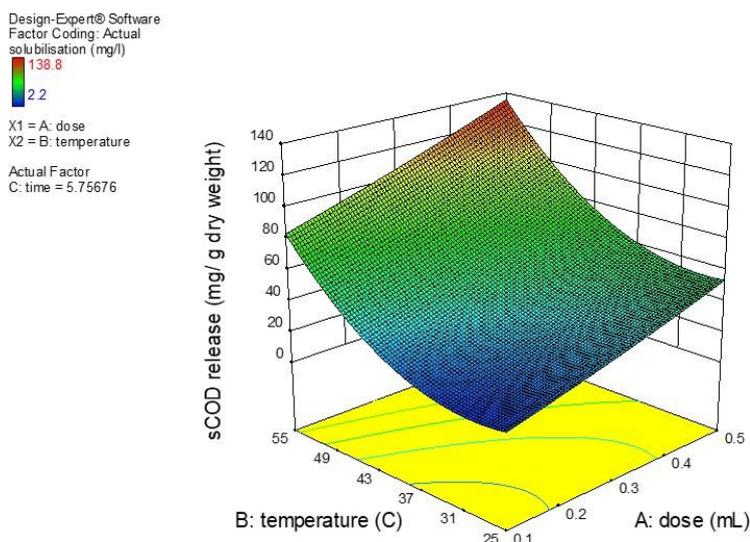
Figure 4.14. The interaction between cellulase dose and temperature on sCOD release from *Scenedesmus sp*. throughout cellulase pretreatment

Figure 4.15 indicates interaction between time and protease dose on sCOD release from *Scenedesmus sp.* According to the figure, both time and protease dose had linear effect on sCOD release and contact time was more significant than enzyme dose. The highest increase was observed at 9 hours. Figure 4.16 shows the interaction between time and temperature on sCOD release. It was observed that both parameters were effective in sCOD release. However, the effect of temperature was more significant than the effect of time when protease was used. As the interaction between temperature and protease dose was investigated (Figure 4.17), it was clear that with increasing temperature, the effect of enzyme on sCOD release increased. At low temperature, insignificant effect on sCOD release was observed with increasing enzyme dose.
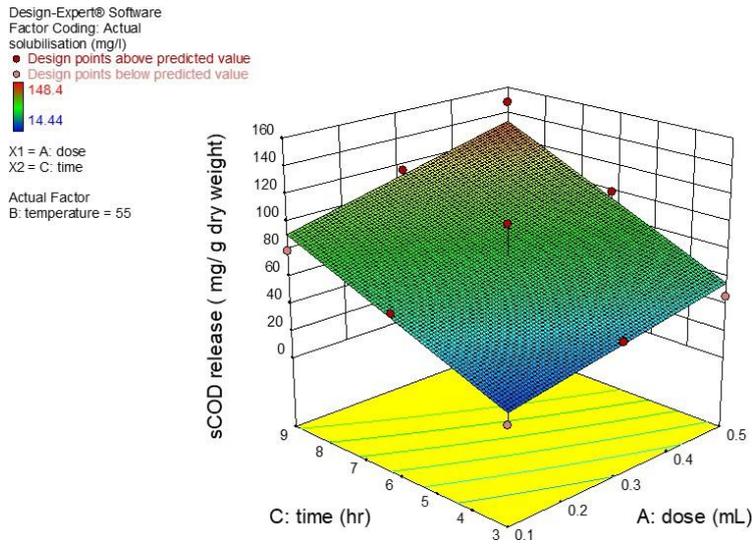
Figure 4.15. The effect of time and enzyme dose on sCOD release from *Scenedesmus sp.* throughout proteasepretreatment
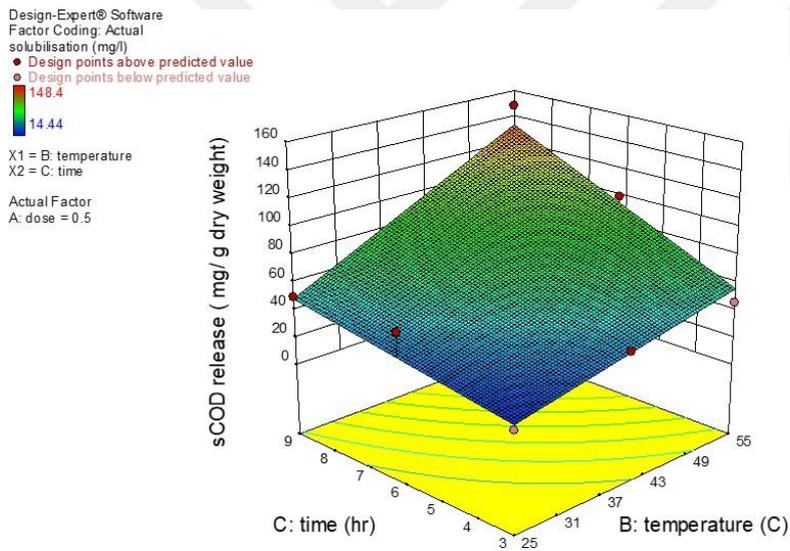

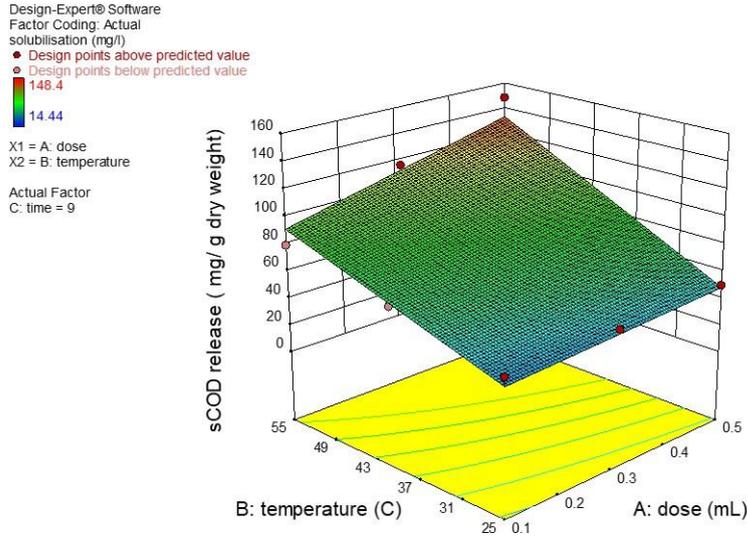Figure 4.16. The effect of time and temperature on sCOD release from *Scenedesmussp.* throughout protease pretreatment

Figure 4.17. The effect of temperature and enzyme dose on sCOD release from *Scenedesmus sp*. throughout protease pretreatment

Pretreatment conditions of viscozyme pretreatment had exhibited effects of sCOD release from *Scenedesmus sp.* biomass as other enzymes. As time-enzyme dose interaction was examined (See Figure 4.18), the effect of viscozyme dose was found as more significant than contact time. As the dose increased, the sCOD release also increased linearly. At the lowest enzyme dose, there was no significant increase in sCOD between 3 and 9 hours. The same trend was also observed for the highest enzyme dose applied. Figure 4.19 shows the time-temperature interaction. As can be seen, sCOD release ascended as temperature increased. For time, the increase in sCOD followed similar trend. As the enzyme dose-temperature interaction was investigated (See Figure 4.20), it was clear that the relationship both of two variable had a significant effect on sCOD release.
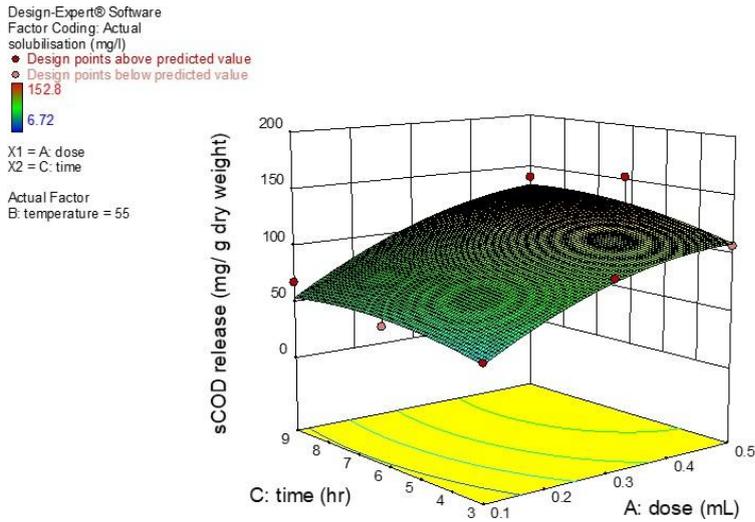
Figure 4. 18.The effect of time and enzyme dose on sCOD release from *Scenedesmus sp.* throughout viscozymepretreatment
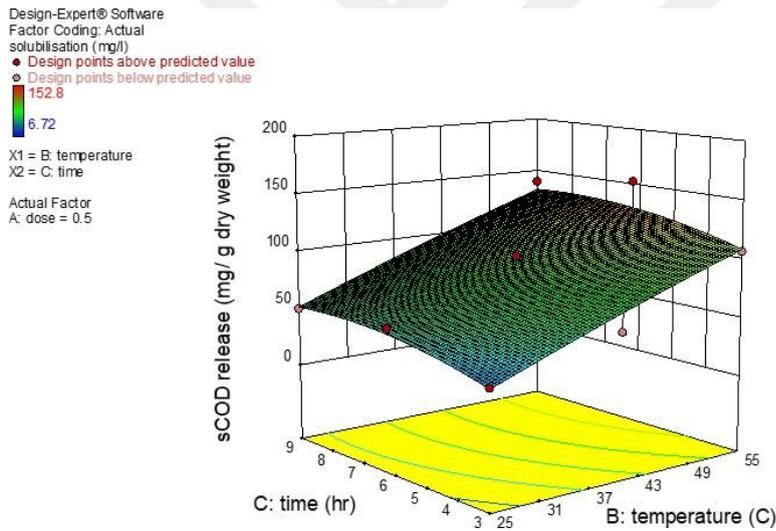

Figure 4. 19. The effect of time and temperature dose on sCOD release from *Scenedesmus sp.* throughout viscozyme pretreatment
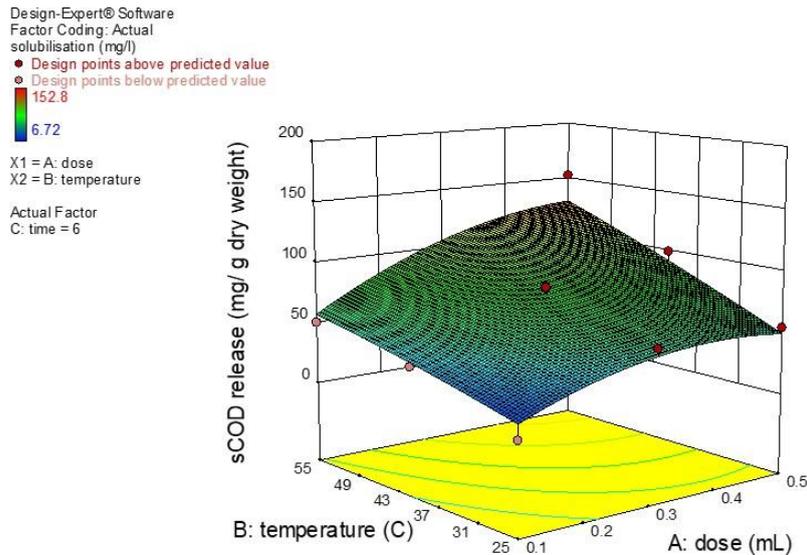
Figure 4. 20. The effect of enzyme dose and temperature dose on sCOD release from *Scenedesmus sp.* throughout viscozyme pretreatment

RSM provides to set a model that figures out the relationship between sCOD release and pretreatment conditions. Model selection in RSM is dependent on choosing highest polynomial model with additional significant terms. Model should not be aliased, which means there is not enough unique design points to estimate all coefficients. For all the cases in this study, cubic models were aliased, so they were not suitable for modeling the effect of pretreatment conditions on sCOD release. Moreover, suggested model also should have an adjusted R^2 and a predicted R^2 in which the difference between them is less than 0.2. p-values are also important in terms of choosing model. p-value stands for the probability that the model values are some or greater than actual results. When p-value is low, it means the model is more significant. Table 4.1. shows the models suggested by RSM for this study. According to the results, quadratic model was suggested for cellulase and viscozyme pretreatment. Quadratic model includes the effect of factors solely, interactions of two factors, and quadratic effect of factors (square of factor). For protease pretreatment, the model was suggested as 2 FI which is two factor interaction model and only includes one factor and two factor relationship.

Table 4.1.Suggested Models and R^2 values for enzymatic pretreatment of *Scenedesmus sp.*

	Cellulase Pretreatment	Protease Pretreatment	Viscozyme Pretreatment
Model type	Quadratic	2FI	Quadratic
Sequential p-value	<0.0001	0.0036	<0.0001
Predicted R^2	0.9428	0.8450	0.8584
Adjusted R^2	0.8993	0.7781	0.7837

Analysis of variance (ANOVA) test was also applied to determine significance of independent variables (pretreatment conditions). According to ANOVA test, for all enzymatic pretreatments, the models were found to be significant (high F values). A coefficient to be significant, the p- value of this coefficient should be less than 0.05. Being 'significant' means that small change on these variables can change sCOD release and has a considerable effect on the result.

For cellulase pretreatment of *Scenedesmus sp.*, significant independent variables were found as dose, time, temperature, and quadratic effect of time and temperature (Table 4.2). These results indicated that the interaction between factors were insignificant. Changing time, dose, and temperature had a significant effect as one factor. Moreover, temperature had a quadratic effect on sCOD release and increasing temperature provided increase in sCOD release. For protease pretreatment of *Scenedesmus sp.*, dose, temperature, time, dose-temperature and temperature- time interaction were determined as significant (Table 4.3). All these variables had a positive effect on sCOD release as the values are increased; Finally, for viscozyme pretreatment of *Scenedesmus sp.*, significant independent variables were determined as dose, time, temperature, dose-temperature interaction and quadratic effect of dose. (Table 4.4). These results showed that all factors were significant to some extent for solubilization of *Scenedesmus sp.*, however; the effect of factors changes with respect to different types of enzymes. This result showed that enzymatic pretreatment had different responses on sCOD release

since each enzyme behave differently under different dose, temperature, and contact times. So, a general conclusion could not achieve in terms of the most important pretreatment condition for enzymatic pretreatment.

Table 4.2. ANOVA for cellulase pretreatment of *Scenedesmus sp.*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	40900.79	9	4544.53	48.64	< 0.0001	significant
A-dose	9637.35	1	9637.35	103.15	< 0.0001	
B-temperature	25568.14	1	25568.14	273.66	< 0.0001	
C-time	1463.41	1	1463.41	15.66	0.0010	
AB	91.85	1	91.85	0.98	0.3353	
AC	19.00	1	19.00	0.20	0.6577	
BC	126.75	1	126.75	1.36	0.2602	
A^2	14.21	1	14.21	0.15	0.7014	
B^2	3529.99	1	3529.99	37.78	< 0.0001	
C^2	450.09	1	450.09	4.82	0.0424	
Residual	1588.31	17	93.43			
Cor Total	42489.10	26				

Table 4.3.ANOVA for protease pretreatment of *Scenedesmus sp.*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	22536.98	6	3756.16	24.62	< 0.0001	significant
A-dose	1802.00	1	1802.00	11.81	0.0026	
B-temperature	8370.45	1	8370.45	54.87	< 0.0001	
C-time	9503.61	1	9503.61	62.30	< 0.0001	
AB	775.38	1	775.38	5.08	0.0355	
AC	101.50	1	101.50	0.67	0.4243	
BC	1984.04	1	1984.04	13.01	0.0018	
Residual	3051.09	20	152.55			
Cor Total	25588.07	26				

Table 4.4. ANOVA for viscozyme pretreatment of *Scenedesmus sp.*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	31963.67	9	3551.52	18.51	< 0.0001	significant
A-dose	*11858.00*	*1*	*11858.00*	*61.81*	*< 0.0001*	
B-temperature	*15737.75*	*1*	*15737.75*	*82.04*	*< 0.0001*	
C-time	*1093.25*	*1*	*1093.25*	*5.70*	*0.0289*	
AB	*1276.38*	*1*	*1276.38*	*6.65*	*0.0195*	
AC	*133.47*	*1*	*133.47*	*0.70*	*0.4158*	
BC	*6.45*	*1*	*6.45*	*0.034*	*0.8566*	
A^2	*1380.17*	*1*	*1380.17*	*7.19*	*0.0157*	
B^2	*25.46*	*1*	*25.46*	*0.13*	*0.7201*	
C^2	*452.75*	*1*	*452.75*	*2.36*	*0.1429*	
Residual	3261.12	17	191.83			
Cor Total	35224.79	26				

The polynomial equations were also set by fitting the experimental data with the suggested model and they can be used to predict sCOD release at different pretreatment conditions. The polynomial equations for enzymatic pretreatment of *Scenedesmus sp.* were derived which explained the relationship between sCOD release and input variables as (A: enzyme dose, B: temperature, C:time):

sCOD release by cellulase pretreatment (mg/ g dry weight) = +46.67 + 23.14*A+37.69*B+9.02*C+2.77*AB+1.26*AC-3.25*BC+1.54*A^2+24.26*B^2-8.66*C^2
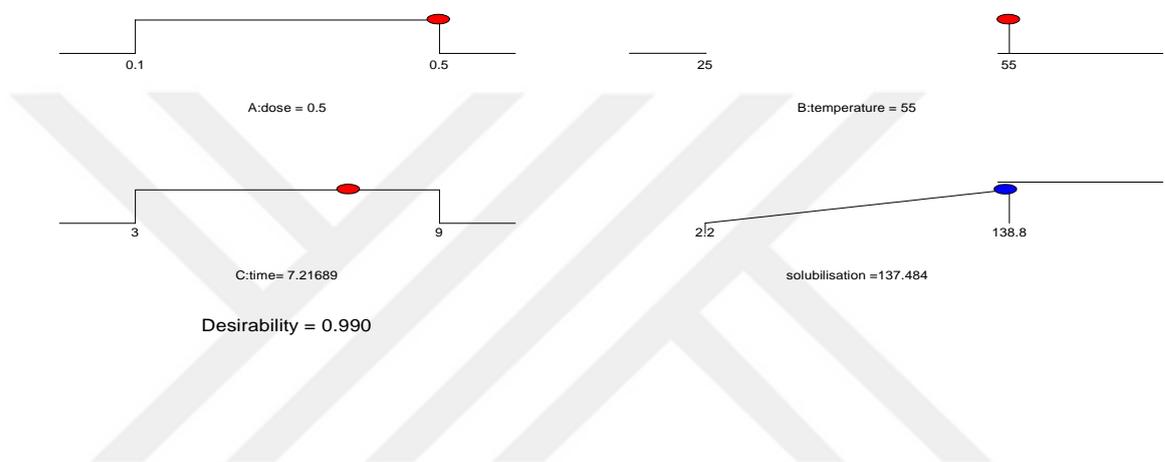
sCOD release by protease pretreatment (mg/g dry weight) =+55.17 + 10.01*A+21.56*B+22.98*C+8.04*AB+2.91*AC+12.86*BC

sCOD release by viscozyme pretreatment (mg/g dry weight) =+81.15 + 25.67*A+29.57*B+7.79*C+10.31*AB+3.33*AC+0.73*BC-15.17*A^2-2.06*B^2-8.69*C^2

Optimization scenario was also conducted to achieve an optimum pretreatment condition for maximized sCOD release. So that, the best pretreatment conditions were

selected and applied prior to BMP tests. For the optimization scenario, input parameters including dose, temperature, and contact time were chosen as 'in range' (between experimental values) and ultimate goal was selected as 'maximization of sCOD release', which was the output. Importance of the input parameters and the output was weighted as the same. After optimization run, the maximum desirability that can be achieved was '1' (means that the results are the closest to the constraints). The results of the optimization scenario were given in Figure 4.21.

a. *Optimum pretreatment conditions for cellulase pretreatment of* Scenedesmus sp.

A:dose = 0.5

B:temperature = 55

C:time= 7.21689

solubilisation =137.484

Desirability = 0.990

b. *Optimum pretreatment conditions for protease pretreatment of* Scenedesmus sp.

A:dose = 0.5

B:temperature = 55

C:time=9

solubilisation = 133.52

Desirability = 0.889
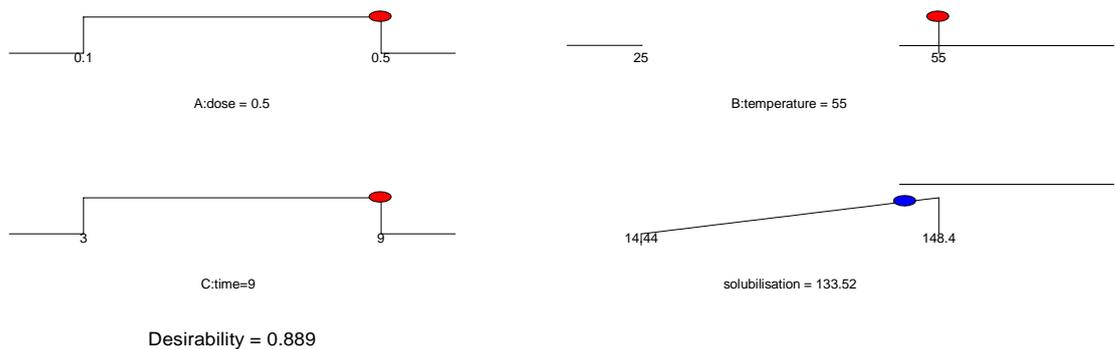
c. *Optimum pretreatment conditions for viscozyme pretreatment of* Scenedesmus sp.
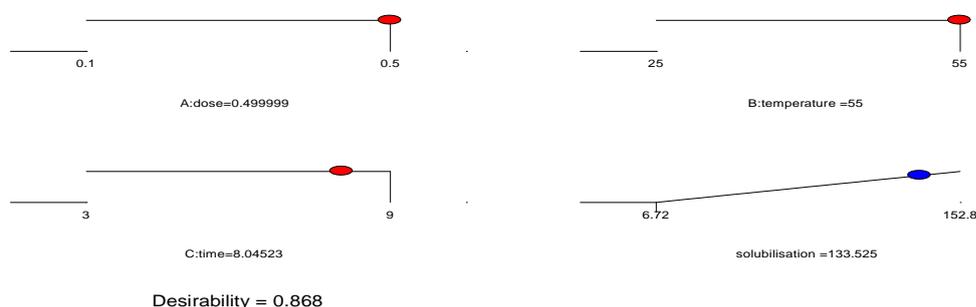
Figure 4.21. Optimized enzymatic pretreatment conditions for *Scenedesmus sp.*

As can be seen from optimization results, for all types of enzymes, the optimum conditions for temperature and enzyme dose were found as 55°C and 0.5 mL, respectively. Moreover, optimum time conditions were determined as between 7 and 9 hours. These results showed that high temperature enzymatic pretreatment (55°C) were the most effective in terms of sCOD release. According to results of experimental studies and optimization studies by RSM, further experiments for biomethane potentials was conducted for pretreated *Scenedesmus* sp. including enzymatic pretreatment conditions at 55ºC for 9 hours, however; all enzyme doses were chosen for further experiments to determine the effect of enzyme dose on methane yield. The operational conditions directly affect the cost of enzyme pretreatment which is an important issue to be considered.

4.1.7. Comparison of Enzymatic Pretreatment Conditions for *Scenedesmussp*.

Figure 4.22 shows the visual interpretation of *Scenedesmus sp.* after different enzymatic pretreatment conditions. The pictures were taken by a digital camera attached to LEICA DM 5000 B. *Scenedesmus sp.* has rigid cell wall characteristics and after thermal pretreatment at 55°C, live cells were observed, showing that complete disruption of cells were not achieved. However, depigmentation was observed after thermal

pretreatment. The color changed from green to brownish green. After application of enzymatic pretreatment, cell disruption to some extent was observed. Depigmentation was also observed for all types of enzymatic pretreatment as after thermal pretreatment. Cellulase pretreatment showed similar characteristics with thermal pretreatment. For cellulase pretreatment, partial cell wall degradation may occur which could not be observed via microscopy. After protease and viscozyme pretreatment, partial degradation was observed. After enzyme mix pretreatment the amount of solubilized cells were more than other pretreatments. Solubilized cells can be seen easily after enzyme mix pretreatment. These results were also convenient with solubilization increases (%). Enzyme mix pretreatment showed the highest solubility increase as 37.8% when compared to single enzyme pretreatment and the amount of solubilized cells were more than single enzyme pretreatment. Aggregation was also observed between cells especially after pretreatment. It is stated that depigmentation and aggregation are the indicators of cell disruption [149]. Depigmentation was observed for all treated biomass and aggregation was observed especially for viscozyme, protease, and enzyme mix pretreatment. It is important to notice that complete solubilization of *Scenedesmus sp.* was not observed for all types of pretreatment; however, partial cell wall degradation could occur after pretreatment.
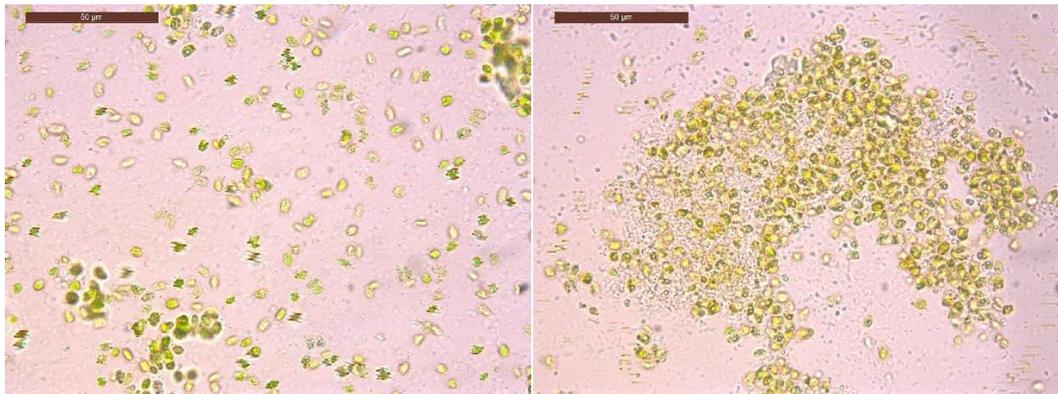
Raw *Scenedesmus sp.*   After thermal pretreatment (55°C)
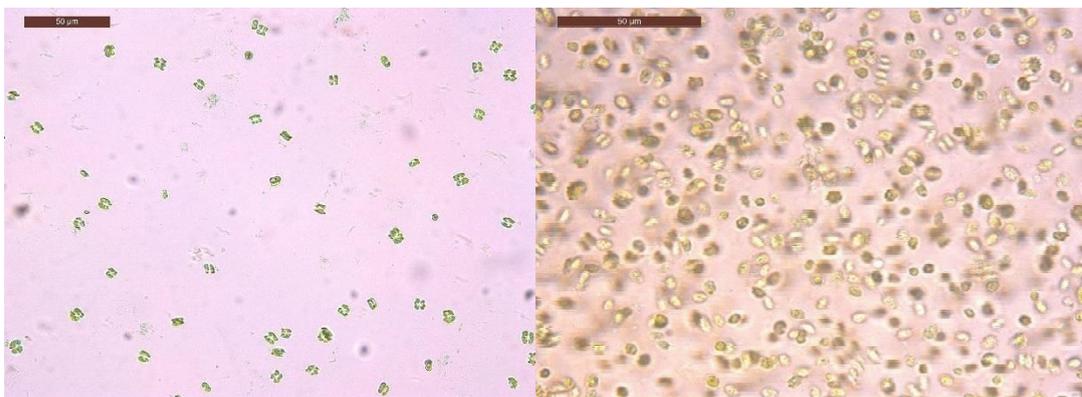
After cellulase pretreatment (0.5 mL)   After protease pretreatment (0.5 mL)

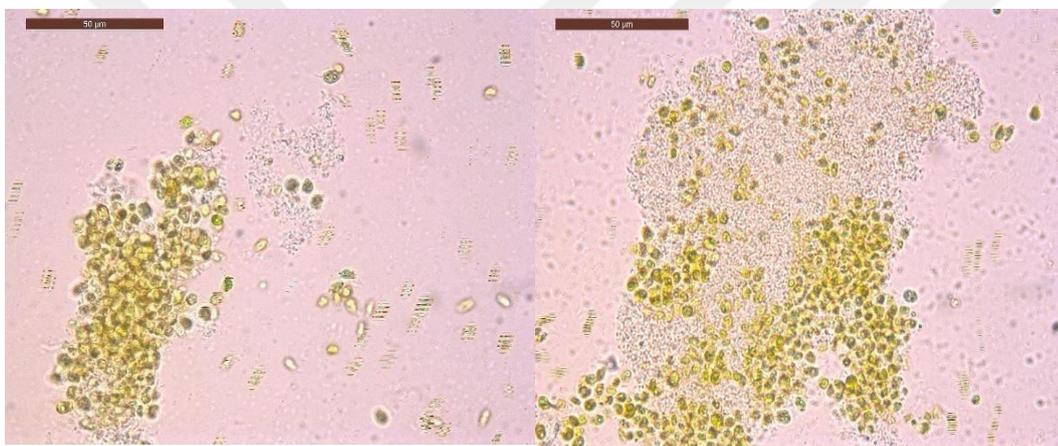After viscozyme pretreatment (0.5 mL)   After enzyme mix pretreatment (0.5 mL)

Figure 4.22. Visual interpretation of *Scenedesmus sp*. biomass after various enzymatic pretreatment

Table 4.5 indicates the highest sCOD releases, soluble protein, and carbohydrate increases (%) of *Scenedesmus sp.* after enzymatic pretreatment. According to the results, it was concluded that enzyme mix had the highest efficiency among other pretreatments than single enzymes. These results approved synergistic effect of enzyme mix. Single enzymes showed similar solubilization efficiencies and high temperature (55°C) was found to be more effective than lower temperature pretreatments. The possible reasons for that 55°C pretreatment provided partial cell wall disruption to some extent. Moreover, activity of applied enzymes is generally higher at high temperatures than at low temperatures. This was also reported by several researchers indicating that at low temperatures the activity of enzymes is relatively low. For instance, Cordova et al.[150]stated that for enzyme mixtures highest enzymatic activity was at 50°C and

lower at 21°C. Another important conclusion derived from the results is that after 9 hours solubilization did not increase significantly. Especially, for high temperature pretreatment, the heating requirement is higher and longer periods increases the energy demand. As carbohydrate and protein solubilization were compared, highest protein solubilization was observed after enzyme mix pretreatment followed by protease pretreatment. On the other hand, highest carbohydrate solubilization was observed for both enzyme mix and cellulase.

Table 4. 5. Highest sCOD release of *Scenedesmus sp.* after different enzymatic pretreatment conditions

	Cellulase	Protease	Viscozyme	Enzyme mix
Dose	0.5	0.5	0.5	0.5
Temperature	55	55	55	55
Reaction time	9	9	6	9
sCOD release	138.8	154.8	152.8	268.8
Solubility increase	12.3	15.4	16	37.8
Soluble protein release	10	78.7	26	102
Soluble carbohydrate release	89	63.3	52	89

(Dose: ml /g biomass, Temperature (°C), contact time (hours), sCOD release (mg/g dry weight), solubility increase (%), soluble protein release (mg/g dry weight), soluble carbohydrate release (mg/g dry weight))

4.1.8. BMP Results for *Scenedesmus sp.* at Mesophilic and Thermophilic Temperatures

BMP assays for both untreated and pretreated *Scenedesmus sp.* biomass were applied to observe methane potentials in batch reactor and make a discussion about the effect of enzymatic pretreatment on methane production. BMP assays were carried out at

mesophilic and thermophilic temperatures, so that, temperature impact on methane production was also investigated.

BMP Results for Scenedesmus sp. Biomass after Mesophilic Digestion

According to the results of pretreatment and optimization studies, 55°C for 9 hours was selected as optimum pretreatment conditions for enzymatic pretreatment for all types of enzymes in order to have maximum sCOD release. Therefore, next studies were focused on investigation of effects of enzymatic pretreatment (55°C, 9 hours) with various enzymes on methane production. Cellulase, protease, viscozyme, and enzyme mix pretreated biomass were used in biomethane potential tests (BMP) since each enzyme had posed different effects on biomass solubilization. The effect of different enzyme doses (0.1, 0.3 and 0.5 mL) was investigated. These investigations will provide information on best conditions like enzyme type and dose which can be used in pretreatment of *Scenedesmus sp.* for improved methane production.

In this part, BMP tests were applied for both pretreated and untreated biomass to observe the relationship between solubilization and biogas improvement in terms of enzyme dose. The BMP tests were continued for 43 days until there was no methane production. Average methane content was found to be around 74%. According to BMP tests, untreated(raw) *Scenedesmus sp.* biomass yielded 105.9 mL CH_4/g VS. There are a few studies showing methane yields potentials from *Scenedesmus sp.* Zamoalla et al. [84] found out that after BMP test, methane yield of *Scenedesmus obliquus* was 0.21mL CH_4/ g VS with an inoculum/substrate ratio of 3 and volatile solids concentration was 0.2%. Astals et al. [85] determined methane yield of *Scenedesmus obliquus* as 163 mL CH_4/gVS.In this study,VS loading was 3% and substrate to inoculum ratio (S:I)was 2. In another study, methane yield of *Scenedesmus* was found as 170 mL CH_4/ g VS with 6% VS loading and S: I ratio as 0.5. Mahdy et al. [87] also achieved 140.3 mL CH_4/ g VS with S: I ratio of 0.5 and 14.5 g VS/L. In this present study, VS concentration was 4.2 g/L and S: I ratio was 1. As can be seen from the previous studies, depending on the different S: I ratios and VS loadings, methane yields showed different yields for *Scenedesmus sp.* biomass. Theoretical methane yield for *Scenedesmus sp.* is calculated as 600 mL CH_4/ g VS (if 100% anaerobic biodegradability is achieved) and untreated *Scenedesmus sp.* yielded lower methane

production potential when compared to theoretical methane yield. So, application of pretreatment prior to anaerobic digestion is found essential to provide better methane yields from *Scenedesmus sp.* biomass.

Acceding to BMP results, thermal pretreated biomass had also similar methane production potential with untreated biomass. Thermal pretreatment at 55°C had increased solubilization, however; it did not show any positive effect on cumulative methane yield. With enzymatic pretreatment of *Scenedesmus sp.* biomass by cellulase, methane yields were achieved 17-67% more than untreated biomass. The highest dose (0.5 mL cellulase) pretreated biomass had a net methane yield of 177.1 mL CH_4/ g VS with 67% improvement in methane yield. With 0.3 mL cellulase pretreatment, net methane yield was 141.1 mL CH_4/g VS with 41.8 % improvement when compared to untreated biomass. With 0.1 mL cellulase pretreatment, the methane yield was the lowest 120.4 mL CH_4/g VS but it was 17% more than untreated biomass (Figure 4.23.a-b). Highest cellulase dose resulted in the highest sCOD release after pretreatment which also gave highest enhancement on methane production (67%). These results showed that there was a positive correlation between cellulase dose and methane yields. As daily methane productions were evaluated, it was observed that methane production was found to be highest at 9th day for all cellulase pretreated biomass and highest methane production was observed as 10.6 mL CH_4/ gVS.d for 0.5 mL cellulase pretreated biomass. It may be due to more effective pretreatment with the highest dose resulting in higher accessibility of biomass by anaerobic archea. Similar trend was observed with 0.3 mL cellulase pretreated biomass. For 0.1 mL cellulase pretreated biomass, much time was required to reach highest daily methane production which was 9.4 mL CH_4/ gVS.d (at 15[th]day) (Figure 4.23.b). However, at 9th day, the production rate was 7.3mL CH_4/ gVS.d. For untreated *Scenedesmus sp.,* highest daily methane production was also observed at 15th days as 7.5 mL CH_4/ gVS.d. These results showed that, cellulase pretreatment provided better hydrolysis efficiency during hydrolysis and it could reach highest daily methane production rates earlier depending on the cellulase dose.

In general, it was concluded that cellulase had a positive effect on cell wall disruption and organic matter solubilization. As stated before, *Scenedesmus sp.,* had rigid cell wall components including cellulose and it was obvious that partial disruption of cellulose in

cell walls were achieved and anaerobic microorganisms were able to use organic matter released after cellulase pretreatment easily.

a.

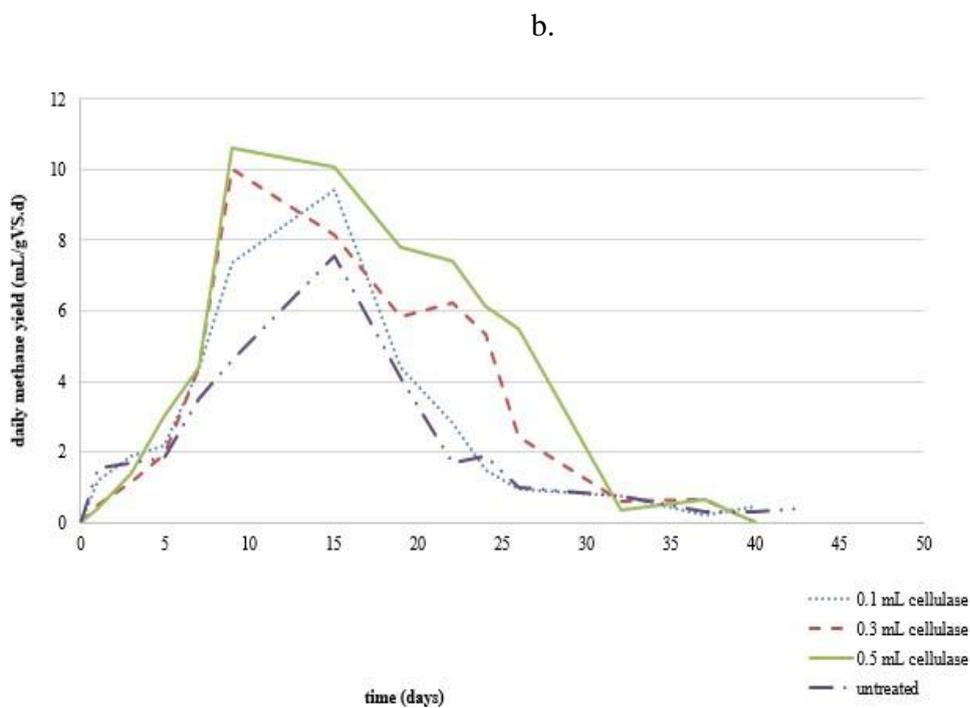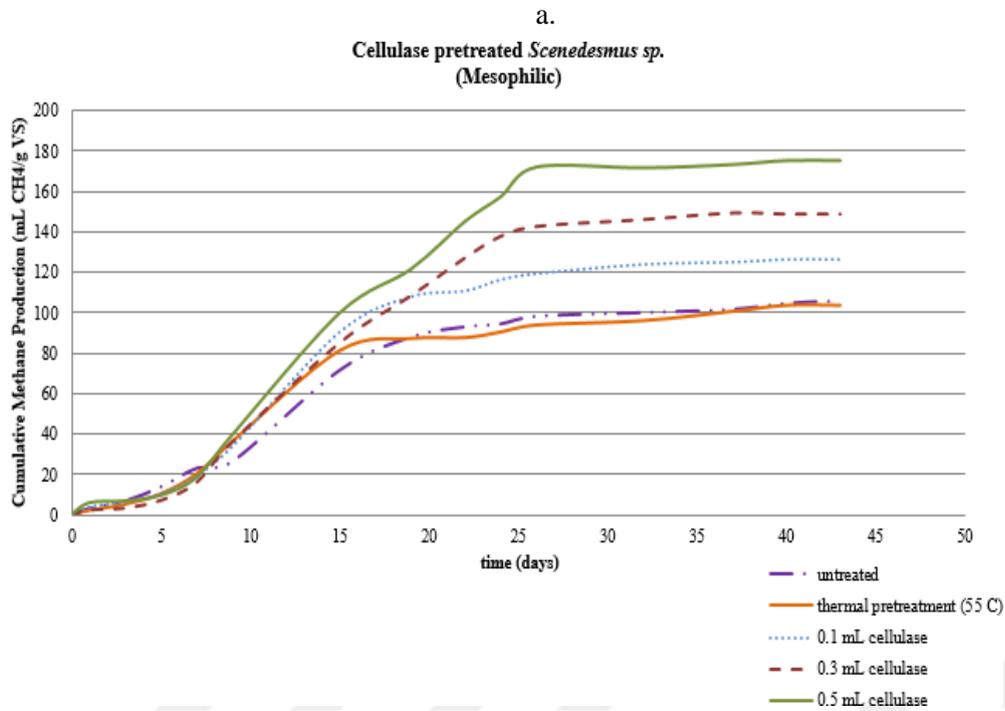Cellulase pretreated *Scenedesmus sp.*
(Mesophilic)

b.

Figure 4.23. Net cumulative methane production and daily methane production of cellulase pretreated *Scenedesmus sp.* biomass after mesophilic anaerobic digestion

The methane potential of protease pretreated *Scenedesmus sp.* was also higher than untreated biomass (Figure 4.24). Highest improvement was achieved by 0.5 mL protease pretreatment as 83% increase. The net cumulative methane yield was found as 193.8mL CH_4/g VS. Methane yield enhancements were from 37% to 61 % for pretreated biomass with 0.1 mL protease and 0.3 mL protease doses, respectively. Figure 4.24.a shows that until 15 days, the methane productions were similar for pretreated biomass at all protease doses. However, after 15 days, the rate of methane production increased for 0.5 mL protease pretreated *Scenedesmus sp.* biomass. Figure 4.24.bshowsthathighestdailymethaneproductionrateswereobservedat15thdayfor 0.1 mL and 0.3 mL protease pretreated biomass. The rates were 11.0 and 10.6 mL CH_4/gVS.d for 0.1 mL protease and 0.3 mL protease pretreated *Scenedesmus sp.*, respectively. For 0.5 mL protease pretreated biomass, the highest daily production was also observed at 15th day as 2-fold increased with a higher rate of 15.5 mL CH_4/ gVS.d. At 9th day, it showed similar rate with other protease doses. This can be attributed to continuous degradation of protein after 9 days at the highest protease dose.

These results showed that application of protease pretreatment was effective in terms of increasing cumulative methane production potential from *Scenedesmus sp.* Exterior cell walls of *Scenedesmus sp.* includes glycoproteins and protease pretreatment provided cell wall disruption of glycoprotein components. Moreover, proteins contained in cell membrane may also be consumed by anaerobic microorganisms with the help of protease. It is known that protein content of *Scenedesmus sp.* is relatively high (up to 56%) (Table 2.1) and its degradation effectively by anaerobic microorganisms as in raw biomass can be difficult. Therefore, application of protease pretreatment provided better methane yields by increasing accessibility of protein like substances to anaerobic microorganisms. In addition, theoretical methane production from proteins (0.851 mL CH_4/ gVS) is higher than carbohydrates (0.415 mL CH_4/ gVS) [45], so degradation of proteins is more advantageous than carbohydrates to have higher methane yields. During anaerobic digestion, firstly carbohydrates are consumed and protein degradation is repressed. In this study, it was concluded that with application of protease, higher hydrolysis of biomass wasobtained.
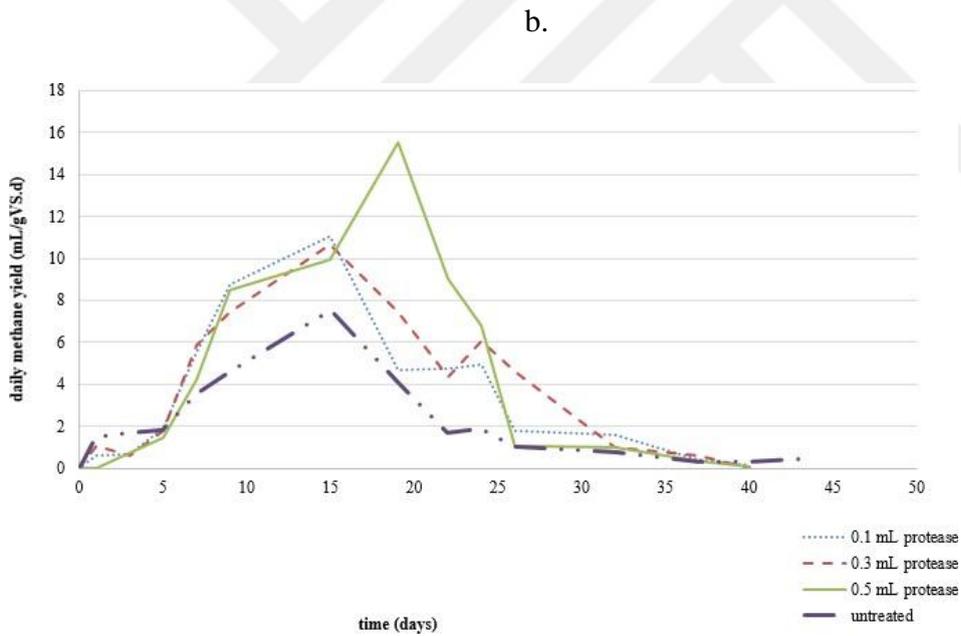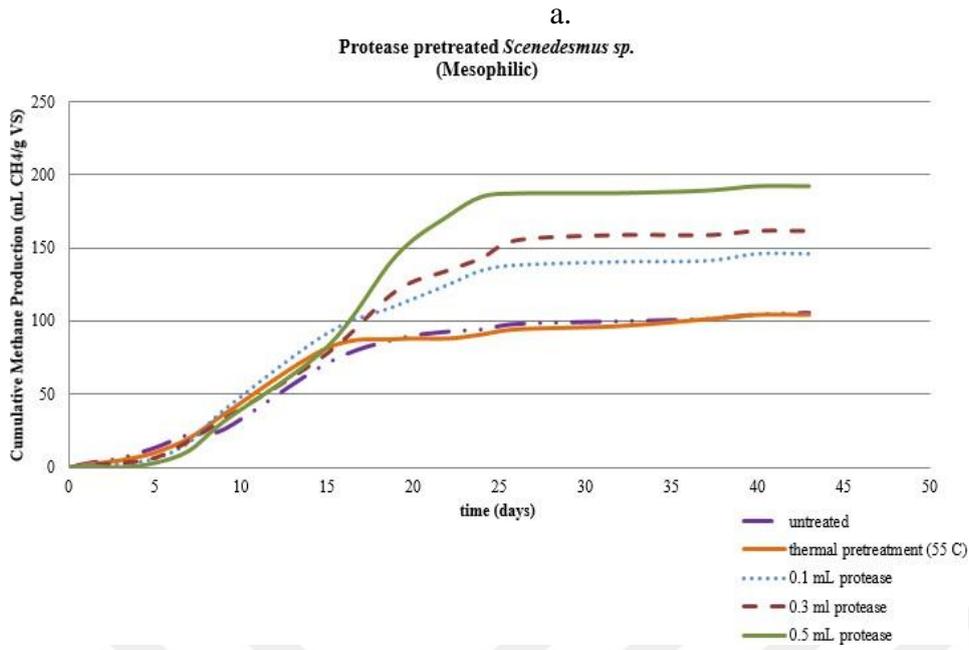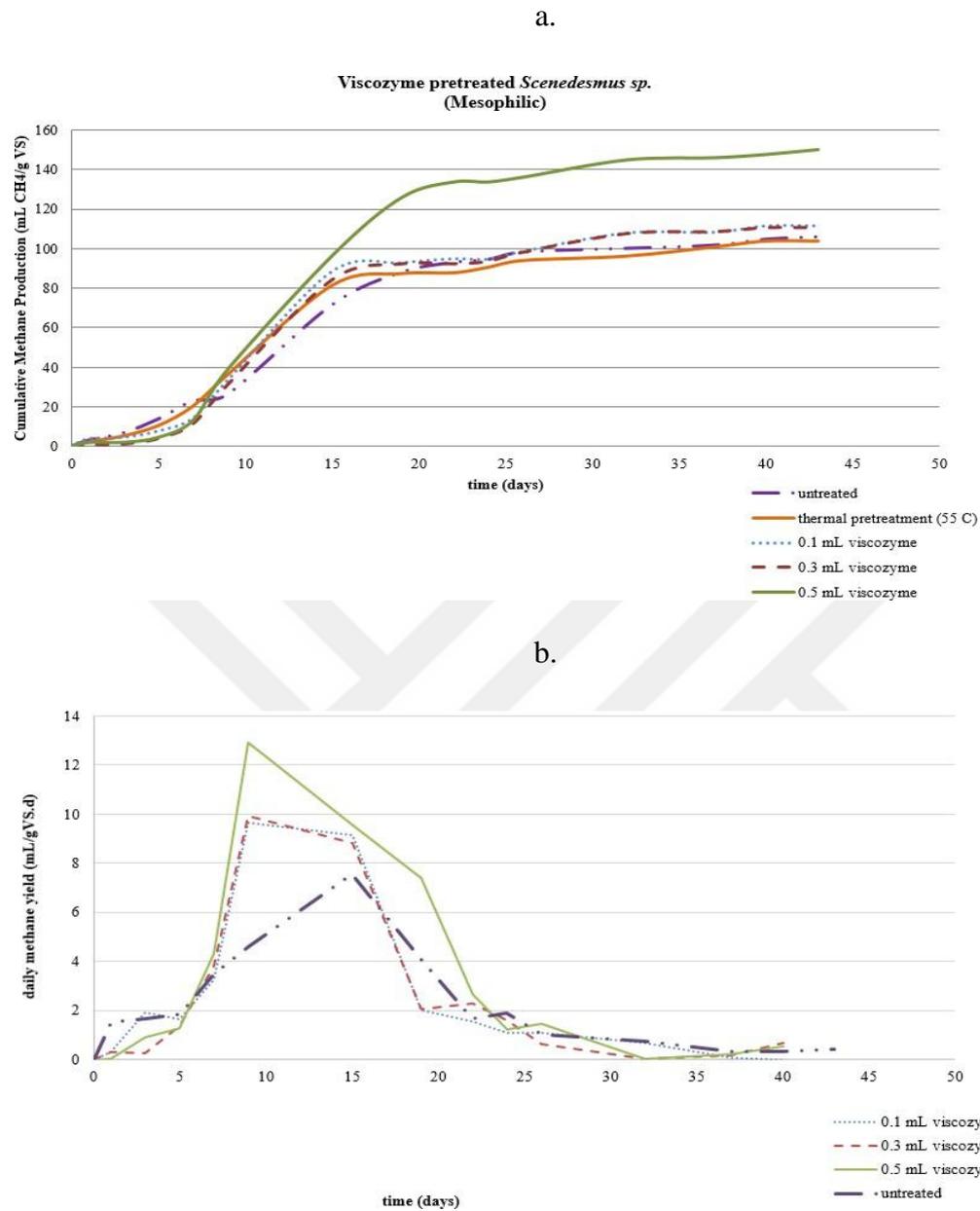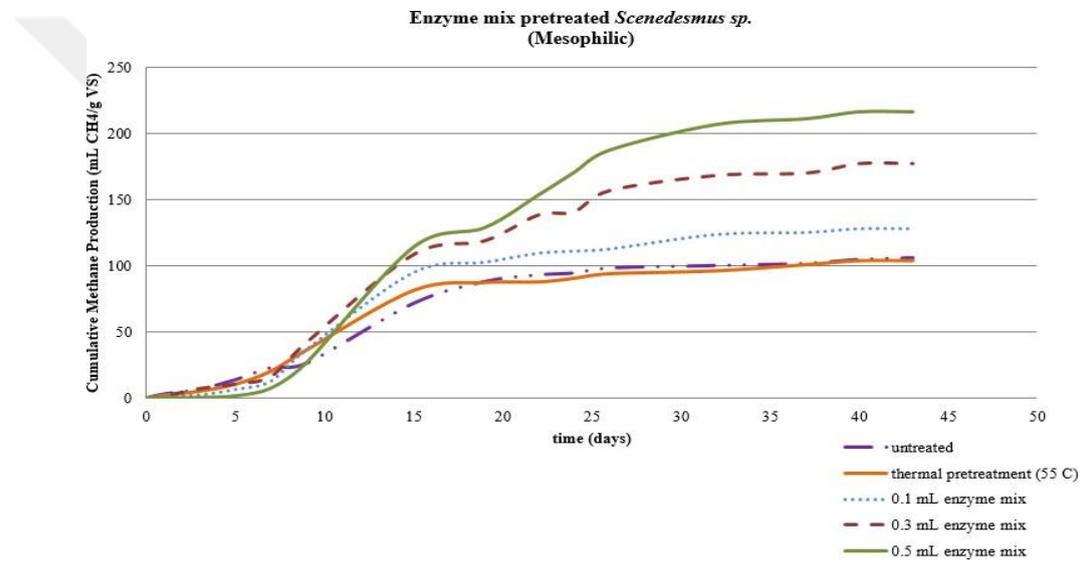
a.

b.

Figure 4.24. Net cumulative methane production and daily methane production of protease pretreated *Scenedesmus sp.* biomass after mesophilic anaerobic digestion

Pretreatment with viscozyme did not enhance methane production effectively in contrast to other enzymes applied like protease, cellulase, and enzyme mix. With 0.1 and0.3mLviscozymedoses,themethaneproductionswereverysimilartomethane

production from untreated biomass and only 4-6% increase in methane production was observed. With 0.5 mL viscozyme dose, 42% of increase compared to untreated biomass was achieved and the net cumulative biogas production was found as 150.1 mL CH_4 /gVS (Figure 4.25.a). 0.5 mL viscozyme pretreatment was more effective than other viscozyme doses in terms of sCOD release and this can be correlated with highest methane yield improvement. Highest daily methane productions were observed at day 9 and after than methane production rate decreased (Figure 4.25.b). Highest methane productionrateswereobtainedas9.6,9.9,and12.9mLCH$_4$/gVS.d,for0.1,0.3,and

0.5 mL viscozyme pretreated biomass, respectively.

As these results are compared, viscozyme pretreated biomass was not as not efficient as pretreated biomass with other enzymes. Solubility increase after viscozyme pretreatment was similar to solubility increase after cellulase and protease pretreatment. However, the same trend was not observed for methane yield improvements from viscozyme pretreated biomass and the methane yields were found to be lower than pretreated biomass with cellulase or protease. This lower improvement may be due to acidic conditions during viscozyme pretreatment. Viscozyme pretreatment was carried out at pH between 4.0-4.5 and it may lead to release of inhibitory compounds such as furans. Although there are some studies related to application of acidic pretreatment and improvement of methane yields, production of inhibitory compounds during acidic conditions were observed in previous studies. Another reason may be the ability of anaerobic microorganisms to degrade carbohydrates without help of enzymes. This may lead to lower improvements of methane production. However, it is important to notice that methane production was increased as 42% for 0.5 mL viscozyme pretreatment. Higher efficiencies can be obtained at higher viscozymedoses.

a.

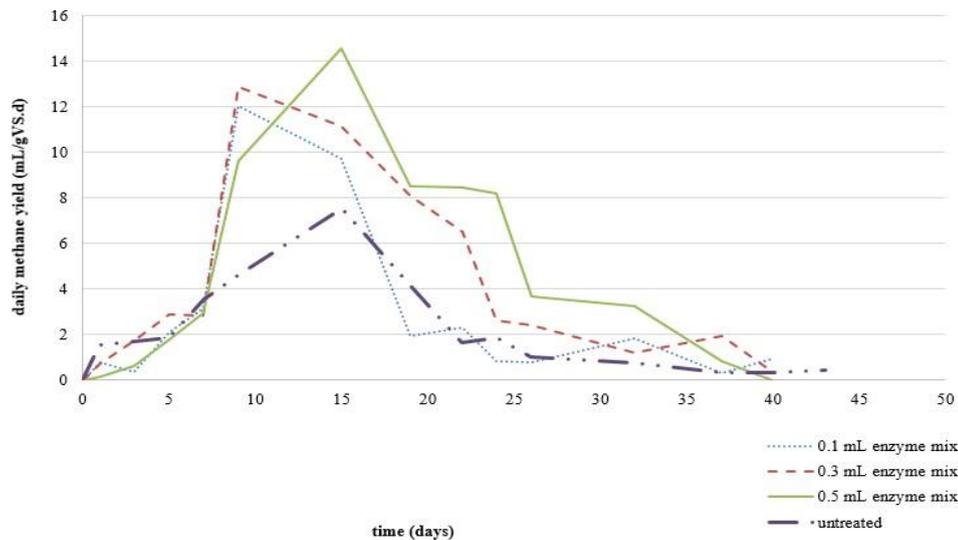**Viscozyme pretreated *Scenedesmus sp.***
(Mesophilic)

b.

Figure 4.25. Net cumulative methane production and daily methane production of viscozyme pretreated *Scenedesmus sp.* biomass after mesophilic anaerobic digestion

Apart from single enzyme pretreatment, *Scenedesmus sp.* was also subjected to enzyme mix pretreatment and BMP tests. Enzyme mix (viscozyme and protease) pretreated biomass increased methane production by 22%, 69% and 109 % for 0.1, 0.3, and 0.5 mL enzyme mix pretreatment of *Scenedesmus sp.* biomass, respectively (Figure 4.26.a). With 0.5 mL enzyme mix pretreatment, methane yield was increased to 216.01 mL CH_4/gVS. Daily methane productions showed the highest methane production rates

110

were observed between 15th day as between 12.0-14.5mL CH_4 /gVS.d (Figure 4.26. b). Nevertheless, at first 5 days, higher methane production was achieved than untreated biomass.

As compared to single enzyme pretreatment, it was obvious that enzyme mix pretreated biomass showed better methane yields. This can be attributed to synergistic effect of enzymes on biomass solubilization. Since *Scenedesmus sp.* cell wall both contains carbohydrates (cellulose, hemicellulose, etc.) and proteins, application of enzyme mix provided better disruption of cell wall and biomass solubilization. So, better methane yields were achieved for enzyme mix pretreated biomass.

a.

b.

Figure 4.26. Net cumulative methane production and daily methane production of enzyme mix pretreated *Scenedesmus sp.* biomass after mesophilic anaerobic digestion

As the methane yields from *Scenedesmus sp.* pretreated with various enzymes were compared, it was concluded that pretreated *Scenedesmus sp.* biomass showed better methane yields than untreated biomass and the improvements were changed between 4%-109%. Table 4.6 shows cumulative methane yields and daily methane yields from both untreated and pretreated *Scenedesmus sp.* According to the results, enzyme mix pretreated biomass showed the highest cumulative methane yield. It can be attributed to the synergistic activity of enzyme mix on cell wall disruption and biomass solubilization, resulting in higher methane production potentials. Since cell wall of *Scenedesmus sp.* includes both complex carbohydrates and proteins in multilayer structure, use of enzyme mix including several types of enzymes (protease, hemicellulase, cellulase, xylanase etc.) provided cell wall and cell membrane disruption of microalgae. Better hydrolysis rates were achieved by anaerobic bacteria resulting in higher methane production. Protease pretreated biomass also showed enhanced methane yields. Exterior cell walls of *Scenedesmus sp.* include glycoproteins and protease pretreatment may provide cell wall disruption of protein components. Moreover, intracellular protein components may also be consumed by anaerobic microorganisms with the help of protease. Cellulase and viscozyme pretreated biomass showed lower improvement efficiencies than protease and enzyme mix pretreatment, and viscozyme pretreated biomass had the lowest improvement. This lower improvement may be due to acidic pretreatment conditions. Viscozyme pretreatment was carried out at pH between 4.0-4.5 and it may lead to production of inhibitory compounds.

Daily methane production improvements were highest for protease (1.4-2 fold) and enzyme mix pretreated biomass (1.6-1.9 fold). In addition, for cellulase (0-1.4 fold) and viscozyme pretreated biomass (1.3-1.7 fold), the highest daily methane production was also higher than untreated biomass. However, the time to reach the highest daily methane production was observed earlier for cellulase and viscozyme pretreated biomass than other pretreated biomass. It may be due to fast degradation of carbohydrates in the digesters. However, for proteins, it takes more time for degradation. Protease pretreatment may disrupt protein content and after a while, anaerobic microorganisms may begin to use degraded proteins in the media. Here, it is

important to notice that at first 9 days, the methane production of protease and enzyme mix pretreated biomass was higher than untreated biomass. This can be an indication of protein consumption in *Scenedesmus sp.* biomass after 9 days. In general, these results suggest that enzymatic pretreatment can provide higher methane productions in a short hydraulic retention time (HRT) which is an important parameter for anaerobic reactor design and cost of the anaerobic digestion process.

Table 4. 6. Comparison of methane yields of pretreated *Scenedesmus sp.* with enzymes after mesophilic digestion

Typeof pretreatment	Methane yield(mL CH$_4$/g VS)	Highest daily methane production (mL/ CH$_4$/ gVS)	
Untreated	105.9	7.5	15th day
Thermal (55°C)	103.8	8.0	15th day
Cellulase	120.4-177.1	7.3-10.6	9th day
Protease	146.0-193.8	10.6-15.5	15th day
Viscozyme	110.1-150.1	9.6-12.9	9th day
Enzyme mix	129.2-216.0	12.0-14.5	15th day

BMP Results for Scenedesmus sp. biomass after thermophilic digestion

After thermophilic digestion, methane productions for pretreated and untreated *Scenedesmus sp.* biomass were observed to be higher than mesophilic digestion. Untreated biomass had a methane production potential as 170.1 mL CH$_4$/ gVS. This value was higher than untreated biomass at mesophilic temperature showing that thermophilic conditions showed better organic matter degradation. It is stated that at thermophilic conditions, cellulose and hemicellulose degradation could occur. Furthermore, at mesophilic temperature *Scenedesmus sp.* could be stay alive and may hamper anaerobic digestion process. However, at thermophilic digestion, their activities are low.

Thermally pretreated (55°C) biomass showed a small increase in methane production as 5% which was insignificant (Figure 4.7a) The same trend was observed at mesophilic conditions. This showed that low temperature pretreatment increased biomass solubilization to some extent, however; there was not a significant methane improvement after low temperature pretreatment.

Scenedesmus sp. had the highest methane production as 237.0 mL CH_4/g VS with 39.3% improvement with 0.5 mL enzyme dose in cellulase pretreatment. 0.3 mL cellulase pretreated biomass showed 21.7% improvement in methane production and 0.1 mL cellulase pretreated biomass improved methane production approximately by16.1% (Figure 4.27.a). As can be seen from the results, there was a positive relationship between cellulase dose and cumulative methane yields. Daily methane production rates for cellulase pretreated biomass increased gradually at first 10 days, and after then, the daily methane production rates were lowered. Cellulase pretreated biomass showed higher methane production at first7^{th}day with a rate between 14.9-17.8mLCH_4/gVS.d. The highestdaily methane production for untreated biomass was 10.5 mL CH_4 /gVS.d at 7^h day. These results showed the better anaerobic degradability of *Scenedesmus sp.* was achieved with cellulase pretreatment in thermophilic digestion. As compared to mesophilic conditions, it was clear that thermophilic digestion of cellulase pretreated *Scenedesmus sp.* biomass showed better cumulative and daily methane yields Furthermore, the time to reach highest daily methane production rate was earlier. It was reduced to 9 days (mesophilic digestion) to 7 days (thermophilic digestion) for cellulase pretreated biomass (Figure4.27.b).
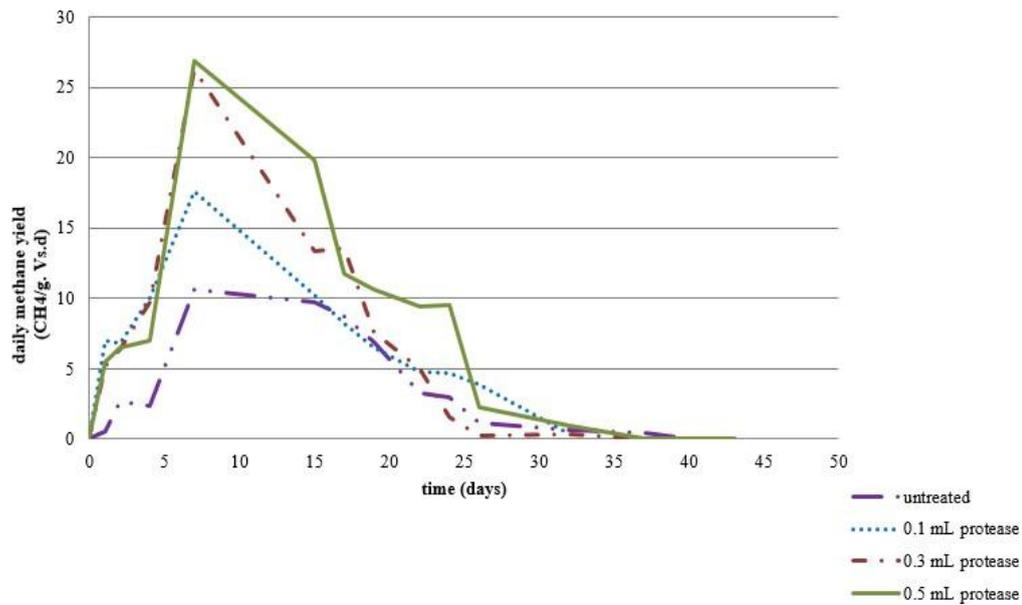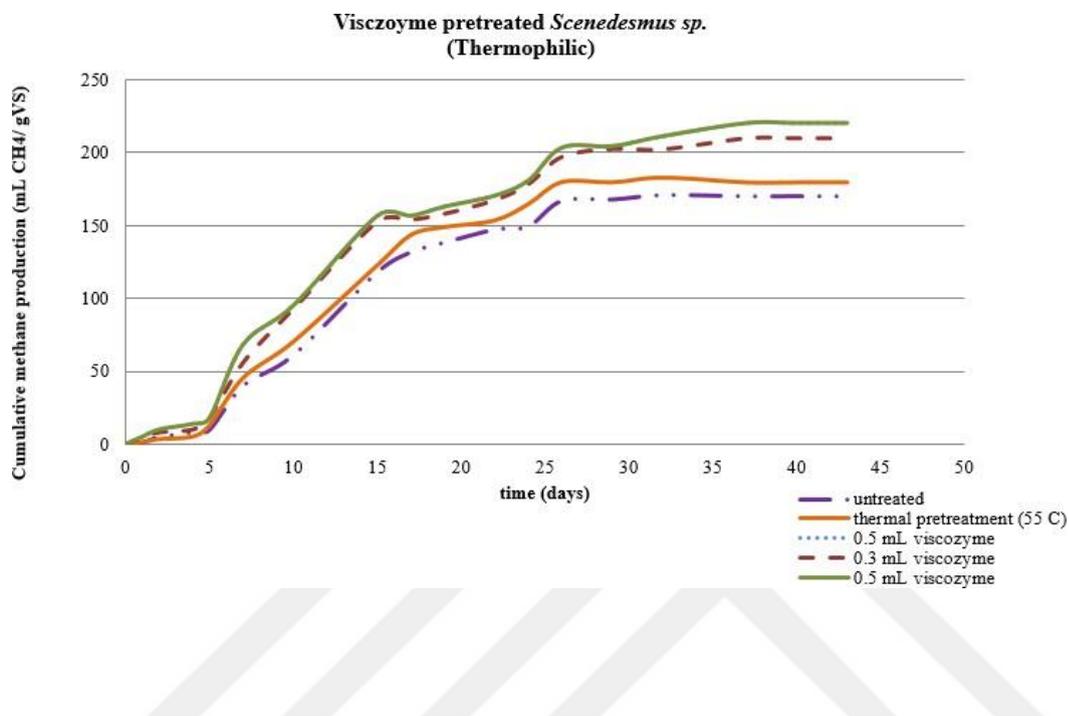
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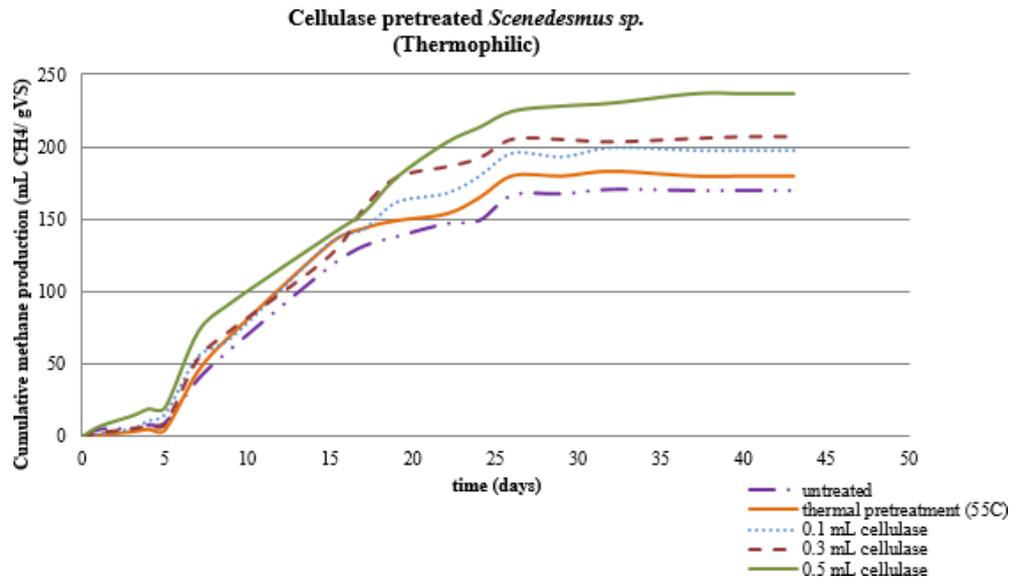
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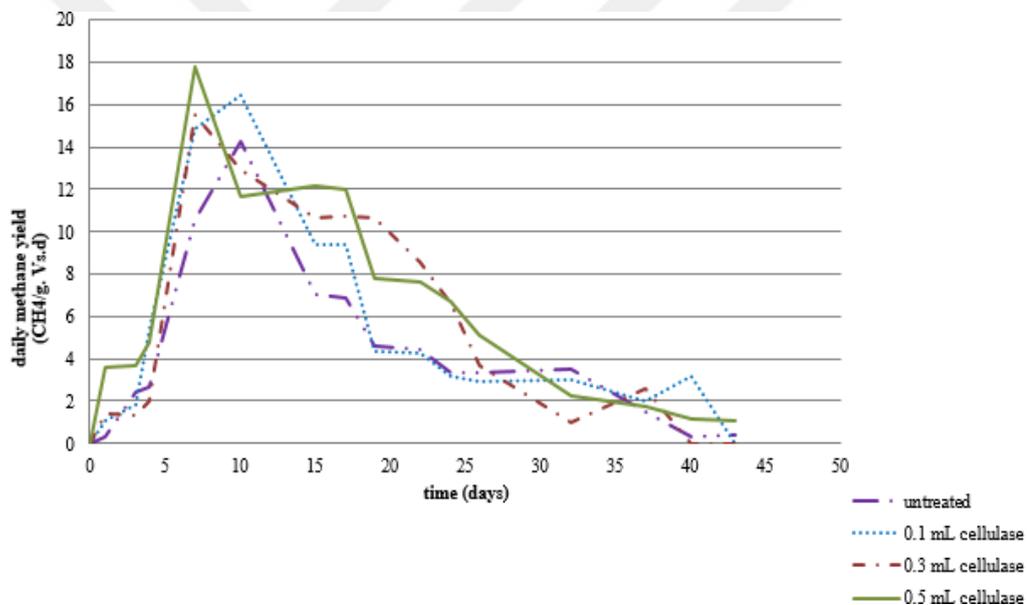
Figure 4.27. Net cumulative methane production and daily methane production of cellulase pretreated *Scenedesmus sp.* biomass after thermophilic anaerobic digestion

Protease pretreated biomass also improved methane production potential from *Scenedesmus sp.* biomass (Figure 4.28.a) at thermophilic digestion. 0.1 mL protease dose increased methane production by 25.5 % when compared to untreated biomass. 0.3 mL protease dose increased by 53.1 % (260.4mL CH_4/g VS). Highest improvement was observed with 0.5 mL dose as 71.4% improvement (291.7 mL CH_4/g VS). As cellulase pretreated biomass, protease pretreated *Scenedesmus* biomass also showed highest

methane production at 7thday (Figure 4.28.b). The highest daily methane yields were found as 17.6, 26.3, and 26.8 mL CH$_4$ /gVS.d for 0.1 mL, 0.3 mL, and 0.5 protease dose pretreatment, respectively. There was a positive correlation was observed between protease dose and daily methane production. As compared to mesophilic digestion, it was clear that thermophilic digestion provided more organic matter degradation and better methane yields. In addition, hydrolysis rate was higher in thermophilic digestion of protease pretreated biomass. However, it is stated that at thermophilic temperatures, ammonia inhibition may occur and can be problematic. In this study, no ammonia inhibition was observed during anaerobic digestion and this should be considered before large scale applications
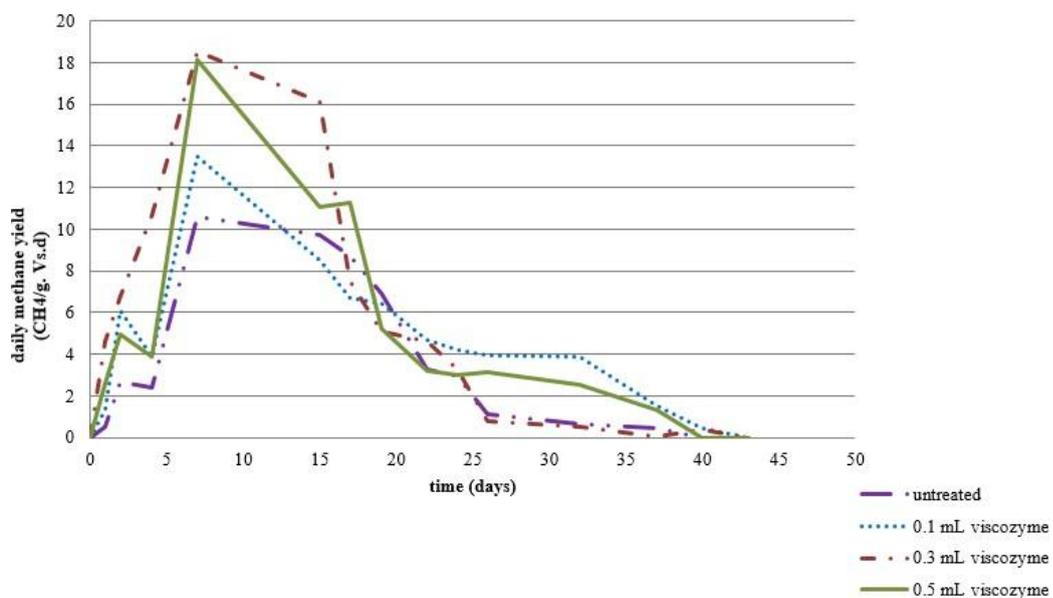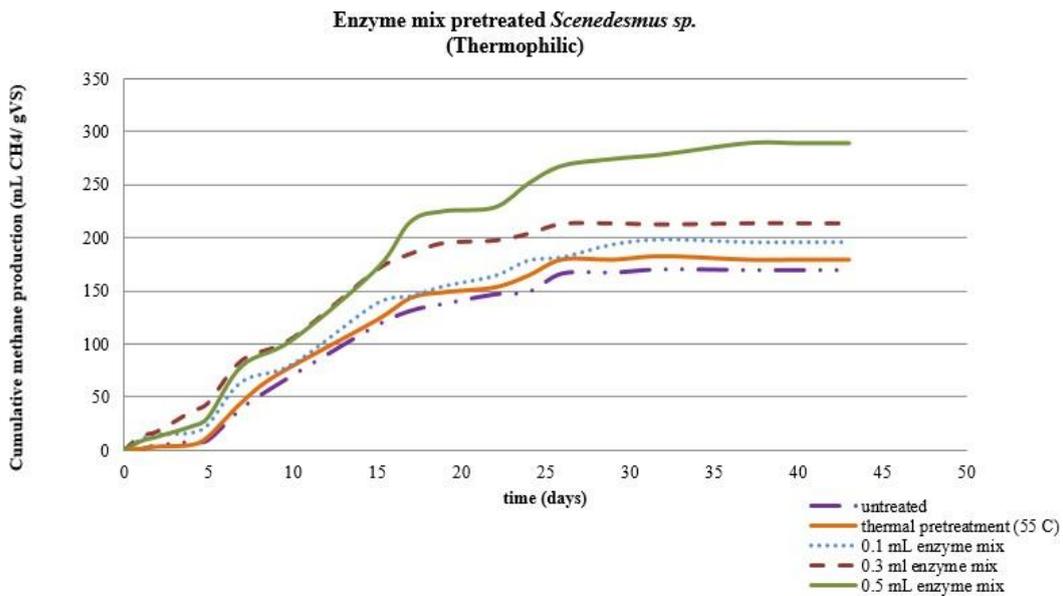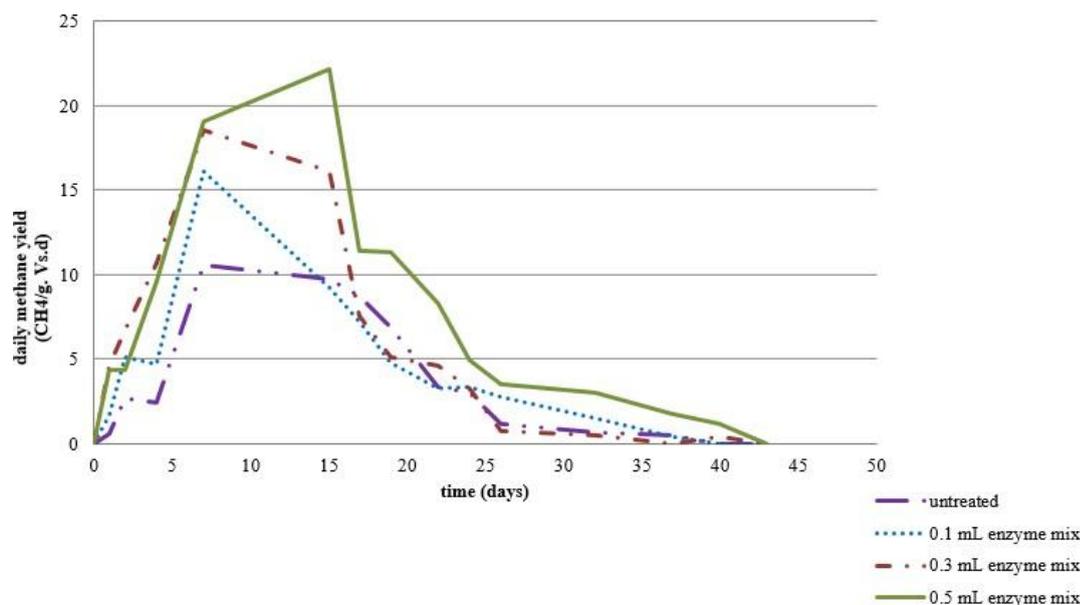
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b.
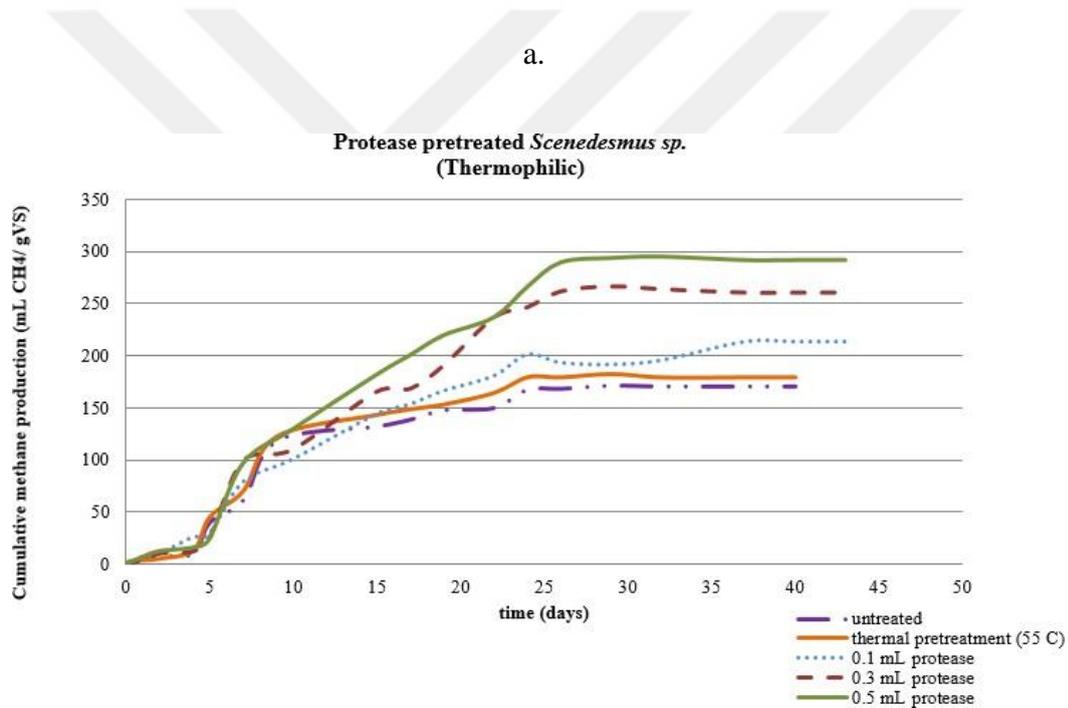
Figure 4.28. Net cumulative methane production and daily methane production of protease pretreated *Scenedesmus sp.* biomass after thermophilic anaerobic digestion

Viscozyme pretreated biomass had also showed higher methane production as comparedtountreatedbiomasswith7.8%,23.5%,and29.7%increasefor0.1,0.3,and

0.5 mL viscozyme pretreatment, respectively. 0.5 mL viscozyme pretreated biomass had a net cumulative methane yield of 220.7 mL CH_4 /gVS.d. (Figure 4.29.a). These results were higher than methane yield results for mesophilic conditions applied to viscozyme pretreated biomass. However, as in mesophilic digestion, the improvements after thermophilic digestion were comparatively lower than pretreated biomass treated with other enzymes. This could be explained with low pH pretreatment conditions of viscozyme. Another reason may be the ability of anaerobic microorganisms to degrade carbohydrates without the help of enzymes. This may lead to lower improvements of methane production. However, it is important to notice that 29.7% of increase was observed for 0.5 mL viscozyme pretreatment. Higher efficiencies can be obtained by increasing viscozyme dose, however; the cost- effectiveness of the produces should be investigated.

Daily methane production rates (Figure 4.29.b) showed that viscozyme pretreated biomass showed higher methane productions during anaerobic digestion than untreated biomass. At first 5 days, methane production was higher than untreated biomass. Highest daily methane production was observed as between 13.5-18.2 mL CH_4/ gVS.d.

for viscozyme pretreated biomass at 7th day. It is obvious that viscozyme pretreatment provided better methane production rates and improved hydrolysis of *Scenedesmus sp.* biomass.

a.

b.

Figure 4.29. Net cumulative methane production and daily methane production of viscozyme pretreated *Scenedesmus sp.* biomass after thermophilic anaerobic digestion

Apart from single enzyme pretreatment, *Scenedesmus sp.* was also subjected to enzyme mix pretreatment and BMP test at thermophilic conditions. Mixed enzyme pretreated biomass after thermophilic digestion increased methane production by 15.3% (196.2 mL CH_4/ gVS) , 25.8% (214.1 mL CH4/ gVS) ,and 70.2% (289.7 mL CH_4/gVS) for 0.1, 0.3, and 0.5 mL enzyme mix pretreated *Scenedesmus* biomass, respectively. Daily methane productions showed highest rate at7[th] day for 0.1 and 0.3 mL enzyme mix pretreated biomass as 16.1 and 18.5 mLCH$_4$ /gVS.d, respectively (Figure 4.30). 0.5 mL enzyme mix pretreated biomass showed a daily methane production yield of 19mLCH$_4$ /gVS.d at 9th day and the rate increased further to 22.1 mLCH$_4$ /gVS.d at the end of 15th day. These results showed that with the highest dose (0.5 mL enzyme mix), the degradation of *Scenedesmus sp.* biomass continued by anaerobic microorganisms which may be an indicator or the efficiency of the pretreatment. 0.5 mL enzyme mix pretreatment provided more disruption of cells than 0.1 or 0.3 mL enzyme mix pretreatment resulting in better biomass degradation during anaerobic digestion.

a.

b.

Figure 4.30. Net cumulative methane production and daily methane production of enzyme mix pretreated *Scenedesmus sp.* biomass after thermophilic anaerobic digestion

The results of BMP tests at thermophilic temperature showed that enzymatic pretreatment was successful to improve cumulative methane yields from *Scenedesmus sp.* (Table 4.7). Enzyme mix pretreated biomass showed one of the highest methane production potential. The other highest methane production potential was belonged to protease pretreated biomass. Enzyme mix contains also protease and protease can be considered the most important enzyme in terms of pretreatment of *Scenedesmus sp.* and methane production improvement. Use of carbohydrases (cellulase and viscozyme) resulted in lower methane improvement when compared to protease or enzyme mix pretreatment. The same trend was also observed in mesophilic digestion that enzyme mix were more effective than viscozyme or cellulase.

Table 4. 7. Comparison of methane yields of pretreated *Scenedesmus sp.* with enzymes after thermophilic digestion

Type of pretreatment	Methaneyield (mLCH$_4$/gVS)	Highest daily methane production (mL CH$_4$/ gVS.d)	
Untreated	170.1	10.4	7th day
Thermal (55°C)	179.6	13.4	7th day

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Cellulase	197.5-237.1	14.9-17.8	7th day
Protease	213.6-291.7	17.6-26.8	7th day
Viscozyme	183.5-220.7	13.5-18.2	7th day
Enzyme mix	196.2- 289.7	12.0-14.5	7th day

Kinetics of the Methane Production from *Scenedesmus sp.*

Mathematical models are commonly used by researchers to analyze anaerobic digestion process, determine kinetic parameters of anaerobic digestion such as specific growth rate, and lag phase. In this thesis, two different models (first order kinetic model and modified Gompertz model) were used to model experimental data and determine kinetic parameters. Determination of hydrolysis rate constant (k) is important to understand the biodegradability of the substrates used in anaerobic digestion. Higher hydrolysis rate constants mean higher availability of substrates and higher organic matter degradation in anaerobic digestion. The rate of hydrolysis increases depending on the concentration of hydrolytic enzymes and availability of adsorption sites and in this study; it was expected to have higher hydrolysis rate constants after enzymatic pretreatment.

The results of the first order kinetic model for *Scenedesmus sp.* showed that and hydrolysis rate(k) values were found to be between 0.038-0.048 day^{-1}and 0.044-0.071 day^{-1} for mesophilic and thermophilic digestion, respectively. R^2values were between 0.93-0.95 for the first order kinetic models (Table 4.8). Hydrolysis rates (k) for pretreated biomass at mesophilic digestion were more than untreated biomass. However, the difference was relatively small. Hydrolysis rates for thermophilic digestion were found to be higher than mesophilic digestion results which showed that better hydrolysis rates were achieved during thermophilic digestion. First order kinetic models are commonly used to describe behavior of hydrolysis process, however, there were some limitations observed in this study. In this study, it was observed that the model did not fit well with the experimental results. (See Appendix 1-1, 1-2). In first order kinetic model, lag phase was not considered which is a natural behavior at the beginning of anaerobic digestion. In addition, and ultimate net methane yields were found to be higher than experimental values for most of the pretreated biomass. In

addition, complex substances including carbohydrate content such as cellulose, hemicellulose, protein and lipids may show different hydrolysis kinetics and in this study, enzymes used in pretreatment hydrolyzed different portions of the biomass. So, hydrolysis rates were different from each other and this could not be correlated directly with methane yields.

Table 4. 8. Results of first order kinetic model for *Scenedesmus sp*.

	Mesophilic				Thermophilic			
	ym	k	R^2	MSE	ym	k	R^2	MSE
Untreated	141.4	0.038	0.940	10.5	220.5	0.044	0.946	16.1
Thermal (55°C)	132.8	0.041	0.936	10.6	228.1	0.046	0.936	18.8
Cellulase (0.1 mL)	162.51	0.041	0.936	12.8	245.8	0.048	0.964	14.6
Cellulase (0.3 mL)	204.28	0.038	0.945	14.2	266.3	0.052	0.964	16.2
Cellulase (0.5 mL)	236.85	0.040	0.960	14.2	279.44	0.054	0.984	10.9
Protease (0.1 mL)	194.4	0.039	0.945	14.0	242.68	0.051	0.977	11.5
Protease (0.3 mL)	225.3	0.041	0.947	16.0	292.2	0.064	0.956	21.0
Protease (0.5 mL)	252.1	0.043	0.937	20.1	320.94	0.069	0.986	12.3
Viscozyme (0.1 mL)	140.6	0.043	0.947	10.1	234.9	0.044	0.984	8.7
Viscozyme (0.3 mL)	138.77	0.046	0.937	11.4	266.2	0.051	0.966	15.5
Viscozyme (0.5 mL)	187.15	0.048	0.938	15.3	270.3	0.051	0.978	12.2
Enzyme mix (0.1 mL)	164.6	0.041	0.938	13.1	235.9	0.054	0.981	10.1
Enzyme mix (0.3 mL)	357.71	0.031	0.964	13.1	249.1	0.059	0.962	16.0
Enzyme mix (0.5 mL)	298.68	0.034	0.980	11.5	31.7	0.071	0.987	11.3

(ym: methane yield potential (mL/gVS), k: First order rate constant (1/d), MSE: mean square error)

Modified Gompertz model was also applied to analyze experimental data obtained from previous parts. This model gave the maximum methane production rates, time of the lag phase, and ultimate methane yield (See Table 4.9). The findings showed that in general, the model was able to predict ultimate methane yields compatible with experimental results. In mesophilic digestion, for the pretreated *Scenedesmus sp.,* lag phases were found as lowered to some extent, which showed that pretreatment did not have a significant effect on reducing lag phase. However, maximum methane production rates were higher for pretreated biomass. In thermophilic digestion, time required for lag phase as found to be lower than mesophilic digestion and maximum methane production rates were higher than mesophilic digestion that resulted in higher methane yields (See Appendix 1-3,1-4).

Table 4. 9. Results of modified Gompertz model for *Scenedesmus sp.*

	Mesophilic					Thermophilic				
	ym	Ue	λ	R^2	MSE	ym	Ue	λ	R^2	MSE
Untreated	103.3	7.13	5.21	0.997	1.9	170.7	11.27	4.11	0.996	3.8
Thermal (55°C)	100.0	7.36	5.00	0.993	3.3	178.4	13.03	4.24	0.993	5.9
Cellulase (0.1 mL)	124.7	8.16	4.91	0.995	3.5	200.4	11.57	2.77	0.995	5.2
Cellulase (0.3 mL)	151.6	9.25	4.72	0.998	2.7	223.6	12.94	2.34	0.991	8.0
Cellulase (0.5 mL)	182.7	9.76	3.93	0.996	4.5	244.9	11.7	1.00	0.982	11.4
Protease (0.1 mL)	145.3	9.18	4.63	0.996	3.5	201.4	11.14	1.86	0.991	7.2
Protease (0.3 mL)	173.7	10.44	4.37	0.997	3.9	252.9	19.53	2.75	0.996	5.9
Protease (0.5 mL)	199.6	12.20	4.33	0.990	8.0	288.5	17.33	1.00	0.978	15.1
Viscozyme (0.1 mL)	109.1	7.35	4.38	0.997	2.4	187.8	8.9	1.7	0.993	5.6
Viscozyme (0.3 mL)	108.5	8.95	4.79	0.993	3.8	219.5	12.96	2.26	0.988	9.2

Viscozyme (0.5 mL)	150.1	11.06	4.52	0.997	3.3	225.6	12.05	1.72	0.988	8.9
Enzyme mix (0.1 mL)	123.5	9.40	4.84	0.991	4.9	201.5	11.12	1.92	0.996	4.4
Enzyme mix (0.3 mL)	178.1	9.36	4.77	0.994	5.2	212.01	15.22	2.08	0.997	4.4
Enzyme mix (0.5 mL)	226.3	12.06	4.86	0.996	5.15	283.63	17.78	1.00	0.983	13.42

(ym: Biogas yield potential (mL/gVS), Ue: Maximum biogas production rate (mL/g VS),λ: Duration of lag phase)

4.1.9. Energy Assessment Results

Application of energy assessment is important in terms of showing applicability of the process. Ratio of energy input (energy requirement of pretreatment) and energy output (methane production improvement by anaerobic digestion) is a good indicator for the net energy balance. In order to have a positive energy balance, this ratio should be less than 1.

The energy assessment results of this study (Table 4.10) showed that energy ratios for pretreated *Scenedesmus* biomass were between 0.68 and 1.41. for mesophilic digestion. Values more than 1 means the energy consumed throughout pretreatment of *Scenedesmus sp.* biomass was higher than energy produced as methane So, according to the results, thermal pretreatment cellulase, protease, and enzyme mix pretreatment at 0.1 mL dose, and viscozyme pretreatment at 0.1-0.3 mL dose had negative energy balances. The lowest energy ratio was obtained from enzyme mix pretreated biomass (0.5 mL enzyme mix) which was 0.68.

At thermophilic digestion all energy ratios were less than 1, indicating a positive energy balance. Lowest energy ratio was belonging to enzyme mix and protease pretreated biomass. The ratios for thermophilic digestion were less than the ratios of mesophilic digestion due to higher methane production yields. Although more methane is produced,

heating of the reactor is the main driver for energy input (not included here to simplify results).

Table 4. 10. Results of Energy Assessment of *Scenedesmus sp.* after various enzymatic pretreatment

	Pretreatment condition	Mesophilic Ein/Eout	Thermophilic Ein/Eout
Untreated	-	-	-
Thermal	55 C	1.41	0.81
Cellulase	0.1 mL dose	1.16	0.74
	0.3 mL dose	0.98	0.70
	0.5 mL dose	0.83	0.62
Protease	0.1 mL dose	1.00	0.68
	0.3 mL dose	0.90	0.56
	0.5 mL dose	0.76	0.50
Viscozyme	0.1 mL dose	1.31	0.80
	0.3 mL dose	1.32	0.69
	0.5 mL dose	0.97	0.66
Enzyme Mix	0.1 mL dose	1.14	0.74
	0.3 mL dose	0.82	0.68
	0.5 mL dose	0.68	0.50

4.2. Effect of Enzymatic Pretreatment on Biogas Production from *P.cruentum*

P. cruentum is generally used due to its potential for polysaccharides, fatty acids, pigment and other chemical compounds such as antioxidants and there is no study found in literature related to investigation of methane potential of *P. cruentum*. So, this study was the first considering investigation of enzymatic pretreatment and methane production from *P. cruentum.* The size of this microalgae is between 6-10 µm and cell

wall of this microalgae is not as rigid as *Scenedesmus sp.* It is encapsulated by polysaccharide complex containing sulfate (EPS) which is composed of glucose, rhamnose, fucose, arabinose, xylose, mannose orthomethyl sugar and acidic residues of glucuronic acid and galacturonic acid. In this part of the study, it was aimed to observe cell wall disruption and increase solubility of intercellular matter of microalgae.

P. cruentum biomass was subjected to cellulase, viscozyme, protease and enzyme mix (protease and viscozyme). The effects of enzymatic pretreatment by using different enzyme doses (0.1, 0.3, and 0, 5 mL/ g biomass) at different temperatures (25°C, 45°C, and 55°C) and at different application periods were carried out for cell wall disruption and organic matter solubilization. After enzymatic pretreatment, biomethane potentials of pretreated *P. cruentum* biomass under mesophilic (35°C) and thermophilic (55°C) conditions were tested in comparison with raw biomass. Two kinetic models were applied to BMP results to determine kinetic parameters of anaerobic digestion. Energy assessment of the pretreatment coupled with methane production was applied to discuss applicability of the enzymatic pretreatment and methane production from *Scenedesmus sp*. The results obtained and comprehensive evaluations are given in the following sections in detail.

4.2.1. Thermal Pretreatment of *P.cruentum*

Thermal pretreatment at 55°C was applied to *P. cruentum* biomass without enzyme addition to observe the impact of temperature on biomass solubility itself. Thermal pretreatment increased sCOD content of the medium as 88.2 mg sCOD/g dry weight in 9 hours and it did not change after 24 hours. The increase in solubility was calculated as 13.6%. This result showed that temperature itself had increased solubilization of *P. cruentum* biomass. At high temperatures as 55°C, cell wall of *P. cruentum* was disrupted and increased biomasssolubilization.

4.2.2. Enzymatic Pretreatment of *P. cruentum* by Cellulase

In this part of the study, it was expected to have no significant solubilization increase after cellulase pretreatment, since *P. cruentum* biomass does not contain cellulose. So,

cellulase enzyme was not expected to degrade cellulose in the medium. At 55°C, for 0.5 mL cellulase dose, sCOD release was achieved, as 125.8 mg sCOD/L in 9 hours and after then, it did not improve further. For 0.3 mL cellulase dose, in 9 hours, sCOD release was found as 128.4 mg sCOD/L. For 0.1 mL cellulase dose, highest release was observed as 106.2 mg sCOD/L (solubility increase as 14.5%) after 9 hours. Thermally pretreated (55ºC) biomass also showed that temperature itself had increased solubilization of biomass and highest increase was observed up to 88.2 mg sCOD/L after 9 hours (Figure 4.30-4.31). As these values were compared, it was observed that cellulase did not have a significant effect on solubilization of biomass. Cellulase pretreatment of *P.cruentum* biomass at lower temperatures showed very low solubilization increase (less than 40 mg sCOD/L), so it was concluded that temperature is the main driving parameter for organic matter solubilization rather than cellulase. Actually, this result was also expected due to lack of cellulose in *P.cruentum*biomass.

As carbohydrate and protein solubilization after cellulase pretreatment were discussed, it was observed that after thermal pretreatment (55ºC), mostly carbohydrate content of the biomass was solubilized and cellulase pretreated biomass showed similar trend (Figure4.32).
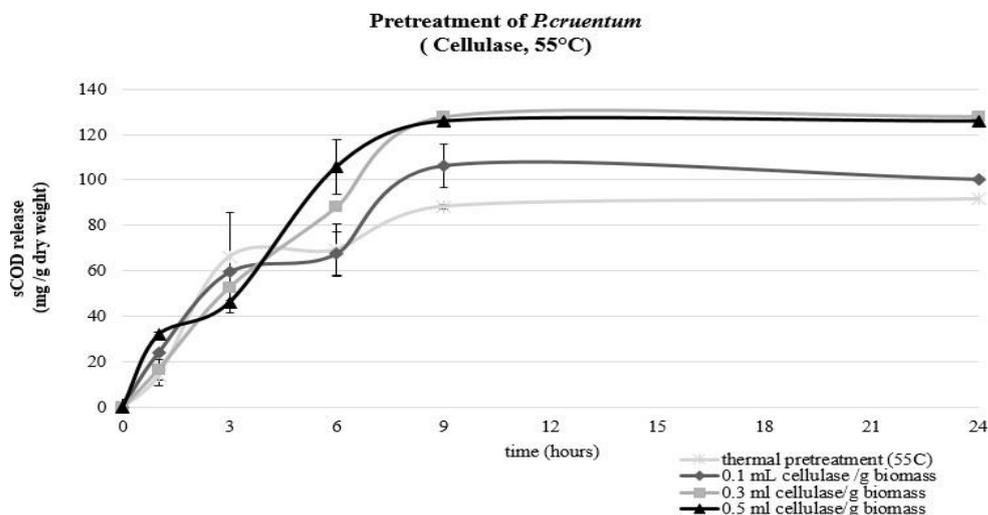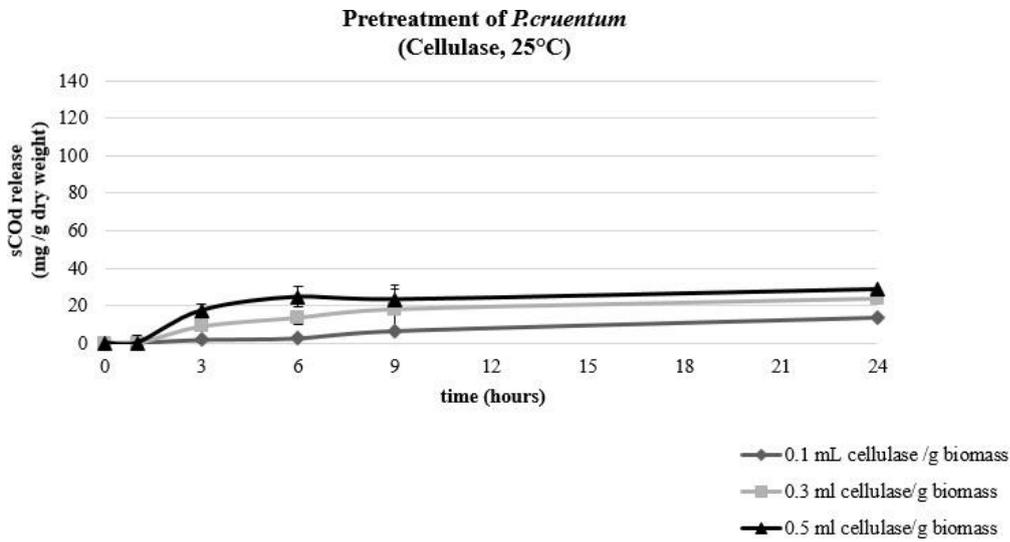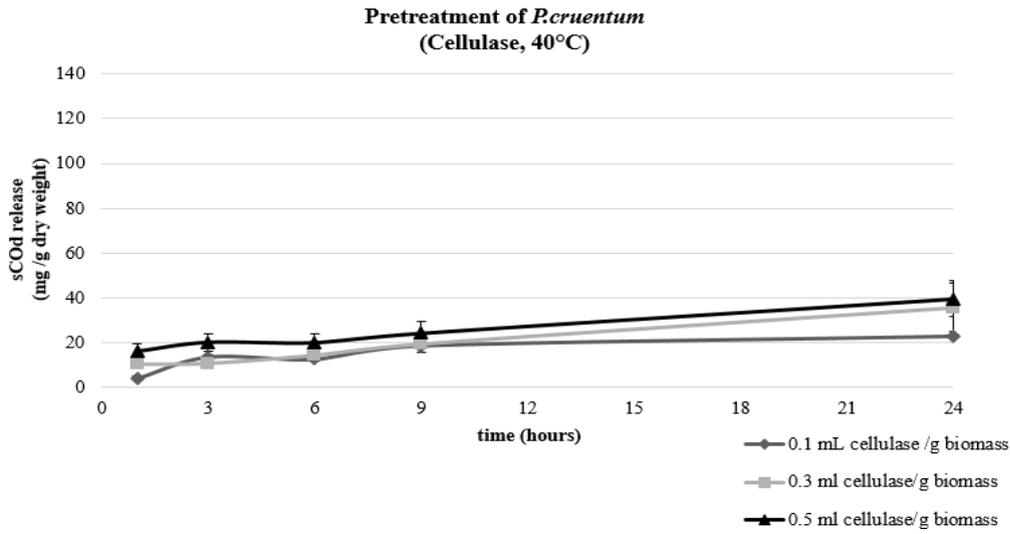
Figure 4.31.The effect of enzyme dose on soluble COD release by cellulase pretreatment at differenttemperatures(*P.cruentum*)
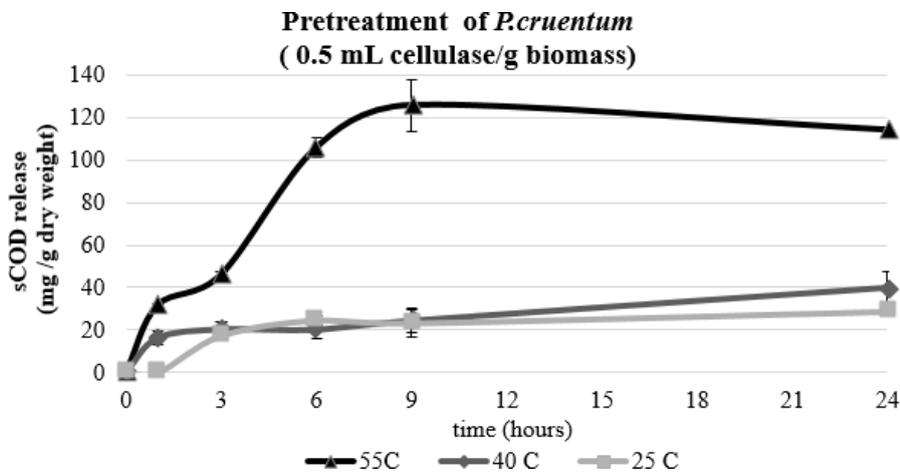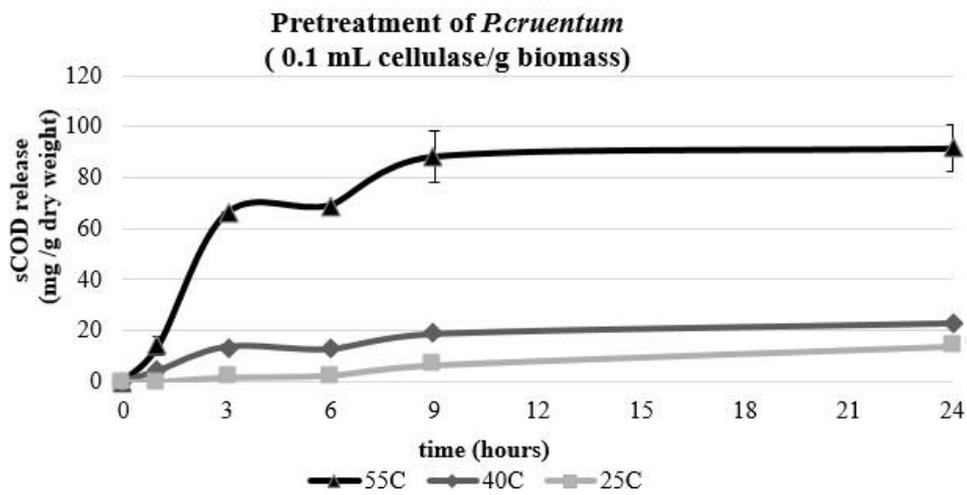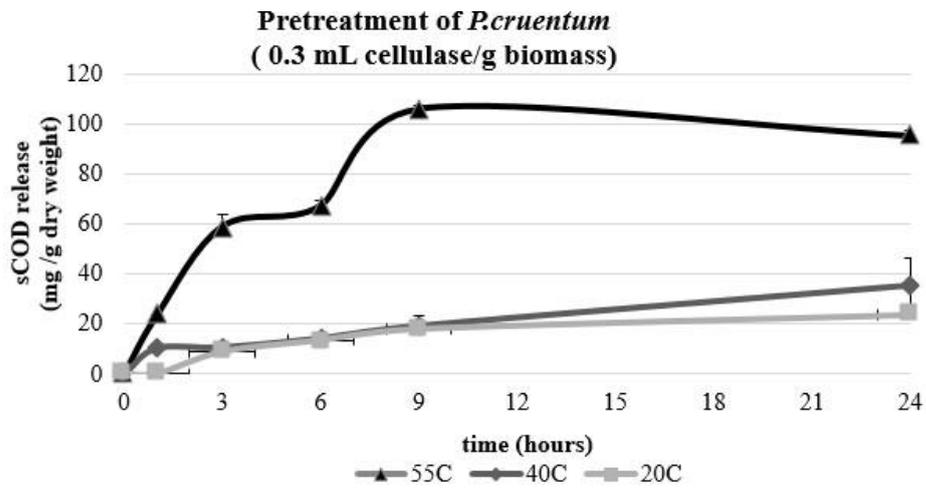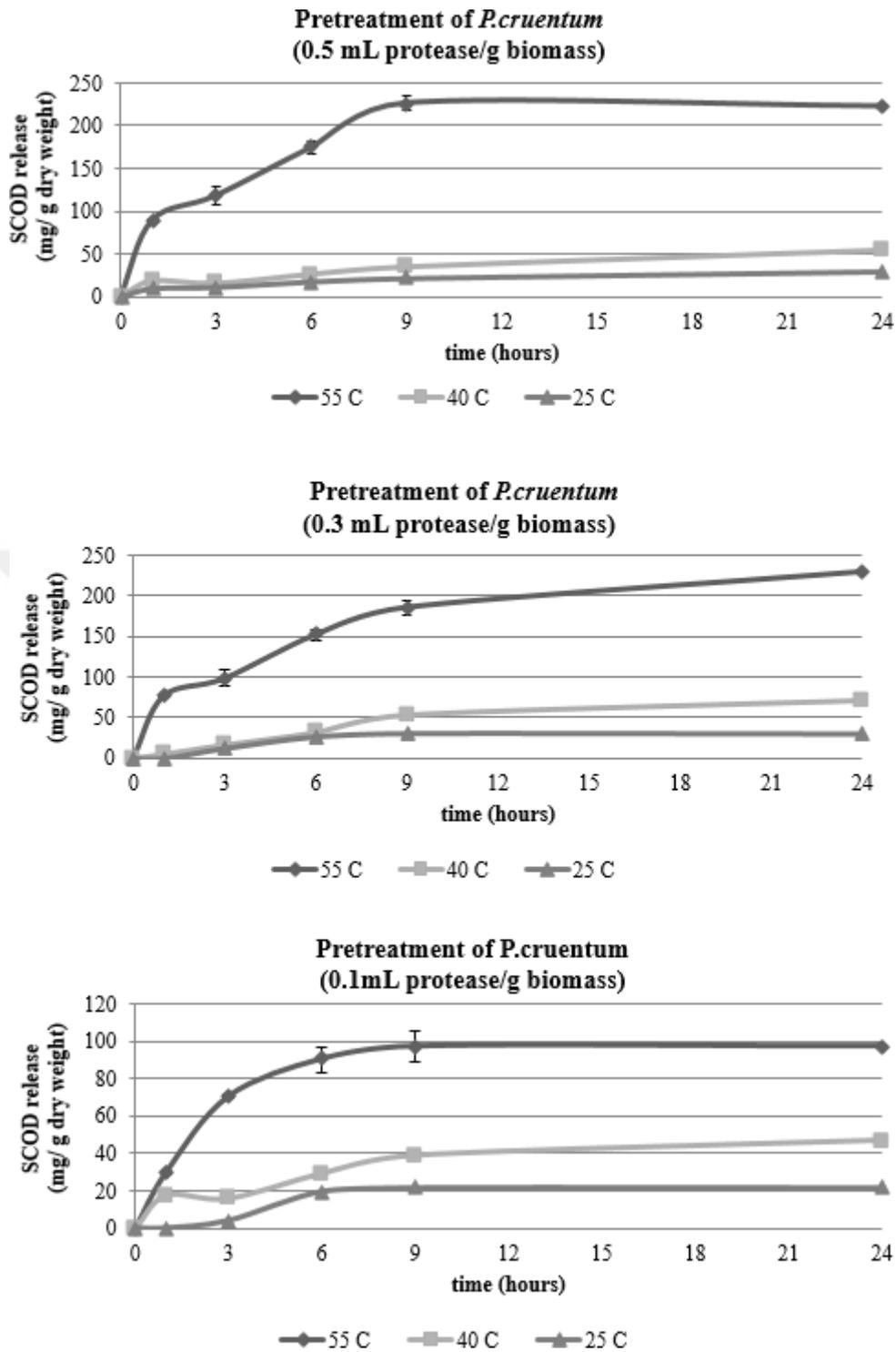
Figure 4.32. The effect of temperature on soluble COD release by cellulase pretreatment at different enzyme doses(*P.cruentum*)
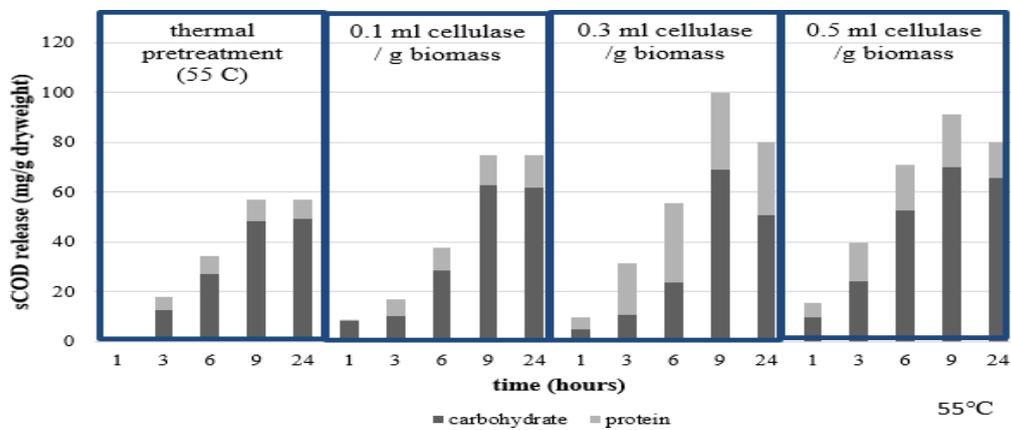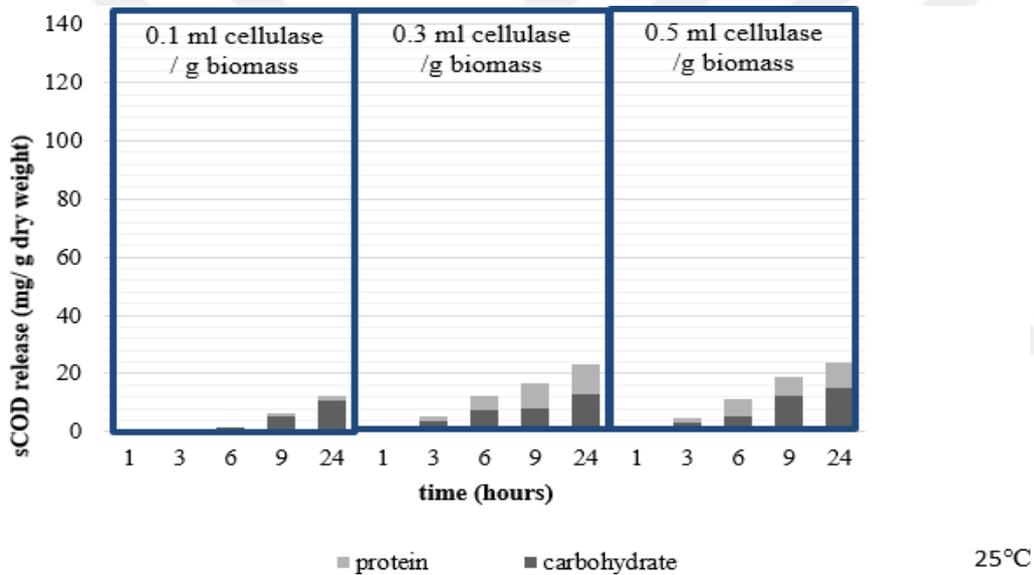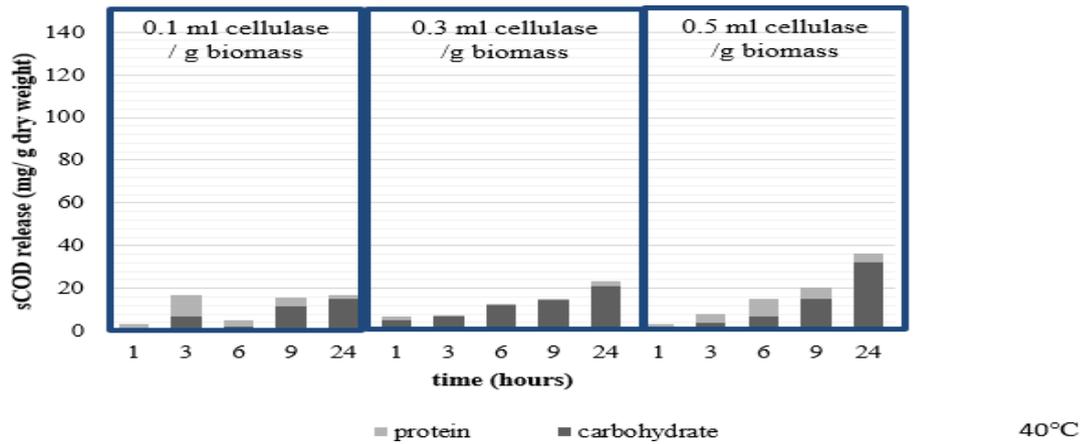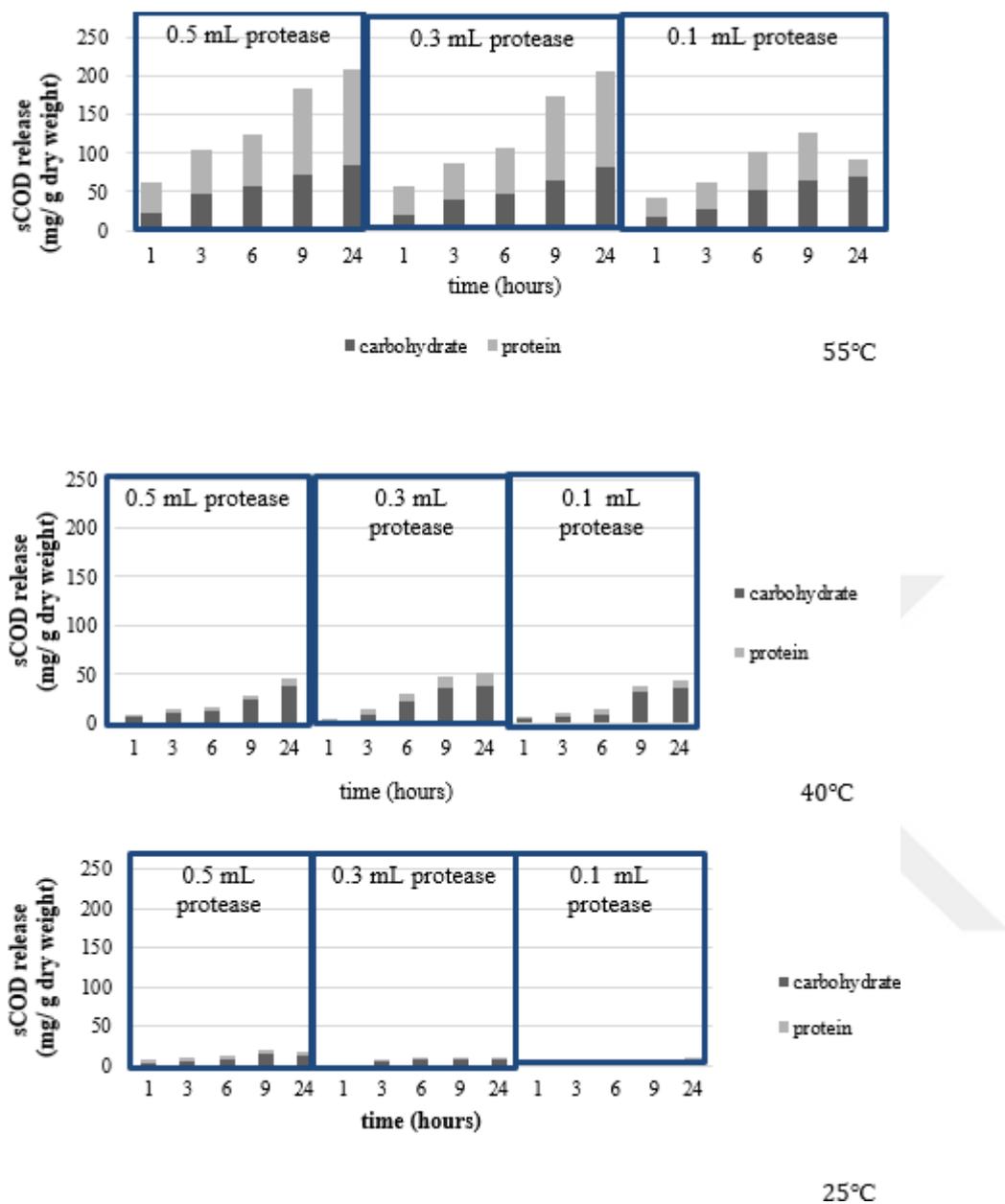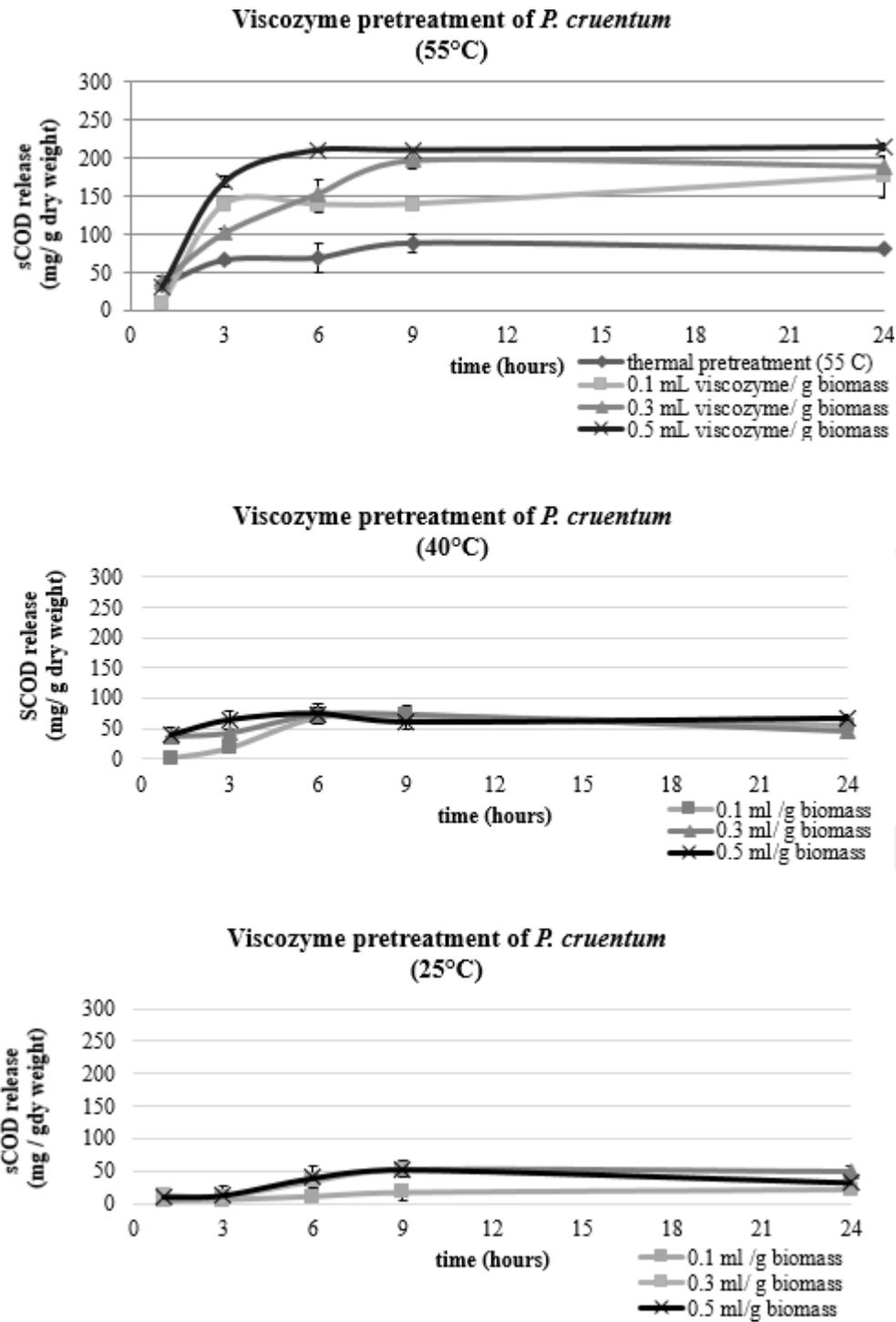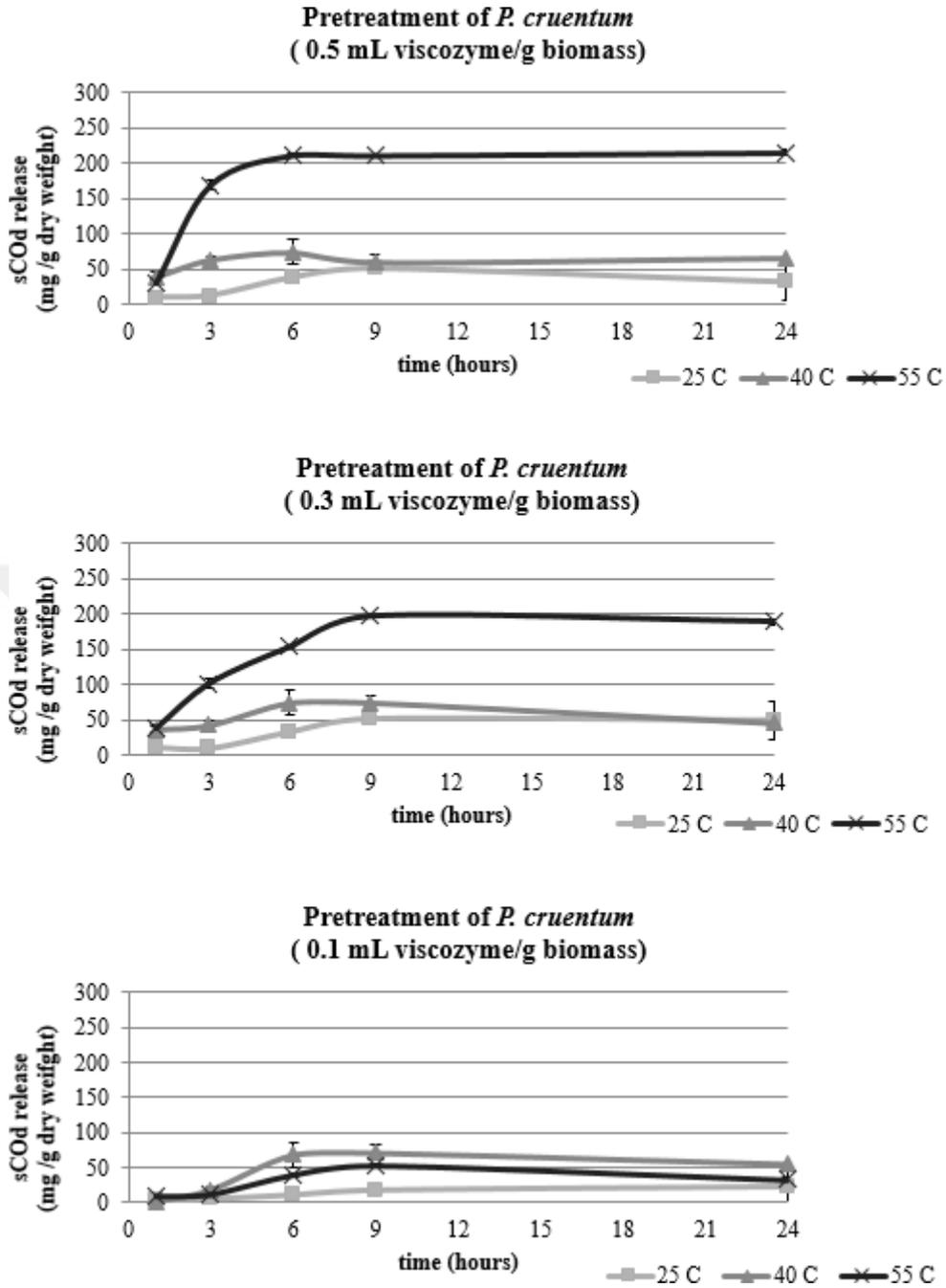
Figure 4.33. Change in soluble carbohydrate and protein content after cellulase pretreatment of *P.cruentum* at various temperatures

4.2.3. Enzymatic Pretreatment of *P. cruentum* byProtease

Protease pretreatment of *P. cruentum* biomass at 55°C, showed highest sCOD increase with 0.5 mL protease dose as 226.4 mg sCOD/g dry weight (solubility increase as 32.2%) in 9 hours (Figure 4.34-4.35). For 0.3 mL protease dose, solubilization increase was observed as 228.8mg sCOD/g dry weight in 24 hours and solubility increase was observed as 32.27%. 0.3 mL protease pretreatment after 24 hours gave similar increase with 0.5 mL protease pretreatment after 9 hours. Moreover, after 9 hours, the difference

was approximately 40 mg sCOD/g dry weight. Highest increase was observed with the highest protease dose, however; after 24 hours, 0.3 mL protease pretreatment showed similar sCOD release as 0.5 mL protease pretreatment. 0.1 mL protease dose was resulted in 97.2 mg sCOD/g dry weight increase (solubility increase as 14.9%) which yielded lower results when compared to 0.3 mL and 0.5 mL protease doses.

Protease pretreatment at 40°C showed lower solubilization increase (less than 40 mg sCOD/g dry weight) for all doses and solubilization after 25°C pretreatment were also found to be lower (Figure 4.33 and Figure 4.34). These results showed that temperature is the most effective parameter for organic matter solubilization and it had a synergistic effect with the protease at high temperatures resulting in higher biomass solubilization. At lower temperatures, protease could not disrupt cell wall of *P. cruentum* biomass, due to its exopolysaccharide cell wall structure. However, at higher temperature, cell wall could be disrupted, and with the help of protease, solubilization of cell components was increased.

As protein and carbohydrate solubilization after pretreatment were examined, it was found out that the ratio of carbohydrate and protein solubilization were similar and as expected, solubilization were highest at 55°C (Figure 4.36).

Pretreatment of *P.cruentum* (protease, 55°C)
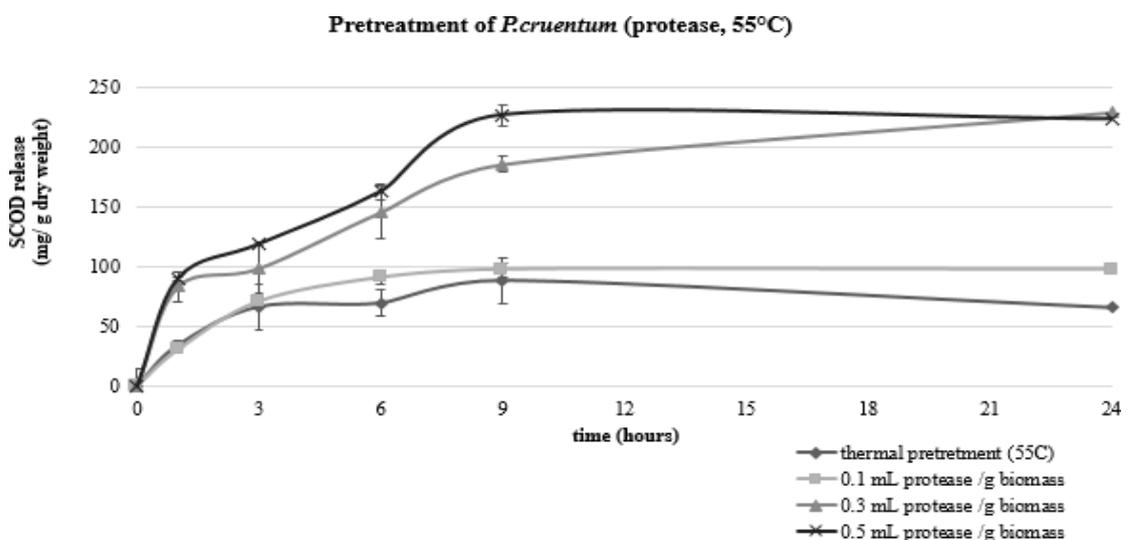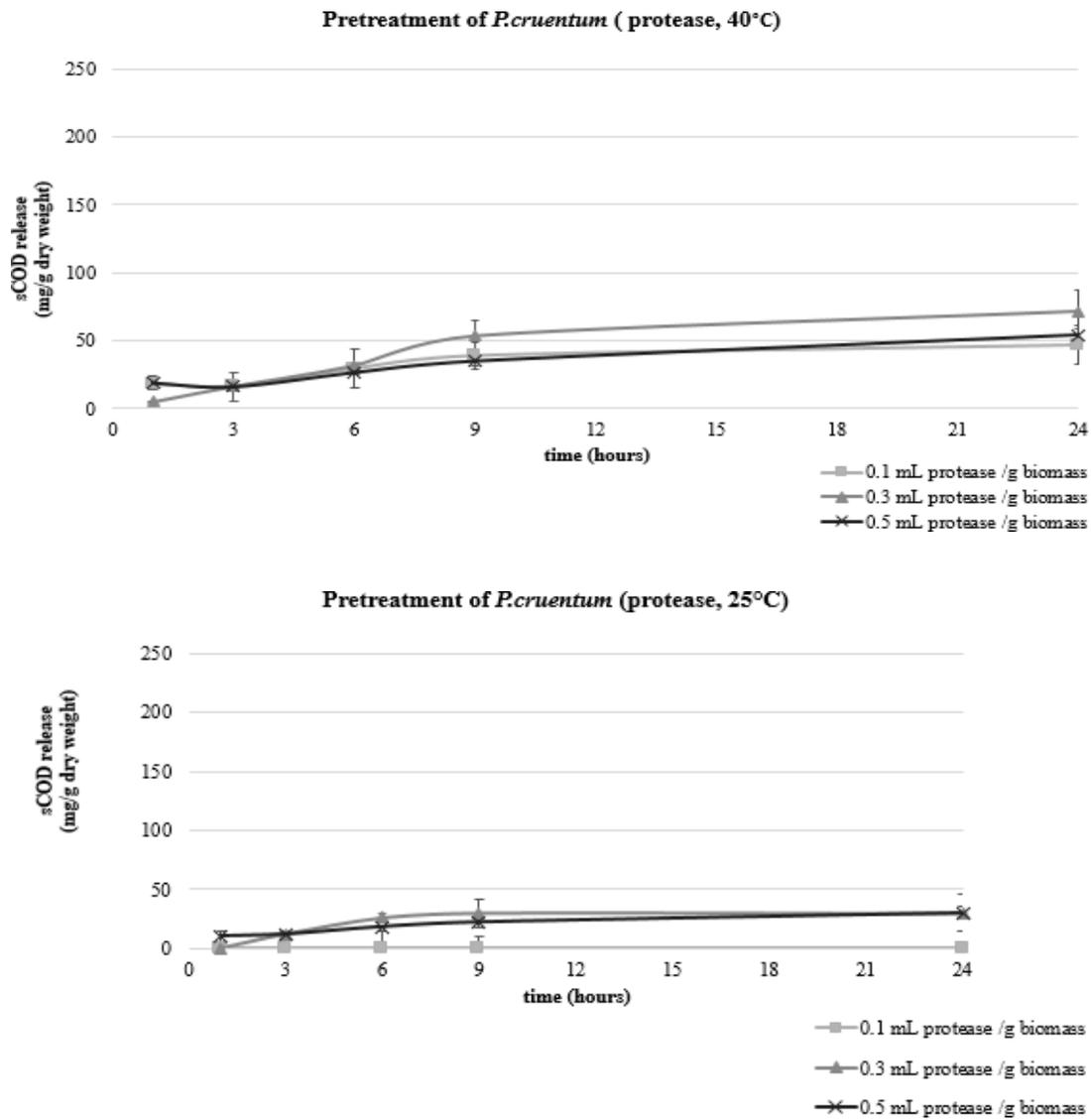
Figure 4.34. The effect of enzyme dose on soluble COD release by protease pretreatment at different temperatures (*P.cruentum*)

Figure 4.35. The effect of temperature on soluble COD release by protease pretreatment at different enzyme doses (*P. cruentum*)
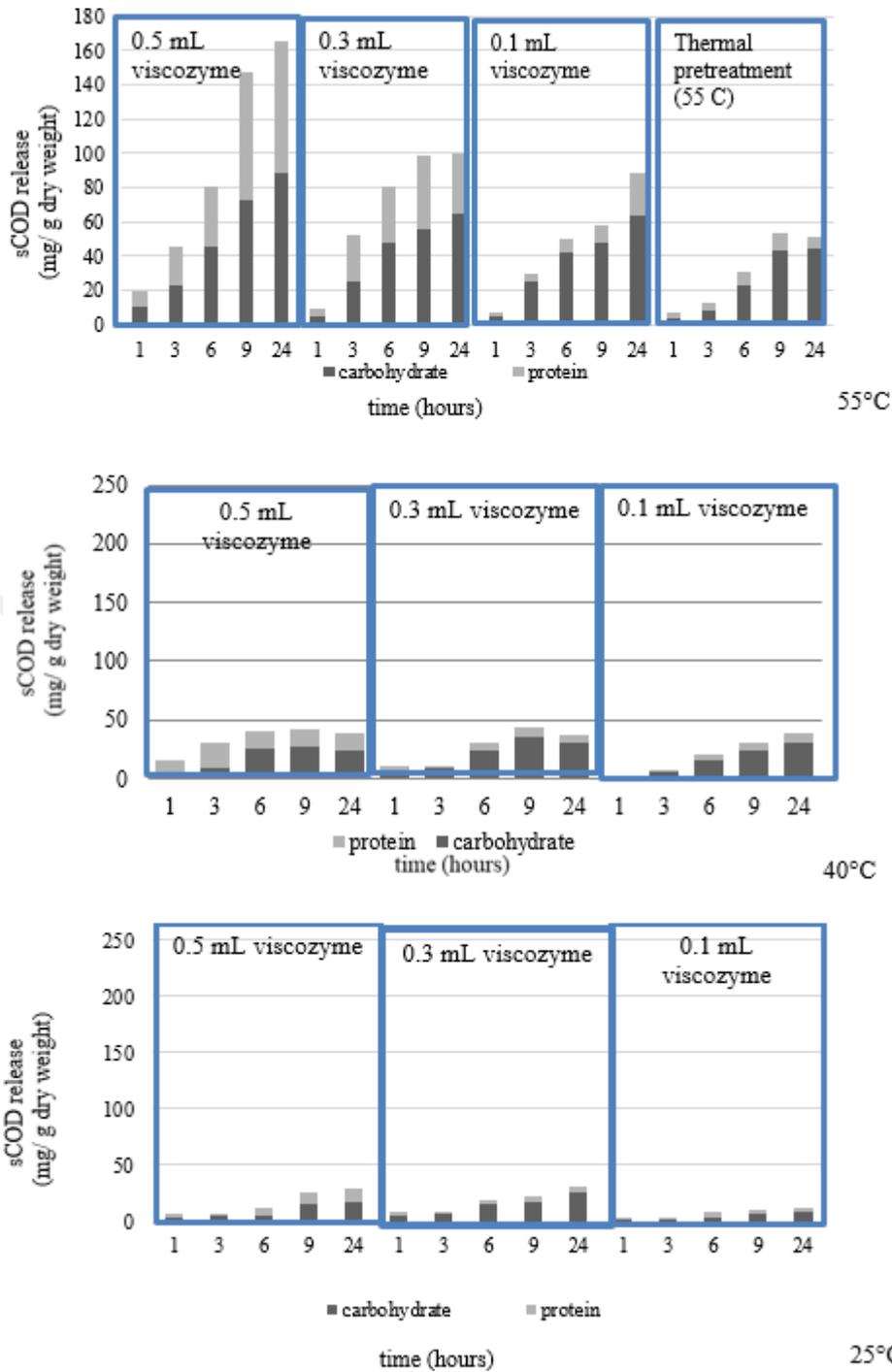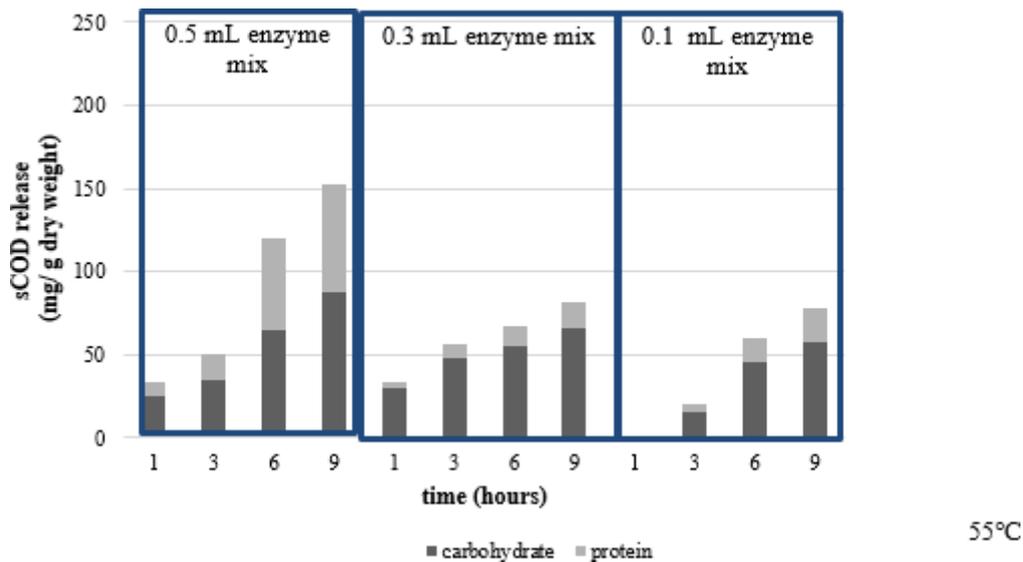
Figure 4.36. Change in soluble carbohydrate and protein content after protease pretreatment of *P. cruentum* at various temperatures

4.2.4. Enzymatic Pretreatment of *P. cruentum* by Viscozyme

Viscozyme pretreatment of *P. cruentum* biomass at 55°C, showed highest solubilization increase at the highest dose (0.5 mL viscozyme) as 211.2 mg sCOD/g dry weight (solubility increase as 32.5%) in 9 hours.197.2 mg sCOD/g dry weight increase (solubility increase as 30.4%) was observed in 6 hours with 0.3 mL viscozyme pretreatment. For 0.1 mL viscozyme dose, 175.6mg sCOD/g dry weight (solubility increase as 27.0%) was observed in 24 hours (Figure 4.37-4.38). It was observed that, after a period of time (24 hours) solubilization reached to similar values for all doses, however, differences were higher until 9 hours. Moreover, the time required to reach maximum solubilization was lower for the highest dose (0.5 mL viscozyme). At lower temperatures (25-40°C), sCOD release values were lower than 100 mg sCOD/ g dry weight. As other enzyme pretreatment results (protease and cellulase pretreatment), temperature was found as the most important parameter However, viscozyme had also impact on biomass solubilization due to its enzymatic mix content including arabinose and xylanase, which are found in *P. cruentum* biomass. Soluble carbohydrate and protein results showed that as dose increases, the protein and carbohydrate solubilization also increase which may be an indicator for an effective pretreatment and release of disrupted intracellular material to the medium (Figure4.39).

Figure 4.37. The effect of enzyme dose on soluble COD release by protease pretreatment at different temperatures(*P.cruentum*)

Figure 4.38. The effect of temperature on soluble COD release by protease pretreatment at different enzyme doses (*P.cruentum*)

Figure 4.39. Change in soluble carbohydrate and protein content after viscozyme pretreatment of *P. cruentum* at various temperatures

4.2.5. Enzymatic Pretreatment of *P. cruentum* by EnzymeMix

After single enzyme pretreatment conditions were investigated, it was concluded that enzymatic pretreatment at 55°C had better solubilization efficiencies. Furthermore, for most of the cases, the maximum solubilization increase was achieved at approximately6-9 hours. So, enzyme mix pretreatment for *P. cruentum* was investigated at 55 °C for 9 hours. According to the results, 198 mg sCOD/g dry weight increase (solubility increase as 30.5%) was achieved for the highest dose in 9 hours. On the other hand, 0.3 mL enzyme mix pretreatment resulted in highest sCOD release as 128 mg sCOD/g dry weight (solubility increase as 19.7%) in 6 hours. 0.1 mL enzyme mix pretreatment achieved highest solubilization increase as 102.6 mg/l (solubility increase 16.1%) after 9 hours (Figure 4.40). Protein and carbohydrate solubilization results showed that protein solubilization was comparatively higher for the highest dose (0.5 mL) than other doses Carbohydrate solubilization was also higher for 0.5 mL enzyme mix pretreatment. This result showed that highest enzyme mix doses provided better cell disruption and release of proteins and carbohydrates (Figure4.41).
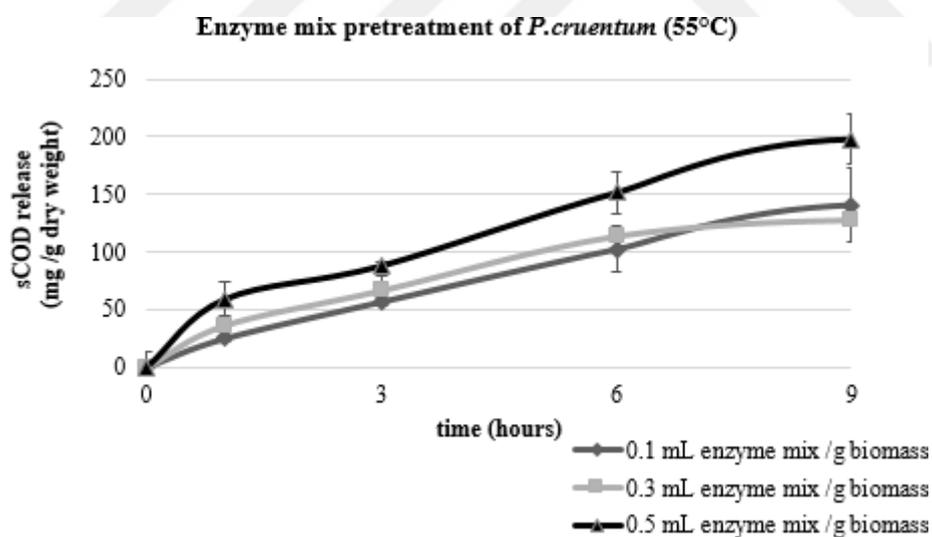
Figure 4.40. Increase in solubilization after enzyme mix pretreatment at 55°C (*P.cruentum*)

Figure 4.41. Change in soluble carbohydrate and protein content after viscozyme pretreatment of *P. cruentum* various temperatures

4.2.6. Optimization Studies for Enzymatic Pretreatment of *P. cruentum* Biomass

In this study, RSM was employed to determine the relationship between pretreatment conditions (enzyme dose, temperature, and time) and solubilization of *P. cruentum* biomass. The ultimate aim was to optimize response to achieve the desired goal which was 'maximize sCOD release'. To analyze experimental data obtained from enzymatic pretreatment studies, Box-Benkhen design was introduced with three levels and three factors for each type of enzyme. Each type of enzyme was analyzed independently since the effect of each enzyme on the *P. cruentum.* biomass was different from each other. The independent variables were chosen as enzyme dose, temperature, and contact time. Therefore, the interactions between these parameters and sCOD release could be determined. The levels of these variables were chosen according to preliminary experiments. Time range were chosen between 3-9 hours instead of 1-24 hours according to experimental results to be able to run RSM.

Figure 4.42 indicates the interaction of time and cellulase dose on sCOD release from *P. cruentum.* As can be seen from the figure, both cellulase dose and time were insignificant for sCOD release due to lack of cellulose in *P.cruentum* biomass.Figure 4.43 shows the interaction of temperature and time on sCOD release. Increasing temperature was very effective in terms of releasing sCOD and the time was found to be less significant. Impact of temperature was independent of enzyme application and high

temperatures were found to be disrupt cell wall of *P. cruentum.* Figure 4.44 indicated that temperature increase from 25°C to 55°C, higher sCOD release was observed. However, increase of cellulase dose did not have any effect on sCOD release.
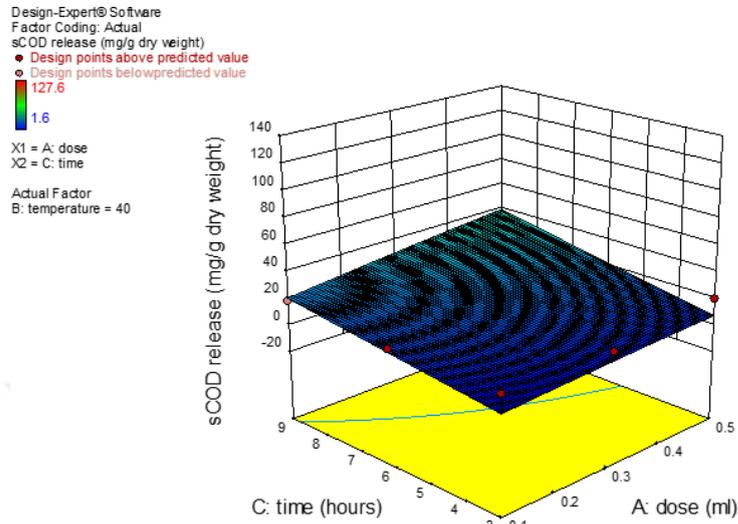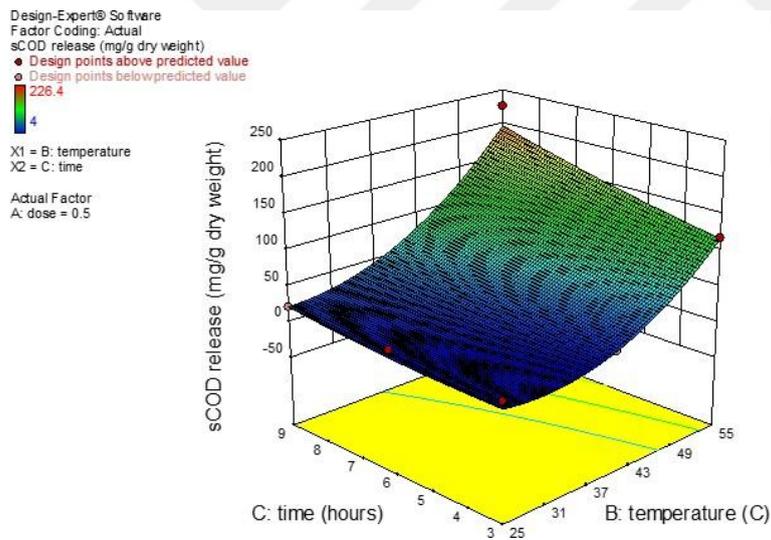

Figure 4.42. The interaction between time and enzyme dose on sCOD release from *P. cruentum* throughout cellulasepretreatment
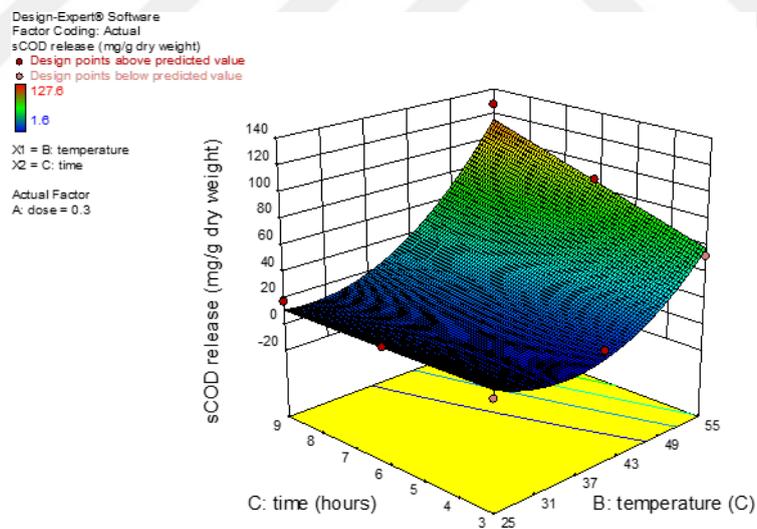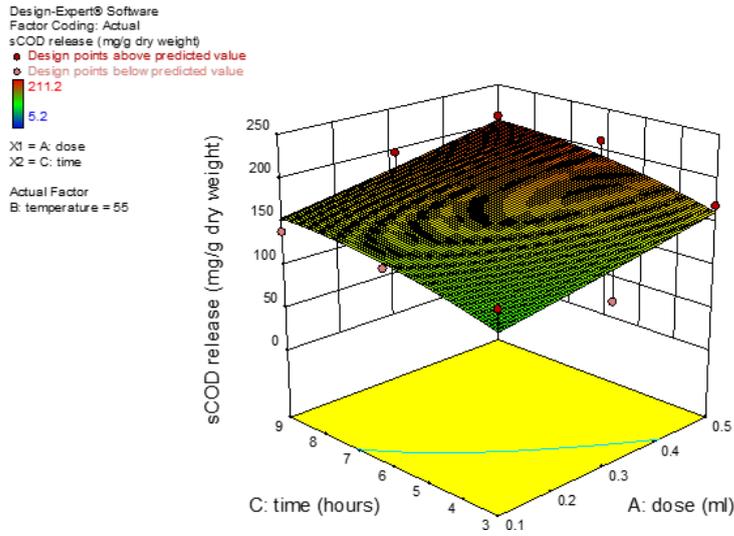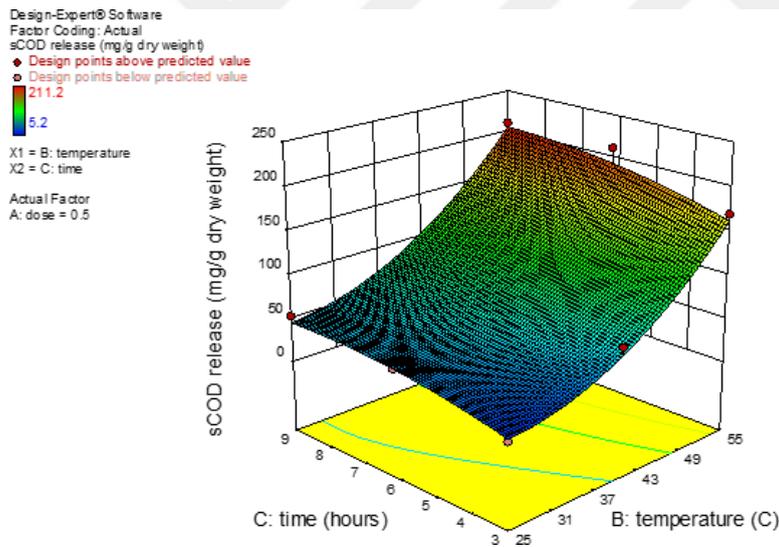

Figure 4.43. The interaction between time and temperature on sCOD release *from P. cruentum* throughout cellulasepretreatment
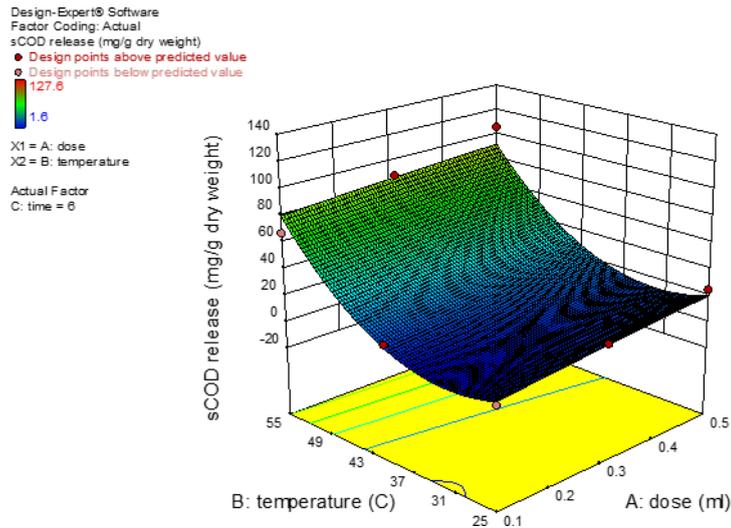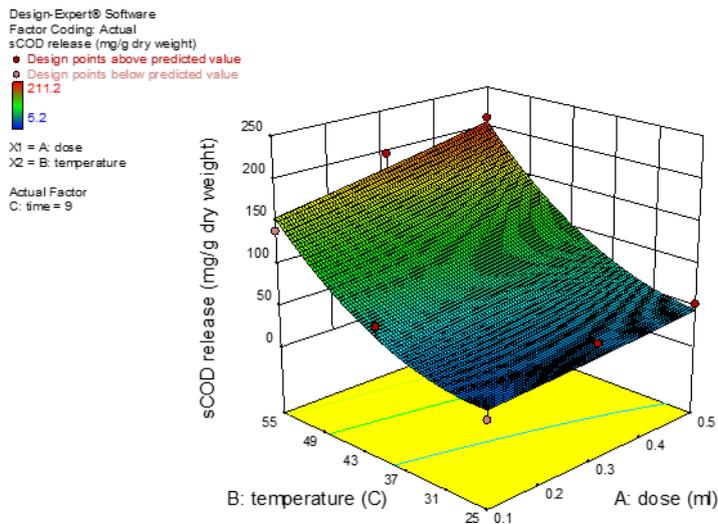
Figure 4.44. The interaction between enzyme dose and temperature on sCOD release from *P. cruentum* throughout cellulase pretreatment

Protease pretreatment of *P. cruentum* showed various responses (sCOD release) to different pretreatment conditions. Figure 4.45 shows that the interaction of protease dose and time. According to the figure, increasing dose did not have a significant effect 3 hours, however; the sCOD become significant as time reached to 9 hours. were found to be not significant. Figure 4.46 shows the interaction between time and temperature. sCOD increase can be observed with the increasing temperature. In case of time, the rate of increase was slower than temperature. Figure 4.47 indicates the interaction between protease dose and temperature and it was clear from the figure that the relationship both of two variables were significant for sCOD release. As temperature and enzyme dose increased, sCOD was also increased and the temperature was observed as more important in terms of release ofsCOD.
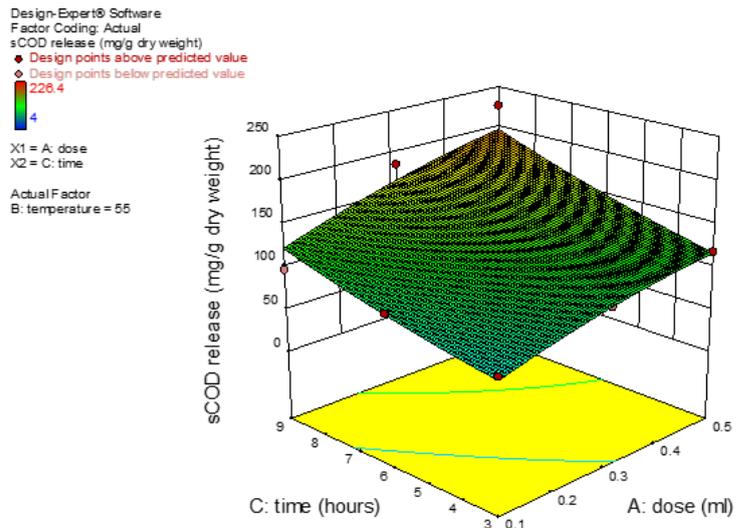
Figure 4.45. The interaction between time and temperature on sCOD release from P. cruentum throughout protease pretreatment

Figure 4.46. The interaction between time and temperature on sCOD release from *P. cruentum* throughout protease pretreatment
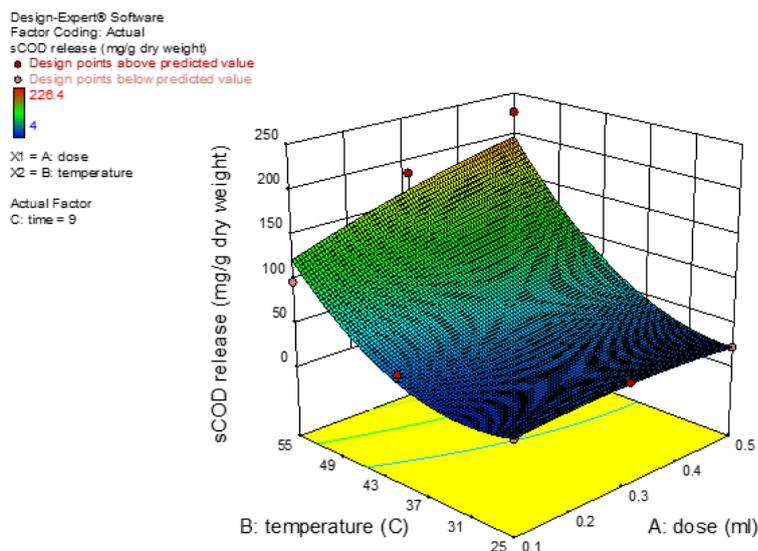
Figure 4.47. The interaction between enzyme dose and temperature on sCOD release from *P. cruentum* throughout protease pretreatment

Pretreatment conditions for viscozyme pretreatment had also variable effects of sCOD release from *P. cruentum*. Figure 4.48 shows time-enzyme dose relationship. According to the figure, the effect of viscozyme dose and time were found to be very small. As the dose increased, the sCOD release also increased to a smaller extent. Same trend was also observed for time. As time increase, there was a small increase in sCOD release. Figure 4.49 shows the interaction between time and temperature. sCOD increase can be observed with the increasing temperature. In case of time, the increase in sCOD followed similar trend, however; the rate of increase was not as sharp as temperature. As the interaction between enzyme dose and temperature effect was investigated (See Figure 4.50), it was clear that the relationship both of two variables were significant for sCOD release. As temperature and enzyme dose increased, sCOD release was also increased and the temperature was observed as more important for release of sCOD.

Figure 4.48. The interaction between time and temperature on sCOD release *from P. cruentum* throughout viscozyme pretreatment

Figure 4.49. The interaction between time and temperature on sCOD release from *P. cruentum* throughout viscozyme pretreatment

Figure 4.50. The interaction between enzyme dose and temperature on sCOD release from *P. cruentum* throughout viscozyme pretreatment

Response surface methodology (RSM) provided models to explain relationship between pretreatment conditions and sCOD release after enzymatic pretreatment. Suggested model should have adjusted R^2 and predicted R^2 in which the difference between them is less than 0.2. lower p-values are also important in terms of choosing model. The RSM results for enzymatic pretreatment of *P. cruentum* suggested that quadratic model was the best to fit with cellulase, protease and viscozyme pretreatment (Table 4.11). As can be seen predicted R^2, adjusted R^2 and p-values proved the significance of the suggested models.

Table 4. 11.Suggested Models and R^2 values

	Cellulase Pretreatment	Protease Pretreatment	Viscozyme Pretreatment
Model type	Quadratic	Quadratic	Quadratic
Sequential p-value	<0.0001	<0.0001	<0.0001
Predicted R^2	0.9246	0.9143	0.9217
Adjusted R^2	0.8671	0.8304	0.8658

Analysis of variance (ANOVA) test was applied in order to determine significance of independent variables. According to ANOVA test, for all enzyme pretreatments, the models were found to be significant. In addition, some independent variables for p-values were less than 0.05 were found to be significant. For cellulase pretreatment, significant independent variables were found to be as time, temperature, the interaction of time-temperature and quadratic effect of temperature (Table 4.12). This result showed that temperature is the main pretreatment condition. For protease pretreatment, time, temperature, dose, dose-temperature, temperature-time, and quadratic effect of temperature were determined as significant (Table 4.13). Finally, for viscozyme pretreatment significant independent variables were found to be as dose, time, temperature, and quadratic effect of temperature (Table4.14).

Table 4. 12. ANOVA for cellulase pretreatment of *P. cruentum*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	35753.80	9	3972.64	36.40	< 0.0001	significant
A-dose	165.62	1	165.62	1.52	0.2348	
B-temperature	20187.10	1	20187.10	184.98	< 0.0001	
C-time	3370.21	1	3370.21	30.88	< 0.0001	
AB	102.08	1	102.08	0.94	0.3470	
AC	40.33	1	40.33	0.37	0.5513	
BC	3113.74	1	3113.74	28.53	< 0.0001	
A^2	4.86	1	4.86	0.045	0.8354	
B^2	8755.44	1	8755.44	80.23	< 0.0001	
C^2	14.42	1	14.42	0.13	0.7208	
Residual	1855.25	17	109.13			
Cor Total	37609.05	26				

Table 4. 13. ANOVA for protease pretreatment of *P. cruentum*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	80817.96	9	8979.77	31.83	< 0.0001	significant
A-dose	2888.00	1	2888.00	10.24	0.0053	
B-temperature	52488.00	1	52488.00	186.05	< 0.0001	
C-time	6681.68	1	6681.68	23.68	0.0001	
AB	4166.41	1	4166.41	14.77	0.0013	
AC	403.68	1	403.68	1.43	0.2480	
BC	2569.61	1	2569.61	9.11	0.0078	
A^2	263.12	1	263.12	0.93	0.3477	
B^2	11220.49	1	11220.49	39.77	< 0.0001	
C^2	136.96	1	136.96	0.49	0.4954	
Residual	4795.95	17	282.11			
Cor Total	85613.91	26				

Table 4. 14.ANOVA for viscozyme pretreatment of *P.cruentum*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.019E+005	9	11316.76	35.01	< 0.0001	significant
A-dose	4512.50	1	4512.50	13.96	0.0016	
B-temperature	83585.98	1	83585.98	258.62	< 0.0001	
C-time	5394.14	1	5394.14	16.69	0.0008	
AB	867.00	1	867.00	2.68	0.1198	
AC	10.45	1	10.45	0.032	0.8594	
BC	147.70	1	147.70	0.46	0.5081	
A^2	8.17	1	8.17	0.025	0.8756	
B^2	6787.21	1	6787.21	21.00	0.0003	
C^2	537.71	1	537.71	1.66	0.2144	
Residual	5494.39	17	323.20			
Cor Total	1.073E+005	26				

The polynomial equations were set by fitting the experimental data with the suggested model and they can be used to predict sCOD release at different pretreatment conditions. The polynomial equations for enzymatic pretreatment of *Scenedesmus sp.* were derived which explained the relationship between sCOD release and input variables as (A: enzyme dose, B: temperature, C:time):

sCOD release by cellulase pretreatment (mg/ g dry weight) $=+226.8+106.38*A+193.78*B+49.46*C+42.17*AB+14.08*AC+2.47*BC-159.70*A^2+157.65*B^2+4.25*C^2$

sCOD release by protease pretreatment (mg/g dry weight) $=+30.39+12.67*A+54.00*B+19.27*C+18.63*AB+5.80*AC+14.63*BC-6.62*A^2+43.24*B^2+4.78*C^2$

sCOD release by viscozyme pretreatment (mg/g dry weight) $=+66.31+15.83*A+68.14*B+17.31*C+8.50*AB+0.93*AC+3.51*BC+1.17*A^2+33.63*B^2-9.47*C^2$

Optimization scenario was conducted to achieve an optimum pretreatment condition for maximized release of sCOD. So that, the best pretreatment conditions were selected for BMP tests. For the optimization scenario, input parameters including dose, temperature, and time were chosen as 'in range' (between experimental values) and ultimate goal was selected as 'maximization of sCOD release', which was the output parameter. Importance of the input parameters and the output parameter were weighted as it. After optimization run, the maximum desirability that can be achieved was '1' (means that the results are the closest to the constraints). The results of the optimization scenario were given in Figure 4.51.

a. Optimum pretreatment conditions for cellulase pretreatment of *P.cruentum*
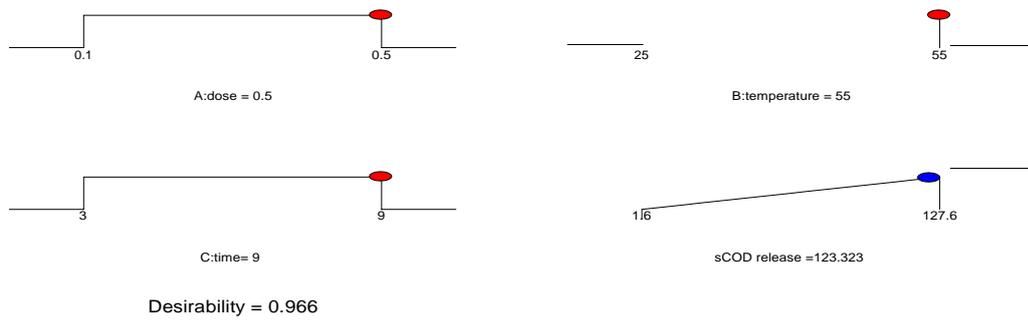
A:dose = 0.5

B:temperature = 55

C:time= 9

sCOD release =123.323

Desirability = 0.966

b. Optimum pretreatment conditions for protease pretreatment of *P.cruentum*

A:dose = 0.5

B:temperature = 55

C:time=9

sCOD release =196.785

Desirability = 0.867

c. Optimum pretreatment conditions for viscozyme pretreatment of*P.cruentum*

A:dose=0.5

B:temperature =55

C:time=9

sCOD release =205.875

Desirability = 0.974
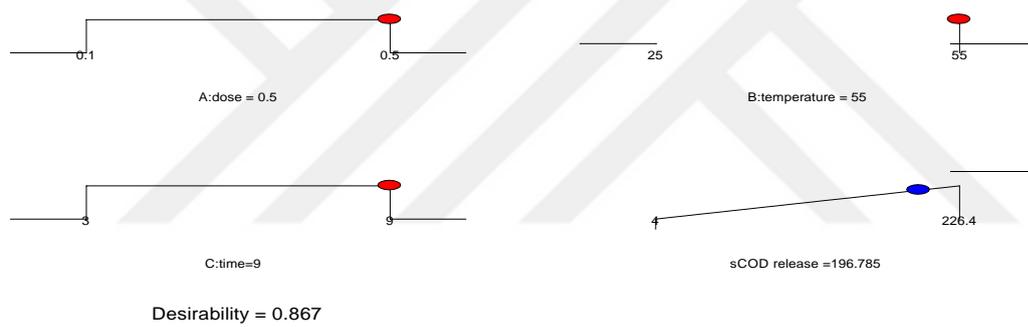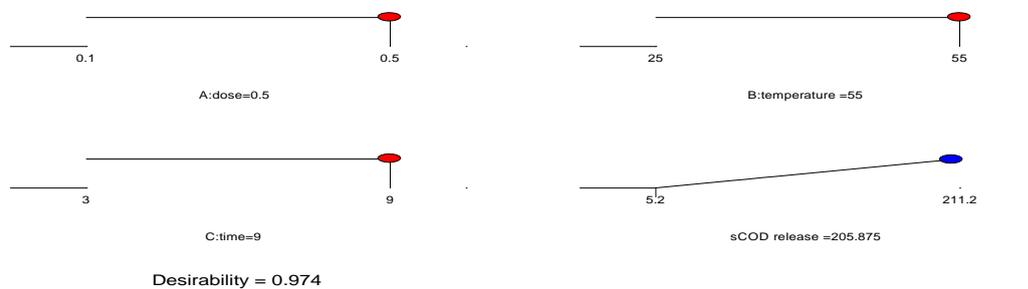
Figure 4.51. Optimized enzymatic pretreatment conditions for *P. cruentum*

As can be seen from optimization results, for all types of enzymes, the optimum conditions for temperature and enzyme dose were found as 55°C and 0.5 mL, respectively. Moreover, optimum time conditions were determined as 9 hours. These results showed that high temperature pretreatment were most effective in terms of sCOD release. Highest enzyme dose was also important parameter for increasing sCOD concentration. According to results of experimental studies and optimization studies by RSM, further experiments for biomethane potentials were decided to be conducted for pretreated *P. cruentum* including pretreatment conditions at 55°C, however; all enzyme doses were chosen for further experiments, to observe the effect of enzyme dose on biomethane production decide whether enzyme dose has a significant effect on methane production.

4.2.7. Comparison of Enzymatic Pretreatment Conditions for *P.cruentum*

In this study, *P. cruentum* biomass was also subjected to cellulase, viscozyme, protease and enzyme mix (protease and viscozyme) After enzymatic pretreatment, it was expected to achieve cell wall disruption and intercellular organic matter solubilization. As can be seen from the Figure 4.52, even after thermal pretreatment, the cell wall of *P. cruentum* was easily disrupted. However, in general, enzymatic pretreatment showed better cell wall disruption and organic matter solubilization. After thermal and enzymatic pretreatment both depigmentation and aggregation were observed, which was stated as the main indicators for cell disruption. In addition to that, after pretreatment at 55°C with and without enzymes, viscosity increase was observed which was an indicator for EPS release to the medium, Since EPS is included in the cell wall of *P. cruentum,* the release to the medium means disruption of cells.

Raw *P. cruentum* biomass
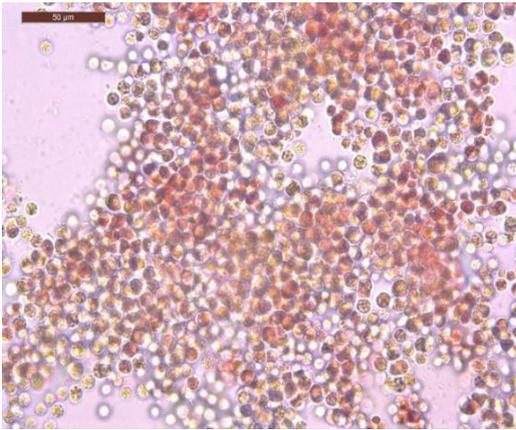
Thermal pretreatment (55°C)
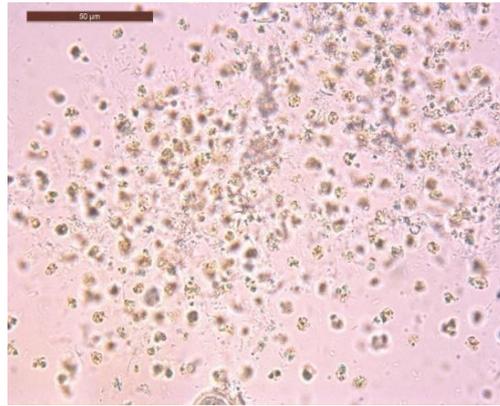
Cellulase pretreatment
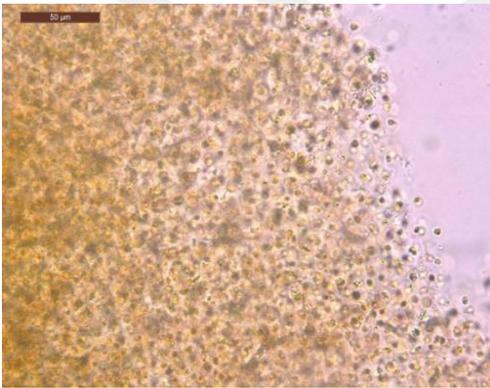
Protease pretreatment
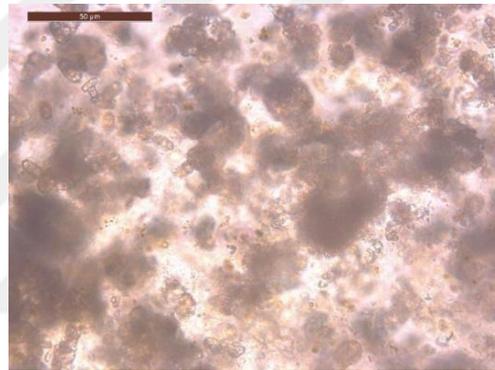
Viscozyme pretreatment
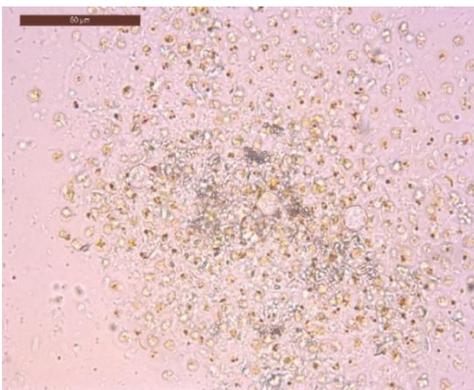
Enzyme mix pretreatment
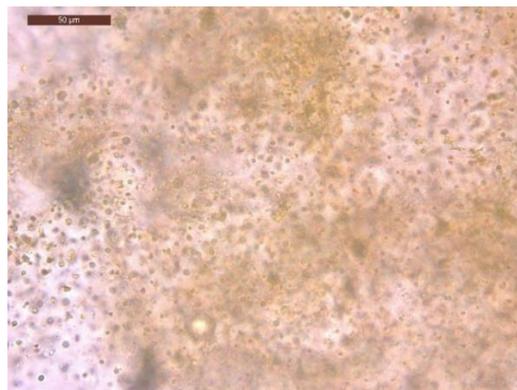
Figure 4.52. Visual interpretation of *P. cruentum* after various pretreatment

According to the enzymatic pretreatment results, the highest solubilization efficiencies were given in Table 4.15. In this study, most efficient pretreatment method for *P. cruentum* was found to be as protease pretreatment at the highest dose (0.5 mL) at 55°C. Viscozyme and enzyme mix pretreatment was also efficient at 55°C. Cellulase pretreatment was not found as effective and most of the sCOD release was due to temperature (55°C) itself. However, low temperature pretreatment had a little effect on biomass solubilization. For this microalgae, temperature was the most effective also in terms of cell wall disruption and temperature was responsible hydrolysis of 13% as itself and it was clear that temperature itself was able to increase biomass solubilization. In terms of protease and carbohydrates solubilization, it was observed that highest carbohydrate solubilization was provided by enzyme mix and viscozyme. Highest protein solubilization was provided by protease pretreatment. In addition, protein release for enzyme mix pretreatment was also high.

Table 4. 15. Highest solubilization efficiencies of *P. cruentum* after different pretreatment conditions

	Cellulase	Protease	Viscozyme	Enzyme mix
Dose	0.5	0.5	0.5	0.5
Temperature	55	55	55	55
Reaction time	9	9	9	9
sCOD release	125.8	226.4	210.8	198
Solubility increase	19.9	34.9	30.5	30.5
Soluble protein increase	21.3	110	50.5	89
Soluble carbohydrate increase	69.7	73.1	82.8	85.2

Dose: mL /g biomass, Temperature (°C), contact time (hours), sCOD release (mg/g dry weight), solubility increase (%), soluble protein release (mg/g dry weight), soluble carbohydrate release (mg/g dry weight))

4.2.8. BMP Results for *P. Cruentum* at Mesophilic and Thermophilic Temperature

BMP assays for both untreated and pretreated *P. cruentum* biomass were applied to determine methane potential in batch reactors and make a discussion about the effect of enzymatic pretreatment. BMP assays were done at both mesophilic and thermophilic digestion, so that, temperature effect on methane production was also investigated in addition to the effect of enzymatic pretreatment.

BMP Results for P. cruentum after Mesophilic Digestion

In mesophilic digestion, untreated *P. cruentum* biomass had a net cumulative methane yield of 130.3 mL CH_4/ gVS. This finding could not be compared with literature results since there is no study related to methane production from *P. cruentum*. Other type of microalgae had methane yields between 24-557 mL CH_4/ gVS. Red seaweeds which are also grown in seawater such as *P. palmate* and *G. verrucosa* have methane yields of 144 mL biogas/ gVS and 279 mL biogas/ gVS, respectively. The main reasons for various methane yields for algae are different cell wall characteristics and cell wall components. In addition, operation parameters in anaerobic digestion are the other important issues.

BMP test results showed that for pretreated biomass showed that enzymatic pretreatment were effective (not for all cases) for improvement of methane production. Cellulase pretreated *P. cruentum* biomass showed various methane yield changes at different cellulase doses. 0.1mL cellulase pretreated biomass showed 3.9% increase in methane yield with a net cumulative methane production of 139.2 mLCH_4/ gVS. However, thermally pretreated biomass showed also 11 % improvement in net methane yield of 147.9 mL CH_4/ gVS. 0.3mL cellulase pretreatment increased 10.4 % of methane yield when compared to untreated biomass and net cumulative methane yield was found to be as 145.3 mL CH_4/ gVS. The net cumulative methane productionafter 0.5 mL cellulase pretreatment was found to be 154.3 mL CH_4/ gVS by 15.2% improvement in methane yield. (Figure 4.53.a) Daily methane production rates also showed that the highest methane production was between 11[th]and 13th days and after then decreases gradually. Untreated biomass showed the highest methane production at

between 11th-13th days as 14.8 mL CH_4/ g VS.d. For cellulase pretreated biomass, results were similar to untreated biomass. These results showed that the contribution of cellulase pretreatment were not significant on methane improvement or increase in daily methane production rates. (Figure4.53.b)
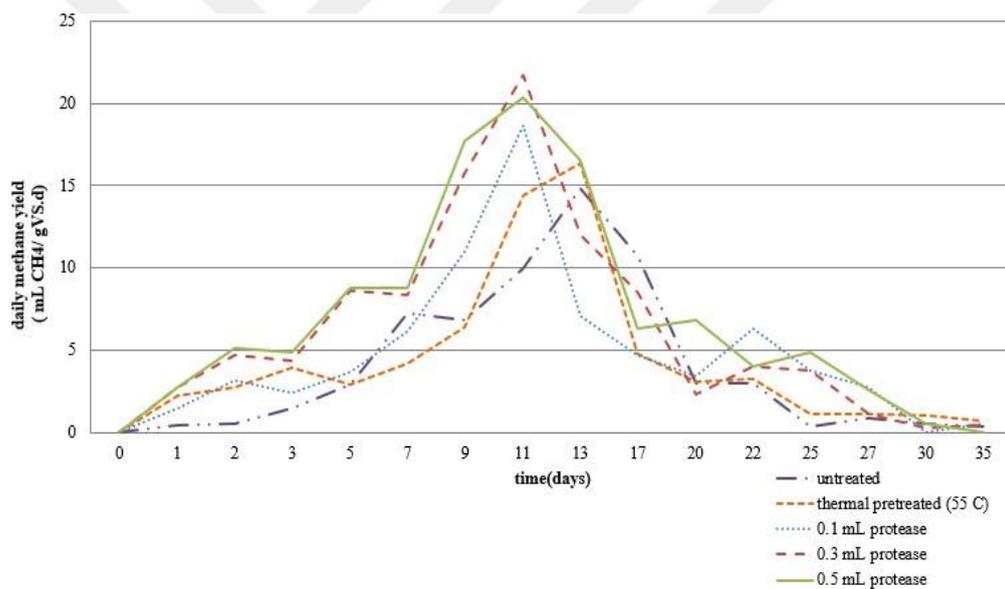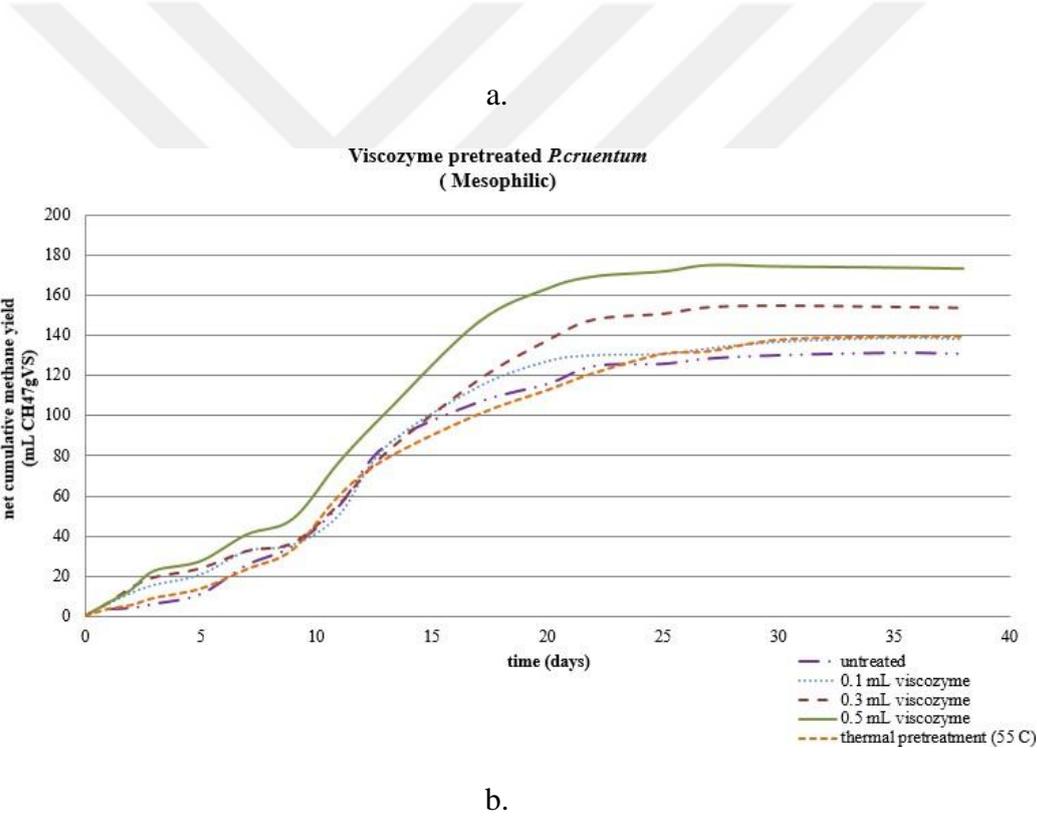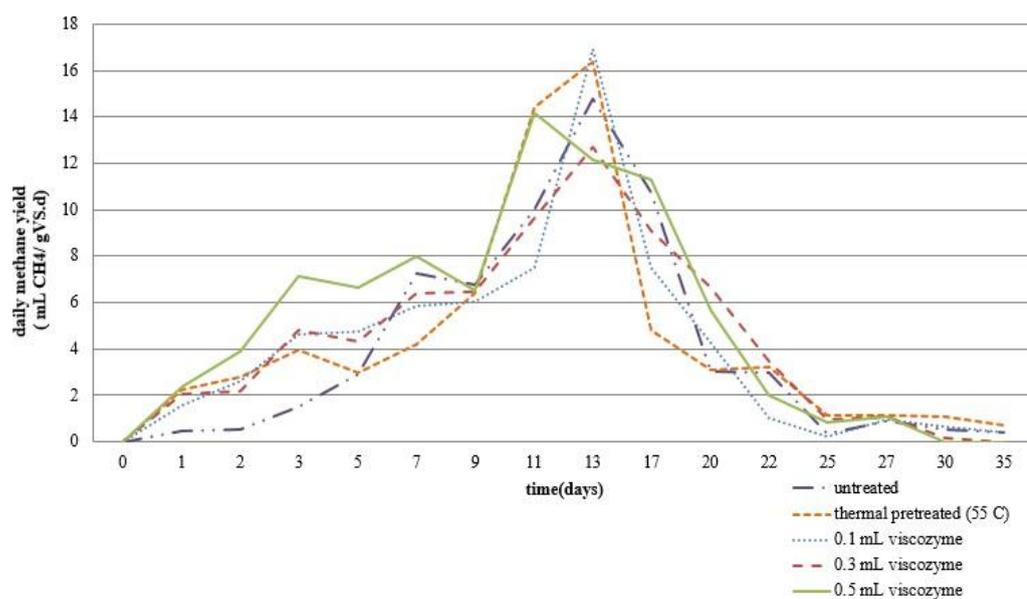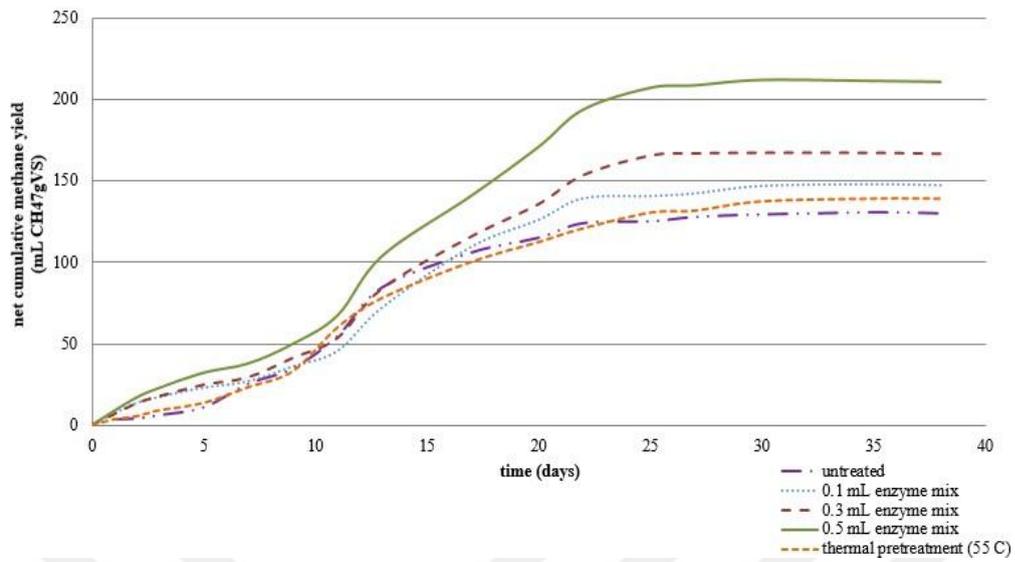
a.

b.
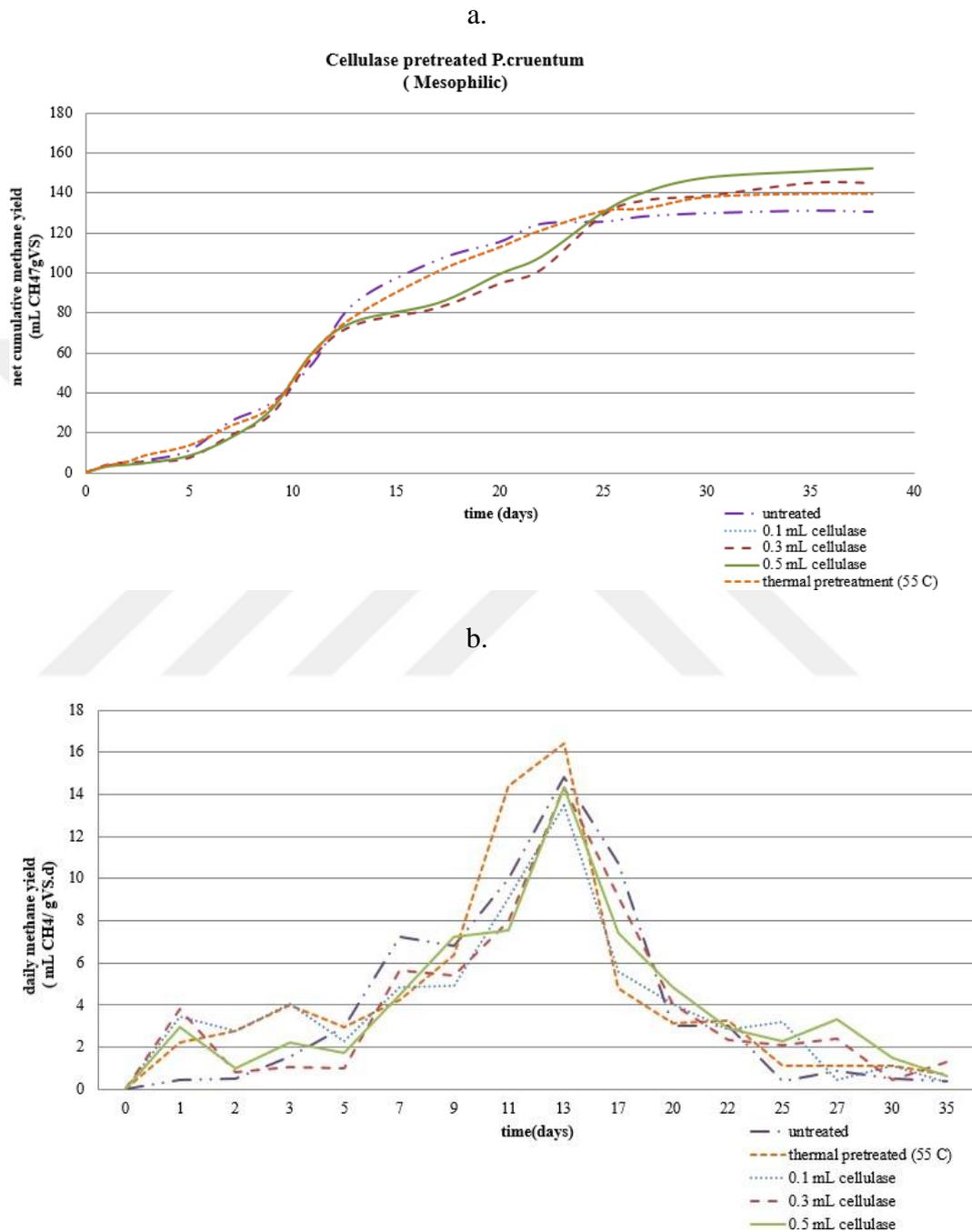
Figure 4.53. Net cumulative methane production and daily methane production of cellulase pretreated *P.cruentum* biomass after mesophilic anaerobic digestion

Protease pretreated biomass showed better methane yield improvement than undertreated and thermally (55°C) pretreated *P. cruentum* biomass. As can be seen from Figure 4.54.a, 0.1 mL protease pretreated biomass showed 26.1% increase in methane yield with a net cumulative methane production of 168.9 mL CH_4/ gVS. 0.3 mL protease pretreatment increased 56% of methane yield when compared to untreated biomass which was 208.8 mL CH_4/ gVS. 0.5 mL protease pretreatment yielded 230 mL CH_4/ gVS (71.7 % improvement). Daily methane production rates also showed that the highest methane production rates for protease pretreated *P. cruentum* biomass was observed between 11th-13th days.0.1 mL protease pretreated biomass showed a daily methane production rate at 11th day as 18.7 mL CH_4/ g VS. d and for 0.3 mL protease pretreated biomass, it was as 15.8 mL CH_4/ g VS.d. However, at 13th day, 0.3 mL protease pretreated biomass produced daily methane of 21.8 mL CH_4/ g VS.d. 0.5 mL pretreated biomass produced 17.7 mL CH_4/ g VS. d at 9th day, and it was increased to 20.3 at 11th day.

The results for the experimental setup showed that at first 15 days, the methane productions were improved by protease enzyme. These findings may be the reason for consumption of proteins degraded by protease enzyme. *P. cruentum* has a protein content of 28-39% (See Table 2.1) and some portion of this biomass could be converted into amino acids during enzymatic pretreatment and after then, they could be easily consumed by anaerobic bacteria. Moreover, cell wall of *P. cruentum* has glycoprotein, which may be also degraded during enzymatic pretreatment. In conclusion, protease pretreatment enhanced methane production from *P. cruentum* biomass and had a significant effect on improving hydrolysis phase.
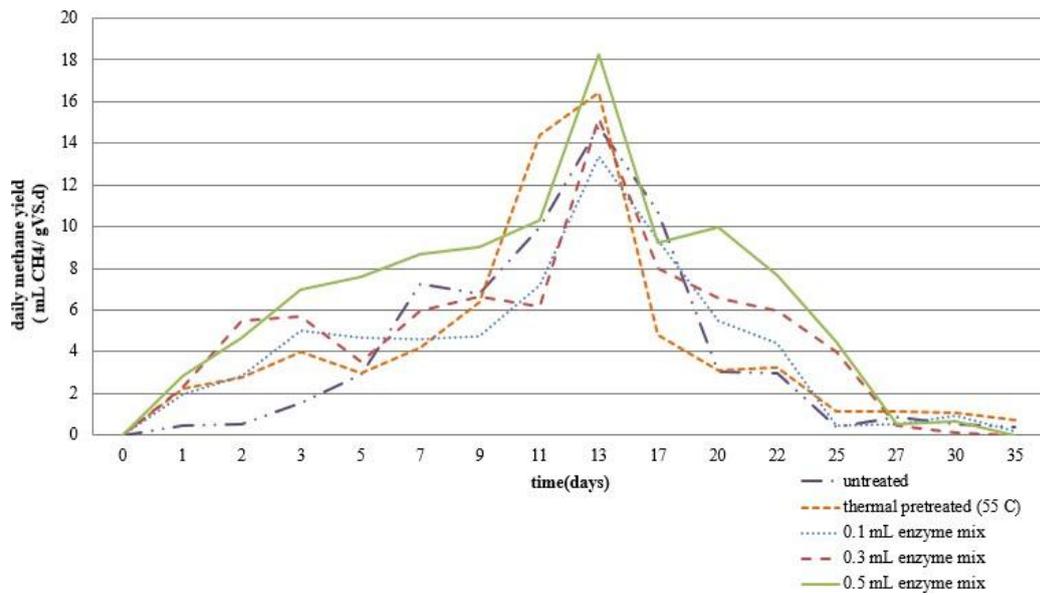
a.
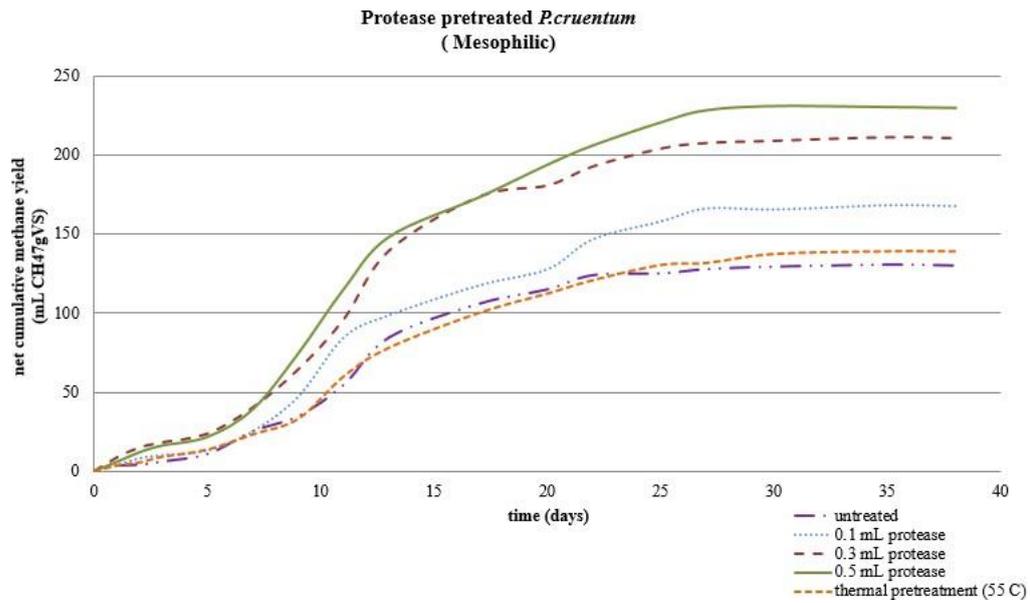
Figure 4.54. Net cumulative methane production and daily methane production of protease pretreated *P. cruentum* biomass after mesophilic anaerobic digestion

Figure 4.55.a shows that viscozyme pretreated biomass had highest methane after 0.5 mL pretreatment with a net methane yield of 173.1 mLCH$_4$/gVS (29.2% improvement). 0.3 mL viscozyme pretreated biomass showed a methane yield of 150.9 mL CH$_4$/ gVS (19.4 % improvement). Daily methane production rates were increased between 11th-13th days and after then decreases gradually. Highest daily methane production rate was observedfor 0.5mLviscozymepretreated biomassas14.2mLCH$_4$/gVS.dat11[th]day.

As untreated biomass, highest methane production rate was similar, however; after 15 days, the methane production rates were higher than raw biomass. In addition, at first 15 days, the methane production was also higher. Viscozyme enzyme is a mix of carbohydrases, and cell wall of *P. cruentum* is composed of different carbohydrates which provided degradation of carbohydrates during enzymatic pretreatment. In addition, the carbohydrate content of this biomass is up to 57% (See Table 2.1), which may be another explanation for the effect of viscozyme pretreatment.

.

a.

b.

Figure 4.55. Net cumulative methane production and daily methane production of viscozyme pretreated *P.cruentum* biomass after mesophilic anaerobic digestion

In literature studies, the effect of enzyme mix pretreatment for organic matter solubilization was found to be effective due to synergistic effect of enzyme mixtures. In this part of the study, for *P. cruentum* biomass, enzyme mix pretreatment was able to increase net methane yields. For instance, 0.5 mL enzyme mix pretreated biomass increased methane yield to 210.7 mL CH_4/gVS (62.3% improvement). It was observed that enzyme mix pretreatment yielded better results than single viscozyme pretreatment, however; as compared to protease pretreatment, the improvement was lower. Figure 4.56.a showed that all enzyme mix pretreated biomass showed better methane yields than untreated and thermally pretreated biomass. 0.1 mL enzyme mix pretreated biomass showed a net cumulative methane yield of 147.7 mL CH_4/gVS and highest daily methane production was observed as 13.4 mL/ CH_4.gVS. d at 13th day. Other enzyme mix pretreatment (0.3 mL and 0.5 mL) showed better daily methane production rates between 15.2 and 18.28 mL/ CH_4.gVS. d at 13th day (Figure 4.56.b). As experimental results showed, enzyme mix pretreatment improved methane production rates at first 10 days. Although, the day at which highest methane production observed did not changed, it helped to increase hydrolysis rate at first 10 days. The same trend could also be observed throughout anaerobicdigestion.

a.
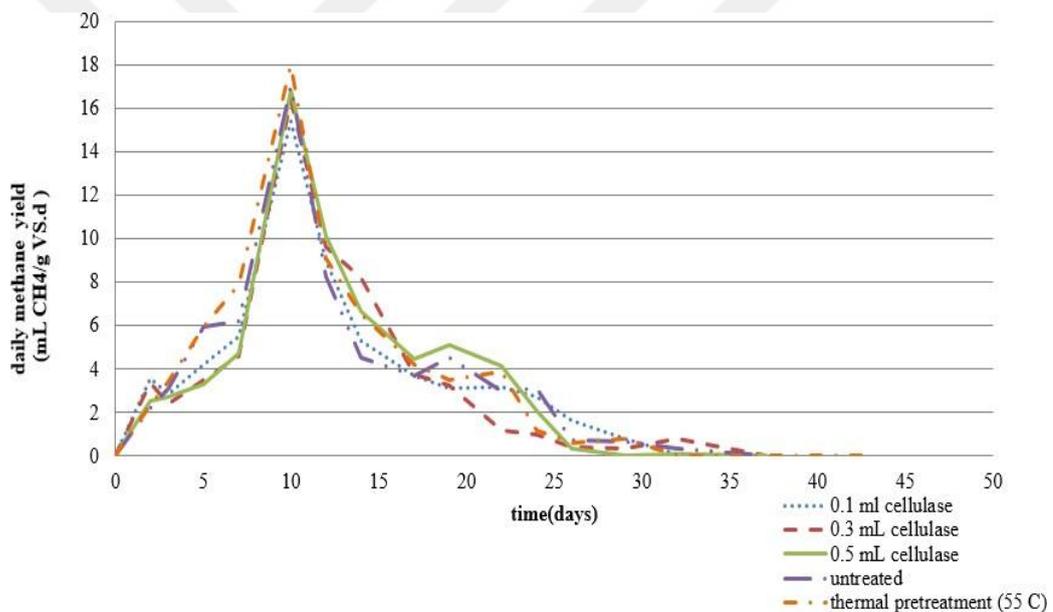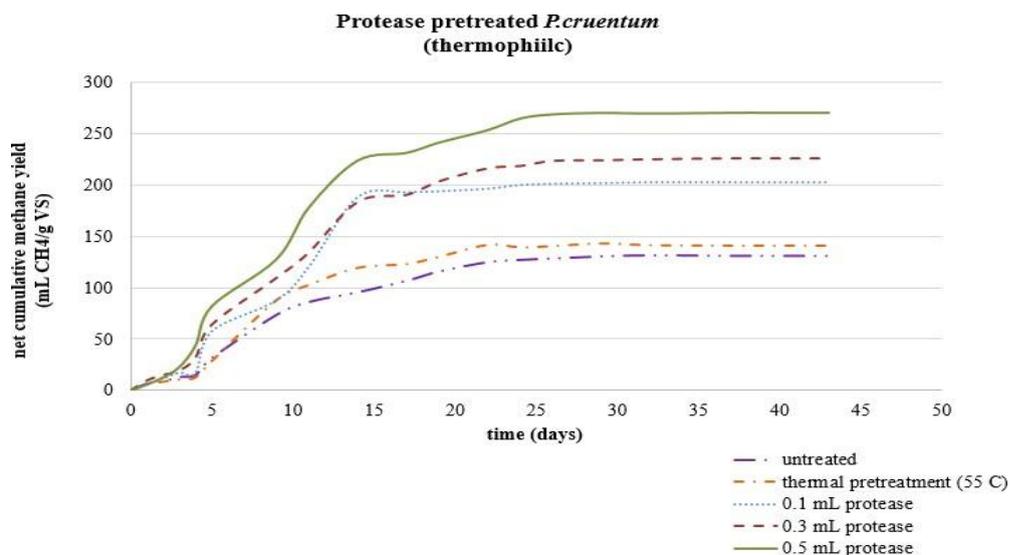
159

Enzyme mix pretreated *P.cruentum*
(Mesophilic)

b.

Figure 4.56. Net cumulative methane production and daily methane production of enzyme mix pretreated *P. cruentum* biomass after mesophilic anaerobic digestion

As the effects of enzymatic pretreatment with various enzymes were compared, it was concluded that pretreated *P. cruentum* biomass showed better methane yields than untreated biomass except for cellulase pretreated biomass and hydrolysis rate was faster than untreated biomass (Table 4.16). As enzyme doses were compared, it was observed that, for most of the cases, lowest dose (0.1 mL) resulted in lowest methane yield improvement. Protease pretreated biomass showed the highest cumulative methane yield and daily methane production improvement. Enzyme mix pretreatment was also found to be effective. As can be seen from Table 4.16, the viscozyme enzyme itself had a limited improvement when compared to protease pretreatment. However, as enzyme mix was used, the improvement was between viscozyme and protease enzyme pretreatment.

Table 4. 16.Comparison of methane yields of pretreated *P.cruentum* with enzymes after mesophilic digestion

Type of pretreatment	Methane yield (mL CH$_4$/ gVS)	Highest daily methane production (mL CH$_4$/ gVS.d)	
Untreated	130.3	14.8	13th day
Thermal (55°C)	147.9	16.4	13th day
Cellulase	139.2-154.3	13.5-14.3	13th day
Protease	168.9-230	15.8-21.3	11th day
Viscozyme	138.4-173.1	14.1-16.9	11th-13th day
Enzyme mix	147.4-210.7	13.4-18.3	13th day

BMP Results for P. cruentum after thermophilic digestion

Thermophilic digestion of *P. cruentum* showed better methane yield potentials than mesophilic digestion for most of the cases (Figure 4.57.a). However, the untreated *Scenedesmus sp.* had a methane yield of 135.2 mL CH_4/ gVS after thermophilic digestion and thermally pretreated biomass had a potential methane yield of 140.7 (4% increase). These results were similar to mesophilic conditions. However, it was observed that, at thermophilic digestion, the hydrolysis rate of *P. cruentum* biomass is higher and highest methane production was at 10th day. Hydrolysis rate of *P. cruentum* biomass at mesophilic temperature was lower and highest methane production was observed at 13th days. So it was concluded that thermophilic digestion of *P. cruentum* did not increase methane yields at the end, however; it decreased the time for anaerobic digestion.

For most of the cases, thermophilic digestion of enzymatically pretreated biomass showed higher methane production yields than untreated biomass. In case of cellulase pretreatment, methane yields were between 152-156 mL CH_4/ gVS which were negligible increase. Due to lack of cellulose in the biomass, these results were expected. They showed similar trend with untreated biomass in terms of cumulative methane production and daily methane production (Figure 4.57.b)

a.

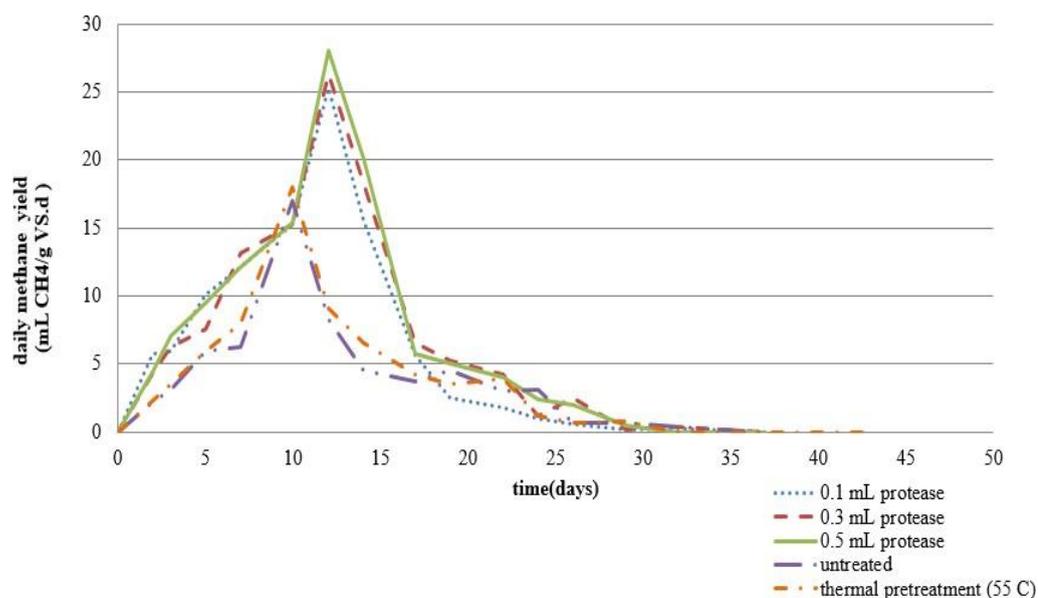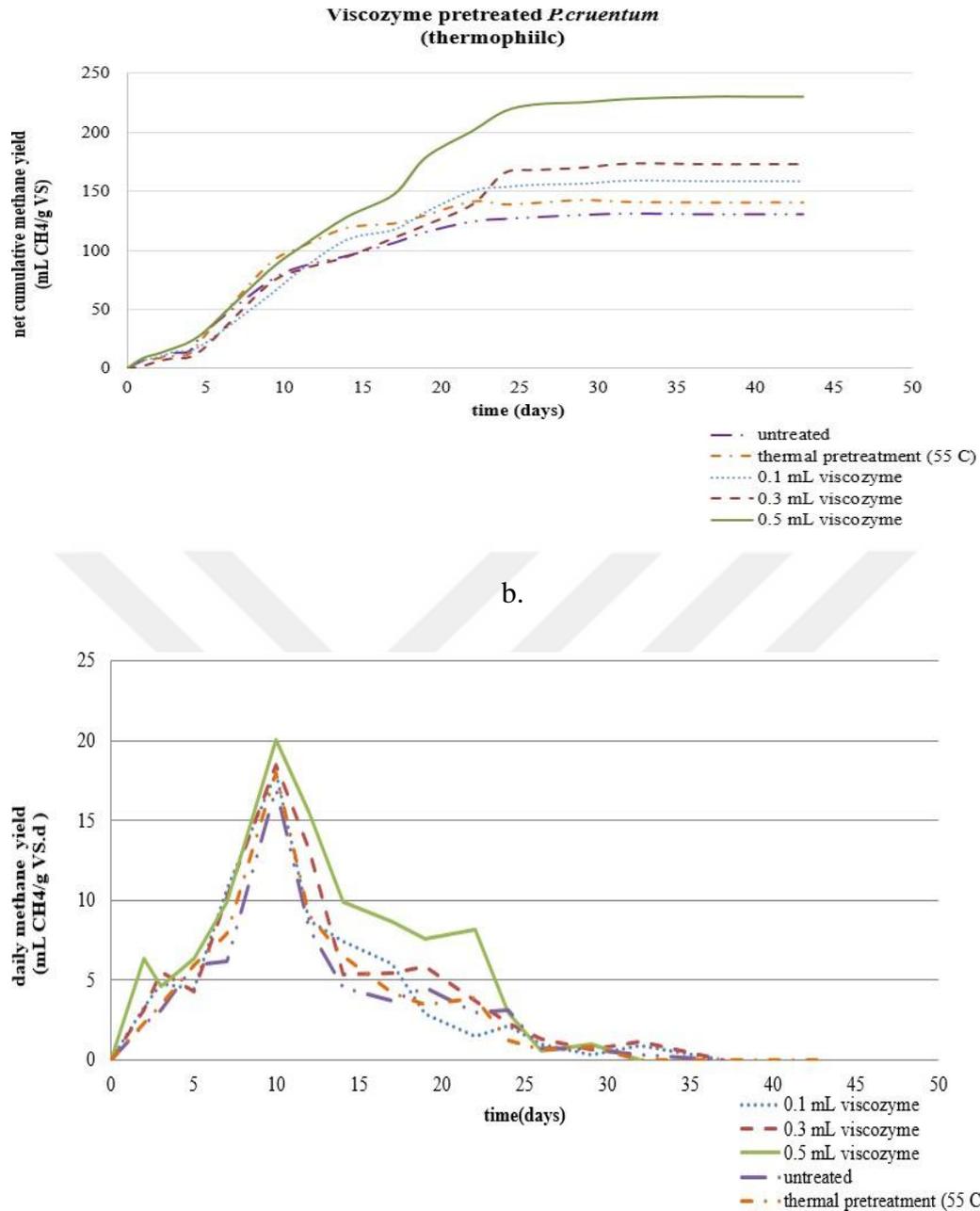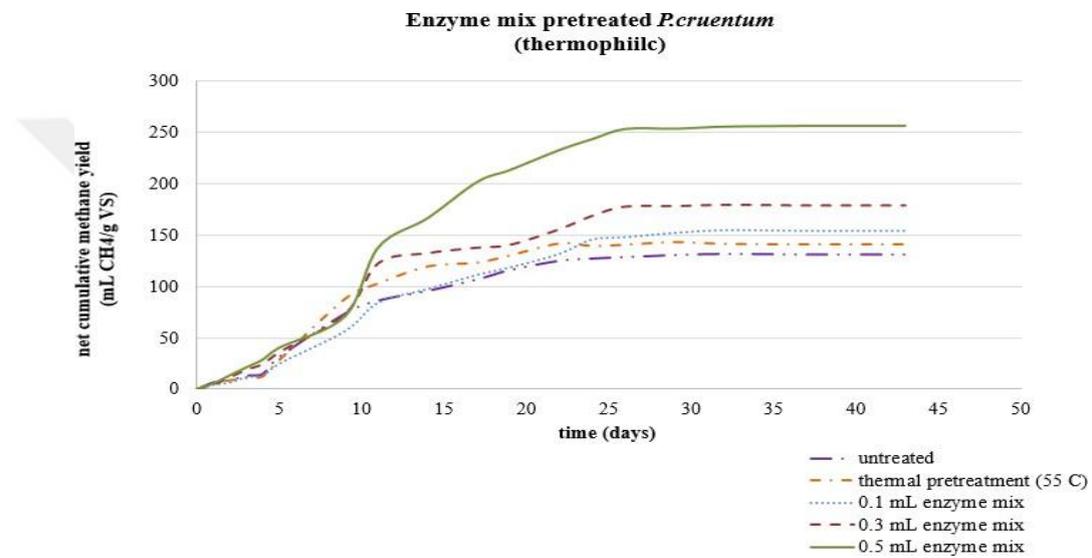Cellulase pretreated *P.cruentum* (thermophiilc)
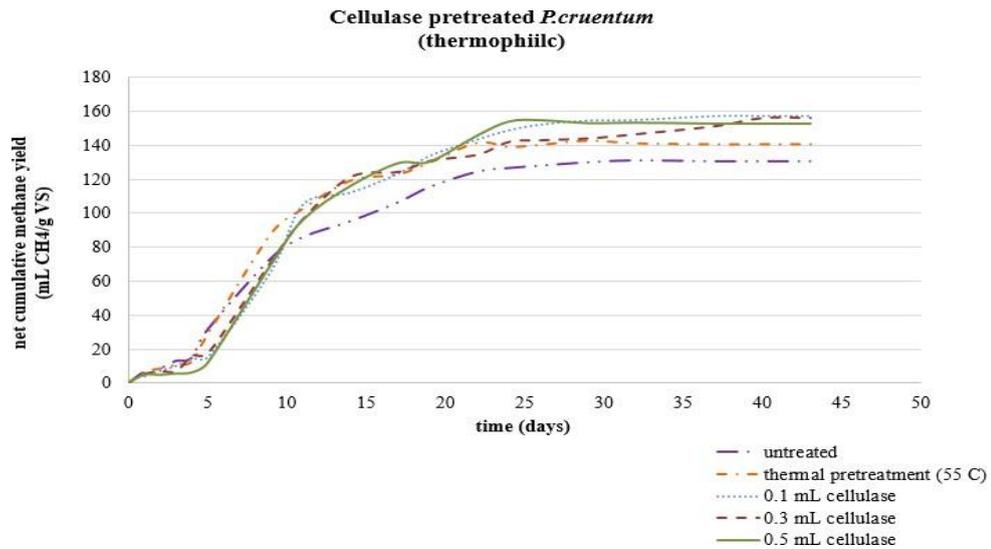
b.

Figure 4.57. Net cumulative methane production and daily methane production of cellulase pretreated *P. cruentum* biomass after thermophilic anaerobic digestion

As at mesophilic digestion, protease pretreated *P. cruentum* biomass at thermophilic digestion showed higher methane yields than untreated biomass. In addition, the net cumulative methane yields were higher than net cumulative methane yields at mesophilic digestion. Highest increase (107%) was observed with 0.5 mL protease dose and net cumulative methane yield was found to be 270 mL CH_4/ gVS. Other protease doses yielded between 202.7-226.3 mL CH_4/ gVS (Figure 4.58.a-b). As can be seen

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from the figures, after 5 days, the methane production rates were increased gradually. Highest daily methane production was observed at 9th day. The rates were similar to each other and they were between 25.3-28 mL CH_4/gVS.d. However, the rate of methane production was significantly higher for 0.5 mL protease pretreated biomass at first 12 days. The highest daily methane production for untreated biomass was observed at 10th day with a rate of 17.3 mL CH_4/gVS.d. As these values are compared, it can be said that with protease pretreatment, the rate was increased and protease pretreatment had improved methane yield potential from *P. cruentum* biomass. For this microalgae, carbohydrates are the main components of the biomass (up to 57%) and carbohydrates. On the other hand, protein portion is observed as 28-39%. Theoretical methane production from proteins (0.851 mL CH_4/ gVS) is higher than carbohydrates (0.415 mL CH_4/ gVS) [45], so degradation of proteins is more advantageous than carbohydrates to have higher methane yields. These findings showed that, application of protease enzyme helped degradation of proteins in the intracellular components and cell wall components of *P. cruentum* and increased methane production. At this point, it is important to notice that, although protease pretreatment was very efficient in terms of improving methane yields, ammonia concentrations should be monitored throughout anaerobic digestion to prevent inhibition of methanogens.

a.

b.

Figure 4.58. Net cumulative methane production and daily methane production of protease pretreated *P. cruentum* biomass after thermophilic anaerobic digestion

Viscozyme pretreated *P. cruentum* biomass, produced higher amount of methane than untreated biomass. The methane yields for viscozyme pretreated biomass were 158.1, 173.2, and 229.8 mL CH_4/ gVS for 0.1 mL, 0.3 mL, and 0.5 mL viscozyme pretreatment, respectively. The methane improvements after viscozyme pretreatment changed between 16.9%-70% after thermophilic digestion (Figure 4.59.a). Highest daily methane yields were observed at 10th day between 18.03 and 20.6 mL CH_4/g VS.d. Due to high carbohydrate content of *P. cruentum* biomass, viscozyme enzyme was effective on enhancement of methane yields from this microalga. As compared to mesophilic digestion, viscozyme pretreated biomass showed higher methane yields after thermophilic digestion. Untreated biomass did not show a significant increase after thermophilic digestion. However, with viscozyme pretreatment, it was observed that methane yields were improved after thermophilic digestion. This may be due the availability of solubilized organic matter in the solution resulting in better degradation rates at thermophilic temperatures.

Figure 4.59. Net cumulative methane production and daily methane production of viscozyme pretreated *P. cruentum* biomass after thermophilic anaerobic digestion

Enzyme mix pretreatment of *P. cruentum* biomass also affect methane production yield in a positive way. The net cumulative methane yield after 0.5 mL enzyme mix pretreatment was 256.8 mL CH_4/ gVS (90 % increase) (Figure 4.60. a-b). The methane yields for 0.1 mL and 0.3 mL enzyme pretreated biomass were 154.4 and 178.4 mL CH_4/ gVS, respectively. It was clear that after application of enzyme mix to *P.cruentum* biomass methane yields gave comparatively better results than viscozyme pretreatment

itself. Enzyme mix pretreatment was able to disrupt both carbohydrate and proteins included in *P. cruentum* biomass and better hydrolysis rates were achieved. Methane production rates were higher than untreated biomass showing better hydrolysis rates. The highest daily methane production rates were observed between 24.2-24.5 mL CH$_4$/ gVS.d at 10th day which was higher than untreated biomass After 9 days, the rate of methane production for enzyme mix pretreated biomass was higher than untreated biomass.

a.

b.
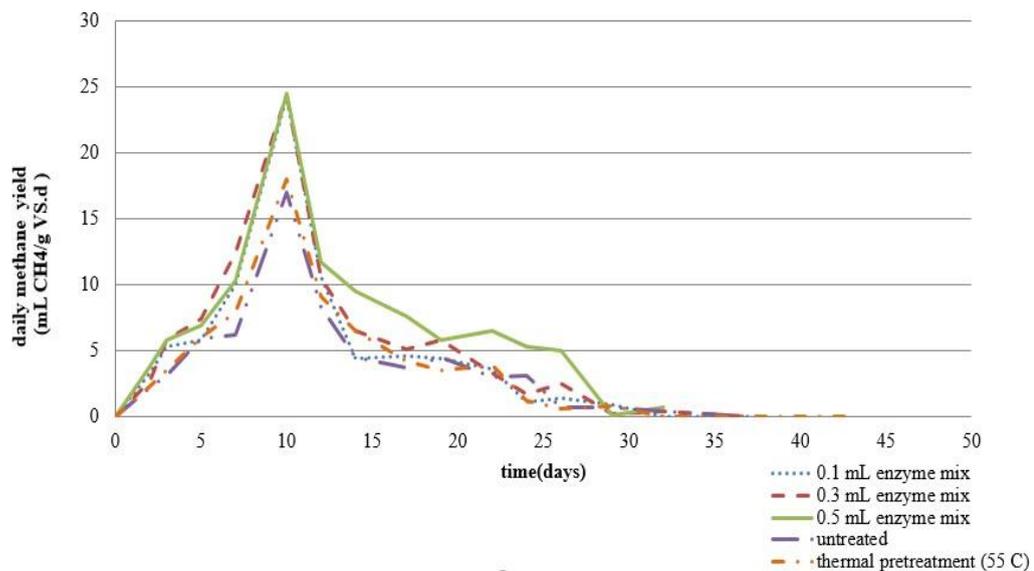
Figure 4.60. Net cumulative methane production of enzymatically pretreated *P. cruentum* biomass after thermophilic anaerobic digestion

As the methane potentials of pretreated biomass and untreated biomass was compared, it was concluded that pretreated *P. cruentum* biomass showed better methane yields than untreated biomass and hydrolysis of pretreated biomass was faster (Table 4.17). Although highest daily methane production of biomass was observed at 9th day for untreated and pretreated biomass, production rate of pretreated biomass was. Protease pretreated biomass showed the highest cumulative methane yield and daily methane production improvement when compared to other enzymes. The viscozyme enzyme itself had a limited improvement when compared to protease pretreatment. However, as enzyme mix was used, the improvement was between viscozyme and protease enzyme pretreatment. For this case, protease was found to be most effective enzyme for methane improvement. During anaerobic digestion, degradation of proteins is more complex than carbohydrates and use of protease enzyme for pretreatment improved the rate of hydrolysis.

Table 4. 17. Comparison of methane yields of pretreated *P. cruentum* with enzymes after thermophilicdigestion

Type of pretreatment	Methane yield (mL CH_4/ gVS)	Highest daily methane production (mL CH_4/ gVS.d)	
Untreated	135.7	17.03	10th day
Thermal (55°C)	140.7	18.1	10th day
Cellulase	152.2-156.9	15.4-18.8	10th day
Protease	202.7-270	25.3-28.0	12th day
Viscozyme	158.1-229.8	18.03-20.06	10th day
Enzyme mix	154.4-256.8	24.2-24.5	10th day

Kinetics of the Methane Production from *P. cruentum* Biomass

In this thesis, two different models were used to model experimental data and determine kinetic parameters for anaerobic digestion of *P. cruentum* biomass (for both untreated and treated biomass). Determination of hydrolysis rate constant (k) is important to

understand the biodegradability of the substrates used in anaerobic digestion. The results of the kinetic model showed that hydrolysis rate (k) values were found to be between 0.015-0.041 day^{-1} and 0.060-0.078day^{-1} for mesophilic and thermophilic digestion. R^2 values are more than 0.94 (Table 4.18). Hydrolysis rates for thermophilic digestion were found to be higher than mesophilic digestion results which showed that better hydrolysis rates were achieved during thermophilic digestion. As *Scenedesmus sp.*, in this part, it was observed that the first order model did not fit well with the experimental results. (See Appendix 1-5,1-6).

Table 4. 18. Results of first order kinetic model for *P. cruentum*

	Mesophilic				Thermophilic			
	ym	k	R^2	MSE	ym	k	R^2	MSE
Untreated	314.7	0.015	0.963	9.16	149.2	0.060	0.960	10.53
Thermal (55°C)	262.9	0.024	0.960	11.17	155.6	0.065	0.955	11.96
Cellulase (0.1 mL)	229.1	0.016	0.958	11.10	180.3	0.062	0.957	13.06
Cellulase (0.3 mL)	381.5	0.015	0.967	9.87	172.6	0.065	0.956	12.69
Cellulase (0.5 mL)	316.7	0.016	0.966	10.54	175.9	0.066	0.952	13.60
Protease (0.1 mL)	269.5	0.030	0.956	13.76	218.9	0.063	0.932	21.50
Protease (0.3 mL)	307.7	0.037	0.945	20.05	248.6	0.077	0.959	18.06
Protease (0.5 mL)	323.0	0.040	0.960	18.03	297.3	0.078	0.970	18.43
Viscozyme (0.1 mL)	210.1	0.035	0.931	13.85	184.6	0.060	0.970	10.79
Viscozyme (0.3 mL)	242.0	0.033	0.945	14.31	226.0	0.062	0.959	13.89
Viscozyme (0.5 mL)	244.1	0.041	0.946	16.07	292.5	0.064	0.970	15.48
Enzyme mix (0.1 mL)	239.4	0.031	0.952	12.63	187.5	0.071	0.972	10.22

Enzyme mix (0.3 mL)	275.7	0.030	0.955	13.89	206.3	0.070	0.972	11.59
Enzyme mix (0.5 mL)	354.8	0.029	0.962	16.01	302.8	0.077	0.976	15.49

The findings (Table 4.19) from modified Gompertz model showed that in general, the model was able to predict ultimate methane yields compatible with experimental results. In thermophilic digestion, time required for lag phase were found to be lower than mesophilic digestion and maximum methane production rates were higher than mesophilic digestion that resulted in higher methane yields. Additionally, enzymatic pretreatment showed lower lag time and higher maximum methane production rates than untreated biomass for both mesophilic and thermophilic conditions (See Appendix 1-7,1-8)

Table 4. 19. Results of modified Gompertz model for *P. cruentum*

	Mesophilic					Thermophilic				
	ym	**Ue**	**λ**	**R^2**	**MSE**	**ym**	**Ue**	**λ**	**R^2**	**MSE**
Untreated (55°C)	130.4	6.25	4.14	0.987	5.4	129.8	9.88	2.49	0.993	4.1
Thermal	146.6	8.14	4.07	0.991	5.2	138.1	12.41	2.53	0.994	4.1
Cellulase (0.1 mL)	146.8	7.52	4.51	0.991	3.7	154.2	11.50	2.63	0.989	6.5
Cellulase (0.3 mL)	155.2	6.76	3.93	0.990	5.4	147.9	11.91	2.59	0.987	6.8
Cellulase (0.5 mL)	157.1	7.59	3.81	0.991	5.4	153.2	11.79	2.63	0.990	6.0
Protease (0.1mL)	170.8	10.17	3.93	0.992	5.6	202.7	19.99	2.10	0.989	8.3
Protease (0.3 mL)	214.1	14.54	3.98	0.994	5.9	222.2	19.72	2.28	0.993	7.2
Protease (0.5 mL)	232.1	14.97	3.56	0.997	4.7	270.2	19.99	1.48	0.994	7.6
Viscozyme (0.1 mL)	141.7	9.75	4.78	0.987	6.1	161.2	9.80	2.31	0.993	5.2

Viscozyme (0.3 mL)	163.6	9.34	3.92	0.987	6.8	180.0	8.85	2.43	0.978	10.2
Viscozyme (0.5 mL)	182.0	11.43	3.63	0.989	7.1	238.2	11.84	2.26	0.990	8.6
Enzyme mix (0.1 mL)	155.8	8.47	3.78	0.990	5.7	157.3	8.74	2.11	0.991	5.5
Enzyme mix (0.3 mL)	177.1	9.52	3.74	0.992	5.9	178.3	11.31	1.95	0.993	5.8
Enzyme mix (0.5 mL)	226.5	11.26	3.34	0.993	6.7	262.2	15.00	1.84	0.996	5.5

As first order model and modified Gompertz model, compared, first order kinetic model did not fit as well as Modified Gompertz model due to natural behavior of anaerobic digestion. The same trend was observed for also *Scenedesmus sp.* Furthermore, first order model was not able to determine lag phase and methane production rate of the digestion which can be considered most important parameters of anaerobic digestion.

4.2.9. Energy Assessment Results

The energy assessment results of this study showed that energy ratios for pretreated P. *cruentum* biomass were 0.69-1.05 and 0.54-1.04 for mesophilic digestion and thermophilic digestion, respectively (Table 4.20). The values mean that the energy consumed throughout enzymatic pretreatment were lower/ equal than energy produced as methane. Lowest energy ratio was obtained with protease pretreated biomass (0.5 mL enzyme mix) for both mesophilic and thermophilic digestion. The energy ratios for thermophilic digestion were lower than mesophilic digestion. However, heating requirements of thermophilic digestion were not taken into account in this study to simplify results and it should be considered. However, for both cases, enzymatic pretreatment of *P. cruentum* was found to have a positive energy balances for most of the cases.

Table 4.20. Result of Energy Assessment of *P.cruentum* after various enzymatic pretreatment

	Pretreatment condition	Mesophilic Ein/Eout	Thermophilic Ein/Eout
Untreated	-	-	-
Thermal	55 C	0.99	1.04
Cellulase	0.1 mL dose	1.05	0.93
	0.3 mL dose	0.99	0.94
	0.5 mL dose	0.95	0.96
Protease	0.1 mL dose	0.86	0.72
	0.3 mL dose	0.70	0.64
	0.5 ml dose	0.63	0.54
Viscozyme	0.1 mL dose	1.05	0.92
	0.3 mL dose	0.91	0.84
	0.5 mL dose	0.84	0.64
Enzyme Mix	0.1 mL dose	0.99	0.95
	0.3 mL dose	0.88	0.82
	0.5 mL dose	0.69	0.57

4.3. Discussion of theResults

Anaerobic digestion of microalgae biomass is a sustainable method to provide bioenergy. Higher energy yield, no biomass extraction requirement, possibility of digestion, possibility of upgrading such as CO_2 bio sequestration, co-digestion, and easiness for integration to biorefinery processes are some of the advantages of anaerobic digestion over other biofuel production processes from microalgae.

Biogas production is influenced by several parameters such as microalgae composition (carbohydrate, protein, and lipid) and cell wall structure. Microalgae composition is affected by cultivation conditions including light intensity and cultivation media. Cell wall structure is strain specific and plays an important role for anaerobic digestion. Microalgae having cell wall characteristics including cellulose, hemicellulose, mannan, xylan and other complex biopolymers such as algeanan are very resistant to degradation by anaerobic microorganisms and they hinder the release of macromolecules in the innercell. During anaerobic digestion, hydrolytic bacteria release various exo enzymes

to disrupt cell wall of microalgae and longer time periods may be required for disruption of cell wall and hydrolysis of intercellular compounds. To decrease time for hydrolysis and increase bioavailability of microalgae biomass to the hydrolytic bacteria, pretreatment methods can be applied before anaerobic digestion. Pretreatment provides disruption/weaken of complex materials retained in the cell wall and degrade complex molecules into monomers, so that; hydrolysis stage of anaerobic digestion is enhanced and consequent methane production could be achieved.

Pretreatment methods are classified as mechanical, thermal, chemical, biological, or combined. Thermal and mechanical methods have commonly e negative energy balance due to higher heating and electricity requirements. Chemical methods induce corrosion and production of inhibitory compounds. Among pretreatment methods, biological pretreatment (enzymatic pretreatment) is considered as promising due to being low energy demand (low temperature and atmospheric pressure), no corrosion and higher selectivity of enzymes.

In recent years, there is a growing interest on application of pretreatment and biogas production from microalgae. There are few studies concerning enzymatic pretreatment of microalgae for enhanced methane production. However, data is scarce in this field. The goal of this study was to investigate biomass solubilization and methane yield after enzymatic pretreatment of microalgae biomass. In this study, *Scenedesmus sp.* and *P. cruentum* were treated with by enzymatic pretreatment at different pretreatment conditions including enzyme type, enzyme dose, temperature, and contact time. For maximization of biomass release during pretreatment, Response Surface Methodology (RSM) was performed to optimize pretreatment conditions in a statistical approach. After optimization of enzymatic pretreatment, pretreated microalgae biomass was subjected to BMP tests to evaluate the increase in methane yields in comparison to untreated microalgae biomass. BMP tests were carried out at two different digestion temperatures (mesophilic and thermophilic). Kinetic models and energy assessment were also studied to evaluate data obtained from experiments.

4.3.1. Comparison of the Effect of Enzymatic Pretreatment on Solubilization of *Scenedesmus sp.* and *P. cruentum* Biomass

Studied microalgae have different characteristics in terms of cell wall, cell components, and cultivation requirements. *Scenedesmus sp.* grows in freshwater and has a multilayer rigid cell wall which is resistant to degradation. The cell wall consists of cellulose and hemicelluloses in addition to glycoproteins, glucosamine containing biopolymers, and algeanan structure. On the other hand, *P. cruentum* grows in saline water and has a cell wall encapsulated by exo polymer substances (EPS). Solubility of raw *Scenedesmus sp.* biomass was around 3% and for *P. cruentum*, it was 5.2%. Solubility of both microalgae is very low and due to cell wall characteristics, hydrolytic bacteria cannot access organic matter easily resulting in longer digestion times and limited methane yields. Methane yields of both untreated *Scenedesmus sp.* and *P. cruentum* biomass showed that pretreatment can enhance methane productions and make anaerobic digestion of microalgae more feasible. So, in this study, the effect of enzymatic pretreatment using different enzymes like cellulase, protease, viscozyme and enzyme mix (protease and viscozyme) were applied to two different microalgae at different pretreatment conditions (dose, temperature, and contact time).

The enzymatic pretreatment results showed that in general, at lower temperatures the solubilization increases were lower. The possible reason for that 55ºC pretreatment provided exopolymer releases from cell wall. Moreover, activity of enzymes is generally higher at high temperatures than at low temperatures. Although low temperature pretreatment requires less energy for heating, the efficiency of the process was found to be unfeasible due to low solubility. Another conclusion derived from enzymatic pretreatment experiments was that for most of the cases, after 9 hours, solubilization increases reached to the maximum level. This result was very important in terms of selecting time of pretreatment since at high temperatures the cost of pretreatment process gradually increases with time. These results were also proved with optimization studies applied by Response Surface Methodology. So, discussion and comparison here are focused on enzymatic pretreatment results applied at 55°C for 9 hours. Enzyme types and enzyme doses are further discussed here, since the behavior of each enzyme is different on both microalgae biomass due to their selectivities.

The comparative summary of the enzymatic pretreatment results was given in Table 4.21. Due to different cell wall characteristics and cell components, enzymatic pretreatment of *Scenedesmus sp.* and *P. cruentum* showed different responses. After thermal pretreatment, solubility of *Scenedesmus sp.* was increased by 6.8%. On the other hand, after thermal pretreatment of *P. cruentum*, solubility increase was observed as 12.9%. The main reason for such difference is the different cell wall characteristics of these microalgae. *Scenedesmus sp.* has a more rigid cell wall that cannot be totally degraded by temperature itself. However, in case of *P. cruentum*, disruption of cells was observed. This can be attributed to release of EPS structure at 55°C. So, it can be concluded that *P. cruentum* has a more fragile cell wall than *Scenedesmus sp.*

Cellulase pretreatment of *Scenedesmus sp.* at different doses (55°C), solubilization increase was observed as between 8.8 and 12.3%. Such increase can be attributed to cellulase degradation in cell wall. On the other hand, cellulase pretreatment did not have a significant effect due to lack of cellulose in *P. cruentum.* At 55°C, the solubilization increases were observed as 14.5-19.3% for most of the solubilization was due to temperatureitself.

Protease pretreatment of *Scenedesmus sp.* at 55 °C provided solubility increase as 14.7-16% after 9 hours depending on the protease dos. Protease pretreatment of *P. cruentum* biomass at 55°C, showed solubility increases between 14.9-32.2% and there was a positive correlation between protease enzyme application and sCOD release. Both microalgae had high protein in their cells and cell walls as glycoprotein, so, protease pretreatment could be effective for hydrolysis of both intracellular cell components and cell wall components. Solubilization increase was better for *P. cruentum* due to more fragile cell wall characteristic than *Scenedesmus sp.*

Viscozyme pretreatment of *Scenedesmus sp.* resulted in 8.2-16% solubility increase at 55ºC and there was a positive correlation between viscozyme dose and solubilization. Viscozyme was found as affective for *Scenedesmus sp.* which provided disruption of cellulosic and hemicellulosic compounds in addition to other carbohydrates such as xylan. Viscozyme pretreatment of *P. cruentum* biomass at 55°C showed solubilization increase as between 27-32.5%. The time required to reach maximum solubilization was

lower for the highest dose indicating positive relationship between enzyme dose and solubilization. Viscozyme treatment was also effective on *P. cruentum.*

Scenedesmus sp. biomass pretreated with enzyme mix had a solubilization efficiency between 22.5 and 37.8%. In case of *P. cruentum,* the increase in solubilization was as between 16.1% and 30%. In case of *Scenedesmus sp.,* enzyme mix pretreatment resulted in highest sCOD release among other pretreatment methods. This can be explained by synergistic effect of enzymes. Viscozyme and protease together provided disruption of both exterior and interior cell wall components and more solubilization of biomass was observed. On the other hand, enzyme mix pretreatment of *P. cruentum* showed similar solubility increases with single enzyme pretreatment. It is mainly due to more fragile cell wall characteristics of microalgae. Temperature itself provided cell wall disruption and addition of enzymes whether as single or mixture did not affect the total sCOD release after pretreatment. However, it is important to notice that the hydrolysis of cell components was better with enzymatic pretreatment.

In conclusion, it was obvious that solubility *P. cruentum* after enzymatic pretreatment was higher than *Scenedesmus sp.* Even in thermal pretreatment cell wall disruption of *P. cruentum* was easily observed. The main reason for such difference was the complex cell wall components of *Scenedesmus sp.* that resulted in lower solubilization of cell wall and cell components. However, application of enzyme mix provided better solubilization efficiencies for *Scenedesmus sp.*and solubility increase was similar with *P. cruentum.*

Table 4. 21. Highest increase in solubility of two microalgae after enzymatic pretreatment with different enzymes (55°C, 9-hour pretreatment)

	Scenedesmus	*P.cruentum*
Thermal (55°C)	6.8%	12.9%
Cellulase	8.8-12.3%	14.5-19.3%
Protease	14.7-16%	14.9-32.3%
Viscozyme	8.2-16%	27.0-30.4%
Enzyme mix	22.5-37.8%	22.3- 30.5%

Carbohydrate and protein solubilization after enzymatic pretreatment are also important since degradation kinetics of carbohydrates and proteins are different in anaerobic digestion. *Scendesmus sp.* is a protein rich (up to 56%) microalgae and *P. cruentum* has considerably lower proteins (up to 38%) than *Scenedesmus sp.* and carbohydrate content is higher than *Scenedesmus sp.* According to the results of the study, use of carbohydrases resulted in higher carbohydrate solubilization for both microalgae. In the same manner, use of proteases resulted in higher protein solubilization. After cellulase pretreatment of *Scenedesmus sp.*, higher carbohydrate release was observed. Cellulase pretreatment of *P. cruentum* increased mostly carbohydrate content of the biomass and it was mainly due to cell wall components disrupted by temperature itself. After protease pretreatment, protein solubilization was higher than carbohydrates for *Scenedesmus sp.* due to cell wall degradation and intracellular proteins. In case of *P. cruentum,* generally, the ratio of carbohydrate and protein solubilization was similar to each other and protein solubilization was comparatively higher for 0.5 mL protease dose. Viscozyme pretreatment of *Scenedesmus sp.* and *P. cruentum* increased solubilization of carbohydrates rather than proteins. In case of enzyme mix pretreatment, highest protein and carbohydrates solubilization was observed after enzyme mix pretreatment for *Scenedesmus sp.* and *P. cruentum.* These results showed that the release of carbohydrates and proteins are dependent of enzyme type and microalgae.

The research related to application of enzymatic pretreatment of microalgae is various, However, in terms of *Scenedesmus sp.* the research is limited. In addition, there was no study found for *P.cruentum.* So, the following discussion is about only *Scenedesmus sp.* Studies conducted by other researchers showed that enzymatic pretreatment increased biomass solubilization of *Scenedesmus sp.* biomass effectively. Mahdy et al. [127] applied enzymatic pretreatment to *Scenedesmus* biomass with Cellulast (0.2 mL/ gDW), Viscozyme (0.3 mL/ gDW), Pectinex (pectinase) (0.25 mL/ gDW) and Alcalase (protease) (0.2 mL/ gDW) for 3 hours at 50°C. The increase in sCOD solubilization efficiency was found to be between 15-20% for enzymatic pretreatment. It was also noticed that after 8 hours with Alcalase pretreatment, the solubilization efficiency was about 30%. In another study, Ometto et al. [104] applied enzymatic pretreatment to *Scenedesmus obliquus* with Lipomod (Endogalactouronase, Esterase, Protease), Depol

(Alpha amylase), Pectinase and Lipomod (Esterase) with different doses (25, 50, 150, 250, and 350 U/mL) for 24 hours at 50°C .Ometto et al. [104] was also applied other pretreatment methods, however, enzymatic pretreatment was found to be most efficient process. Solubilization efficiencies were found to be between 35-45% in this study. Passos et al. [109] applied enzymatic pretreatment to microalgae biomass (mix microalgae culture) with cellulase, glucohydrolase and an enzyme mix for 6-48 hours at 37°C. Biomass solubilization was observed as 550-650 mg soluble VS/L after pretreatment and biomass solubilization were achieved as 110% (for cellulase) and 126% (for enzyme mix) after 6 hours.

Apart from enzymatic pretreatment, various pretreatment methods were also applied to *Scenedesmus* biomass before anaerobic digestion. After hydrothermal pretreatment (170°C, 800 kPa, and 30 min) of *Scenedesmus* biomass, 2.5-10 fold of COD solubilization was achieved [122]. In another study, ultrasonic (35.5 MJ/kg-128.9 MJ/kg for 15 minutes) and thermal (70-80°C) pretreatment increased sCOD concentration as 2.2-3.1 fold (400-600 mg/l increase) for ultrasonic pretreatment and 1.9-2.3 fold (250-400 mg/l increase) for thermal pretreatment [6]. Microwave pretreatment was also applied to this specie and 280-799% increase in solubilization was found in another study [48]. Although, the pretreatment methods mentioned above are also as effective as enzymes in terms of biomass solubilization, the main limitation of these methods is the high energy demand that results in negative energy balance of the total process which makes anaerobic digestion of microalgae unfeasible. However, enzyme application would be feasible alternative pretreatment method with a lower energy requirement.

4.3.2. Comparison of Methane Production Potentials of *Scenedesmus sp.* and *P. cruentum* after Enzymatic Pretreatment

Methane production from microalgae is a promising way to produce bioenergy and application of pretreatment prior to anaerobic digestion enhances methane production potential. In most of the studies, depending on the pretreatment type and conditions, improvement of methane yields from various microalgae was observed to some extent. This study also proved the effectiveness of pretreatment on methane yield enhancement.

Table 4.22 compares net methane yields and increase (%) after enzymatic pretreatment of two microalgae biomass. As can be seen from the table, untreated *Scenedesmus sp.* and *P. cruentum* showed similar methane yields after mesophilic digestion. After thermophilic digestion, methane yield of untreated *Scenedesmus sp.* were higher than after mesophilic digestion. On the other hand, for untreated *P. cruentum,* methane yields after mesophilic and thermophilic digestion were the same. *Scenedesmus* has a rigid cell wall structure that could be degraded to some extent at thermophilic conditions at longer time periods. However, in case of *P. cruentum*, anaerobic bacteria were able to degrade the biomass at both temperatures due to milder cell wall characteristics. As the results of enzymatic pretreatment were compared, it was observed that enzymatic pretreatment provided better methane yields and the enzyme dose was found to be an important parameter for methane yield improvement for both microalgae type. The most efficient pretreatment was found to be enzyme mix and protease pretreatment for both microalgae. They provided release of both cell wall and intercellular components of microalgae biomass Especially, protease can be considered as a key enzyme for protein solubilization. During anaerobic digestion, degradation of protein is complex and it longer times (days) than carbohydrates (hours). So, it was concluded that degradation of proteins into their monomers prior to anaerobic digestion provided better methane yields and degradation of carbohydrates did not have a significant effect as proteins on methane yields. This relation can be observed between viscozyme and protease pretreatment. Although, sCOD increases were similar, net methane yields were higher for protease pretreatment. This could be due to the differences in the amount of protein and carbohydrate releases. However, application of enzyme mix (viscozyme and protease) showed methane yields as high as protease pretreated biomass which had a synergistic effect.

Table 4. 22.Comparison of *Scenedesmus sp*. and *P.cruentum* for methane yield potential after enzymatic pretreatment

	Scenedesmussp.				*P.cruentum*			
	Mesophilic		**Thermophilic**		**Mesophilic**		**Thermophilic**	
	mL CH_4/ gVS	**Increase (%)**	mL CH_4/ gVS	**Increase (%)**	mL CH_4/ gVS	**Increase (%)**	mL CH_4/ gVS	**Increase (%)**
Raw	105.9	-	170.1	-	130.3	-	135.2	-
Thermal (55°C)	103.9	-	178.6	5.00	147.9	11	140.7	4
Cellulase(0.1 mL)	120.4	17	197.5	16.1	139.2	3.9	156.8	16
Cellulase (0.3 mL)	141.1	41.8	207.0	21.7	147.9	10.4	155.8	15.2
Cellulase (0.5 mL)	177.1	67	237.0	39.3	154.3	15.2	152.7	12.9
Protease (0.1 mL)	145.1	37	213.5	25.5	168.9	26.1	202.7	49.7
Protease (0.3 mL)	170.5	61	260.4	53.1	208.8	60.2	226.3	67.3
Protease (0.5 mL)	193.8	83	291.7	71.4	230	71.7	270.8	100
Viscozyme (0.1 mL)	110.1	4	183.4	7.8	138.4	3.3	158.1	16.9
Viscozyme (0.3 mL)	112.2	6	210.7	23.5	159.9	19.4	173.1	28
Viscozyme (0.5 mL)	150.1	42	220.7	29.7	173.1	29.2	229.8	70
Enzyme mix(0.1 mL)	129.2	22	196.2	15.3	147.7	10.3	154.4	14.2
Enzyme mix(0.3 mL)	178.9	69	214.1	25.8	166.5	24.3	178.4	31.9
Enzyme mix(0.5 mL)	216.0	109	289.7	70.2	210.7	62.3	256.8	90

*Increase is given according to the amount produced from untreated biomass.

Generally, the research in literature focused on mesophilic digestion of pretreated microalgae biomass. As enzymatic pretreatment, there was one literature study concerning methane improvement from *Scenedesmus sp.* Mahdy et al [127] found that methane production (mesophilic digestion) was increased as 1.53, 1.3, 1.2, and 1.3-fold after protease, pectinase, cellulase, and viscozyme pretreatment with 0.2-0.3 mL enzyme dose / g dry weight, respectively and the highest efficiency was observed with protease pretreated biomass. On the other hand, in this study, 1.68, 1.84, 1.41, and 2.06-fold improvement in methane were achieved after cellulase, protease, viscozyme, and enzyme mix (protease +viscozyme) pretreatment with 0.5 mL enzyme dose/ g biomass, respectively. The study conducted by Mahdy et al. [87] showed similarities with this study and better efficiencies were achieved in this study. This difference could be due to several parameters such as the enzyme dose, pretreatment time, pretreatment temperature and substrate concentration. Additionally, the cell composition and cell wall of *Scenedesmus sp.* may differ due to cultivation conditions.

Apart from *Scenedesmus sp.,* other species were also investigated by several researchers in terms of enzymatic pretreatment and biogas production (Table 4.23). Gruber-Brunhumer [110] found out that after enzyme mix pretreatment of *Acutodesmus obliquus,* 30.3% methane improvement was achieved. Ehimen et al. [115] found out that use of enzyme mixture including lipase, xylanase, α-amylase, cellulase and protease resulted in highest methane yield improvement as 31%. Cellulase was also effective for this algae due to its cellulose content. Mahdy et al. [125] studied both *C.vulgaris* and *C.reinhardtii* and find out that use of protease enyzme improved methane yields of

C.vulgaris more than viscozyme enzyme or enzyme mix (viscozyme and protease). For *C.reinhardtii,* highest methane improvement was observed with enzyme mix pretreatment including viscozyme and protease (16%). In other study, protease pretreatment of *C.vulgaris* increased methane production by 64% for 0.585 AU g/DW dose pretreatment [126]. Passos et al. [39] achieved 15.4% increase in methane yield of *Oocystis sp.* after enzyme mix pretreatment including cellulase glucohydrolase and xylanase. In these studies, enzymatic pretreatment was found to be promising for increasing methane production yields from microalgae and due to different pretreatment conditions and type of microalgae, the methane improvements vary.

Table 4. 23. Comparison of enzymatic pretreatment studies in literature for various microalgae

Microalgae	Types ofenzymesused	Pretreatmentconditions	Pretreated biomassmethane yield	Methane yield increase (%)	Reference
Scenedesmussp.	cellulase, protease, viscozyme, enzymemix	0.1-0.5 mL/ g biomas , 55°C, 9 hours	110.1-216 mL CH$_4$/ gVS (mesophilic)	4-109%	This study
			183.4-291.7 mL CH$_4$/ gVS (thermophilic)	7.8-71.4%	
P.cruentum	cellulase, protease,viscozyme, enzymemix	0.1-0.5 mL/ g biomass, 55°C, 9 hours	138.4-230 mL CH$_4$/ gVS (mesophilic)	3.3-71.2%	This study
			152.7-270.8 mL CH$_4$/ gVS (thermophilic)	12.9-100%	
Scenedesmussp.	viscozyme, cellulase,pectinase, protease	0.2-0.3 mL/ g DW, 50°C, 3-8 hours	150-200 mL CH$_4$/ gVS (mesophilic)	20-53%	[127]
Acutodesmusobliquus	enzyme mix (cellulase,xylanase, betaglucanase and protease)was	325-1775 U/g DM, 10% w/w, 37°C, 24 hours	150.7Nm^3/tCOD (mesophilic)	30.3%	[110]
Rhizoclonium	lipase, cellulase, xylanase, α-amylase, protease, enzymemix	1% (w/w),48hours	115-145 mL CH$_4$/gVS (mesophilic)	8-31%	[115]
C.reinhardtii	viscozyme, protease,enzymemix	0.2-0.3 mL/ g DW, 50°C,5 hours	255-311 mL CH$_4$/ gCOD$_{in}$ (mesophilic)	0-16%	[125]
C.vulgaris	viscozyme, protease,enzymemix	0.2-0.3 mL/ g DW, 50°C,5 hours	270-299 mL CH$_4$/ gCOD$_{in}$ (mesophilic)	42-58%	[125]
C.vulgaris	protease	0.146, 0.293 and 0.585AU, g/DW, 50°C, 3hours	223.8-255.6 mL CH$_4$/ gCOD$_{in}$ (mesophilic)	39-64%	[126]
Microalgae biomass (Oocystis sp.)	cellulase, glucohydrolase, enzyme mix	0.5 and 1%, 37°C, 6–48 hours	217 mL CH$_4$/gVS(mesophilic)	15.4%	[39]

Apart from enzymatic pretreatment, there are a few studies related to application of other pretreatment methods (Table 4.24). There is no study found related to application of pretreatment methods to *P.cruentum* biomass. In case of *Scenedesmus sp.*, after ultrasound pretreatment of *Scendesmus sp.* up to 87% increase was observed and enhanced methane yield were found to be as 153. 5 mL CH_4/g COD [6]. In another study, ultrasound pretreatment of *Scendesmus sp.* resulted in (266-307mL biogas /VS)2-27% increase [104]. Thermal and hydrothermal pretreatment were also studied by several researchers. Thermal pretreatment of *Scenedesmus sp.* yield a methane production of 128.7 mL CH4/g COD (36.4% increase) [6]. Ometto et al. [104] achieved 10-44% improvement (270-381 mL biogas /VS) and in another study, after thermal pretreatment 60% improvement in methane production was achieved from *Scenedesmus dimorphus* biomass [123]. Alkali pretreatment resulted in 17-20% improvement (130-179 mL/ CH4 /g COD) [114]. The main limitations behind these methods such as ultrasound or hydrothermal pretreatment are their high energy requirements. As can be seen, with this study, higher methane improvements were achieved with lower energy content. Enzymes have high selectivity that target a specific molecule such as cellulose, hemicellulose or proteins. Since cell wall of *Scenedesmus sp.* has both cellulosic and protein like substances, the application of enzymes without high energy requirement provides cell wall disruption. In addition, they are also effective on complex organics. During hydrolysis phase, complex organics such as carbohydrates, proteins and lipids are converted to monomers through exocellular enzymes produced by hydrolytic bacteria. With enzymatic pretreatment prior to anaerobic digestion, the cell components are converted into monomers and showed better methane yields by improving hydrolysis phase.

Table 4. 24 Comparison of different pretreatment studies in literature for *Scenedesmus sp.*

Microalgae	Pretreatment type	Pretreated biomass methane yield	Methane yield increase (%)	This study
Scenedesmus sp.	Ultrasound	153.5	87%	[6]
Scenedesmus sp.	Chemical	130-170mL CH_4/gCOD_{in}	17-20%	[114]
Scenedesmus obliquus	Hydrothermal	313-548mL CH_4/gCOD_{in}	18-107%	[104]
Scenedesmus sp.	Hydrothermal	180-380mL CH_4/gCOD_{in}	58-81%	[122]
Scenedesmus sp.	Thermal	89.3-128.7 mL CH_4/ gCOD_{in}	9-36%	[6]
Scenedesmus obliquus	Thermal	270-381 mL CH_4/ gVS	60-68%	[104]
Scenedesmus dimorphus	Thermal	130-137 mL CH_4/ gVS	60%	[123]

As stated, main research has been focused on mesophilic digestion of microalgae so far. Thermophilic digestion of microalgae after pretreatment is not as well studied as mesophilic digestion. As the only example, Magdalena et al. [95] found out that after thermophilic digestion of *Chlorella sp.,* the methane yield was found to be as 235.6 mL CH_4/ gCOD. In this study protease pretreated biomass with 0.5 mL enzymedose 291.76 mLCH_4/ g VS which showed an improvement in methane production. In literature, there are some studies indicating that thermophilic digestion did not show better methane improvements. On the other hand, some of the studies indicated that thermophilic digestion showed improvement to some extent. This study showed that thermophilic digestion showed better methane production potential from enzymatically pretreated *Scenedesmus sp.* In addition, this study also showed that untreated biomass at thermophilic digestion also had more methane yield than untreated biomass at mesophilic digestion. Thermophilic digestion can be considered more efficient in terms of better methane production yields. However, energy consumption is one of the main concerns for large scale applications. In this study, energy assessment for both mesophilic and thermophilic digestion were applied and given in later. Another consideration for thermophilic digestion is high concentrations of ammonia in the reactor. However, at high biomass concentrations, this could be problematic since

Scenedesmus sp. has protein content up to 56%. At this point, co digestion can be applied to increase C: N ratio.

4.3.3. Comparison of the Relationship between Solubility Increase after Enzymatic Pretreatment and Methane Yields

The aim of the enzymatic pretreatment of microalgae was to provide rigid cell wall disruption and also degradation of intracellular components into monomers. The results of enzymatic pretreatment of *Scenedesmus sp.* and *P. cruentum* biomass showed that there was a positive correlation between solubilization and methane production (Figure 4.61- Figure 4.62). Generally, increase in biomass solubilization increased methane yields as expected. As the dose of the enzymes increased, the methane production was also increased. However, as the types of enzymes are compared, it was observed that, although the solubilization efficiencies were similar, the methane production potential did not show the same pathway. In general, protease pretreatment. showed better methane production potential for both microalgae. In contrast, for viscozyme pretreatment, it was observed that methane yields did not increase methane yields as expected for *Scenedesmus sp.* This result could be related to higher degradability of carbohydrates giving lower methane productions than proteins during anaerobic digestion.
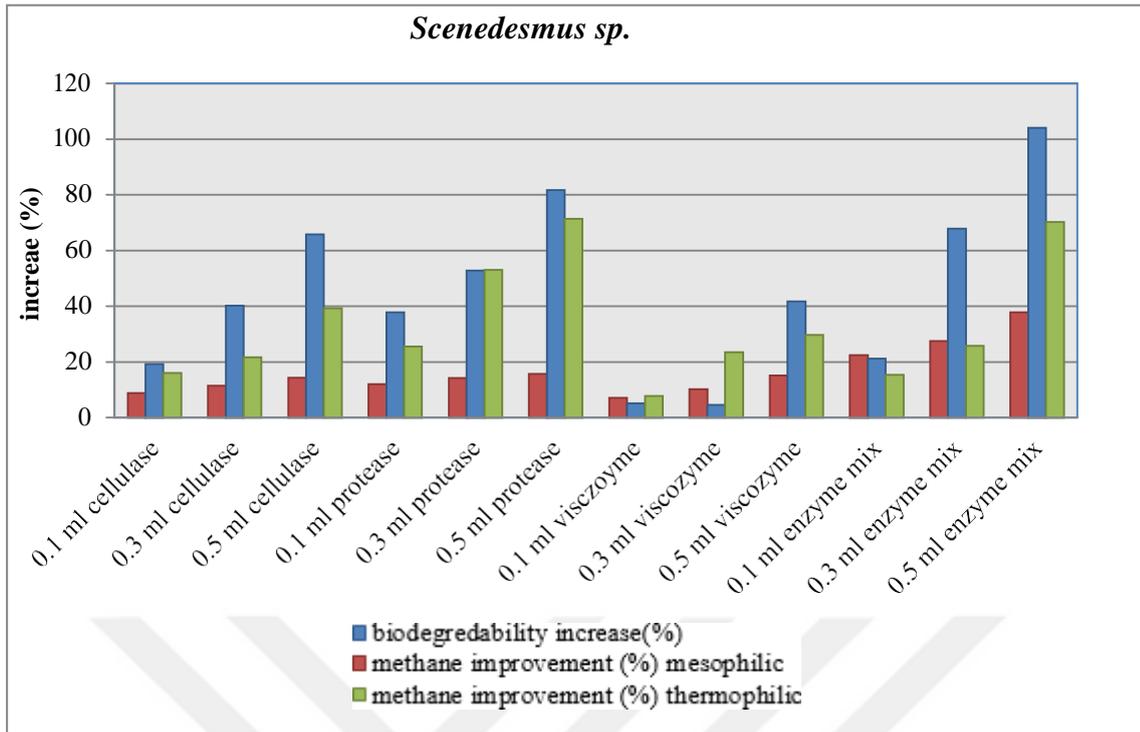
Figure 4.61. Relationship between solubility increase and methane yields for *Scenedesmus sp.*
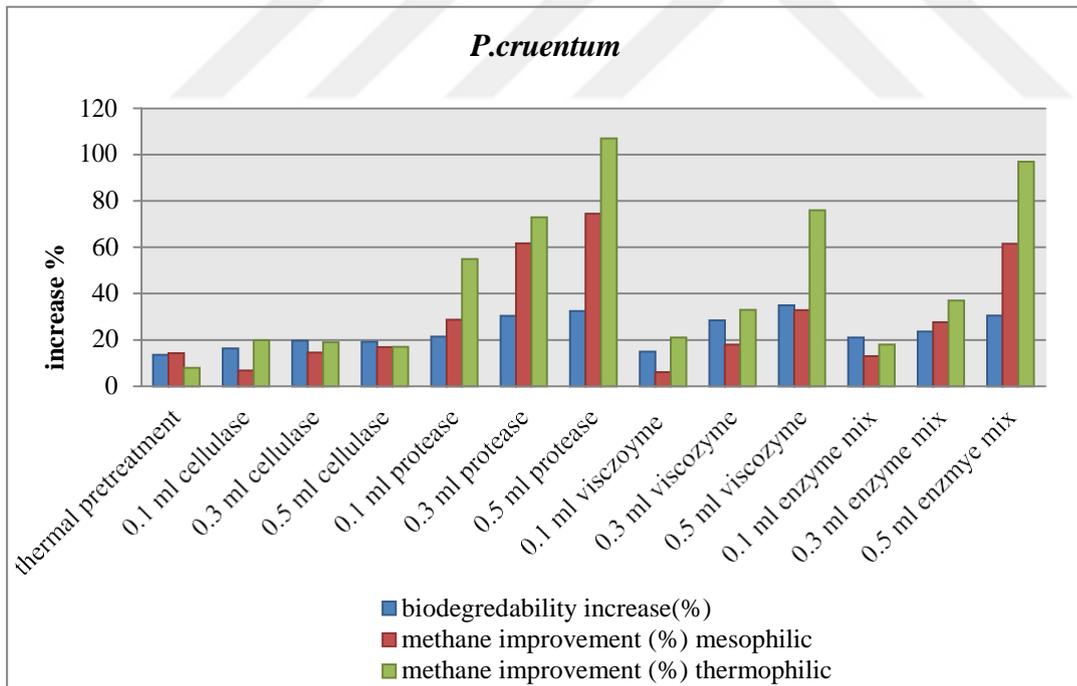
Figure 4.62. Relationship between solubility increase and methane yields for *P. cruentum*

Volatile solids reduction is an important indicator for substrate degradation and methane production in an anaerobic digester. Moreover, it also provides an insight for biodegradability of the substrate. The findings of the study showed that after mesophilic digestion of *Scenedesmus sp.*, volatile solids reduction for pretreated biomass was between 18.5% and 39.3%. For untreated *Scenedesmus* biomass, the VS reduction was found to be around 17%. (Figure 4.63). On the other hand, thermophilic digestion of raw biomass was 33.7% and for enzyme pretreated biomass the values were between 30.4 and 46.2%. It was clear that thermophilic digestion provided higher VS reduction and methane production than mesophilic digestion. As enzymes were compared, it was observed that protease and enzyme mix pretreated biomass showed higher VS reduction that were correlated to methane yields. Figure 4.64 shows VS reduction efficiencies (%) for *P. cruentum* biomass after mesophilic and thermophilic digestion. Untreated *P. cruentum* biomass resulted in 22-27% VS reduction after mesophilic and thermophilic digestion and highest VS reduction was obtained for 0.5 mL protease pretreatment 44% and 67% for mesophilic and thermophilic digestion, respectively. Enzyme mix pretreated biomass also showed 41-57% VS reduction efficiencies after mesophilic and thermophilic digestion. In general, *P. cruentum* showed better VS reduction efficiencies. It was mainly due to carbohydrate contents of microalgae. In addition, cell of this microalgae were as not rigid as *Scenedesmussp.*
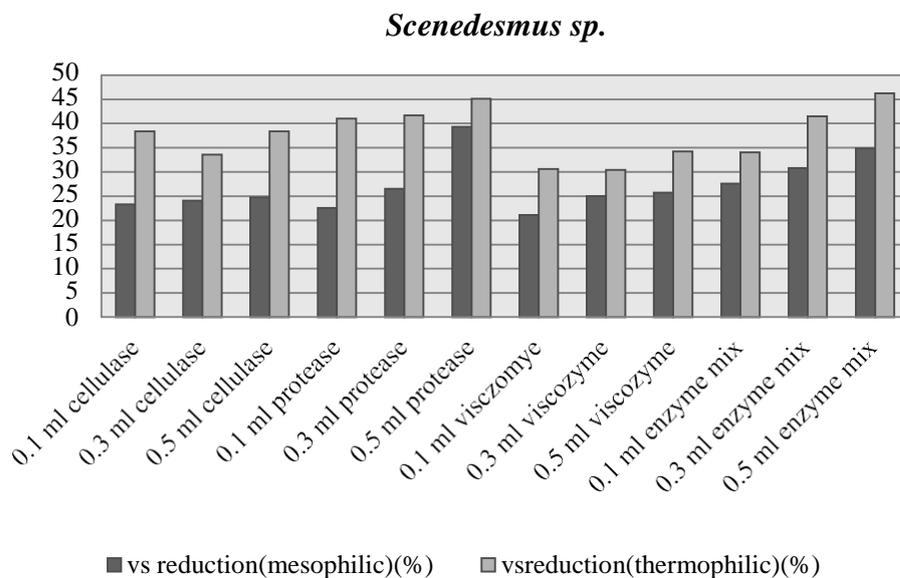
Figure 4. 63. Volatile solids reduction after BMP tests (*Scenedesmus sp.*)
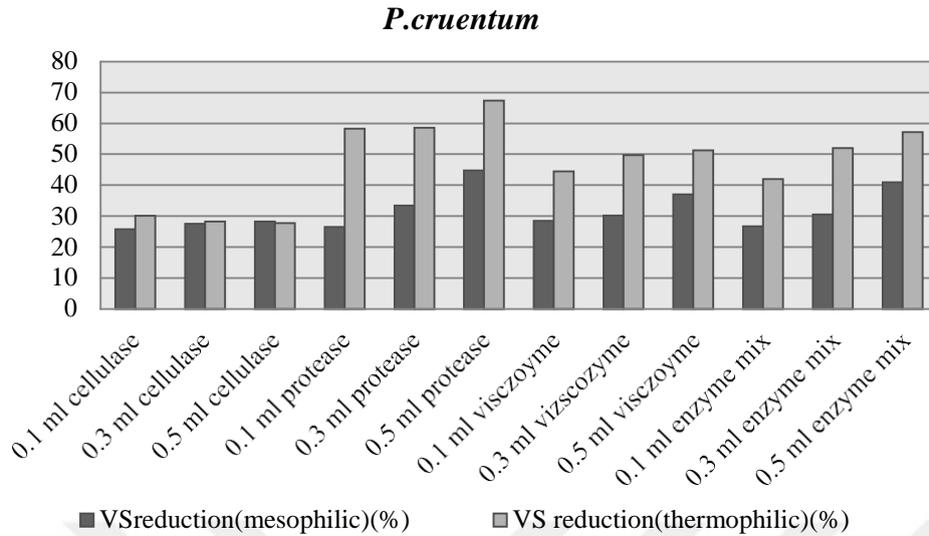
Figure 4. 64. Volatile solids reduction after BMP tests (*P. cruentum*)

As these values are compared with literature findings, it could be noticed that VS reduction efficiencies were still lower. This is mainly due to characteristics of microalgae biomass. Enzymatic pretreatment helped to increase VS reduction and improve methane production. However, other characteristics of microalgae should also be considered. For instance, C: N ratios for both studied microalgae were lower than 10, which was a normal range of anaerobic digestion. To solve this, co-digestion can be applied to improve VS reduction and improve methane production. In addition, substrate concentration may be limiting, so, higher substrate concentrations would be investigated for comparison. In addition, the ratio of substrate to inoculums (S: I) and used of inoculums that is acclimatized to microalgae biomass can be other parameters that should be considered for the improvement of anaerobic digestion performance.

4.3.4. Discussion on Kinetics of Methane production

The findings of the kinetic models showed that, modified Gompertz Model provided better curve fitting than first order kinetic model for both microalgae. General curve for a BMP test is a kind of S- shaped curve. Due to this, first order kinetic model   was   not

able to predict lag phase of the system and the predicted values for the first days were more than experimental values. Moreover, the ultimate methane yields obtained by the first order kinetic model were higher than experimental values. On the other hand, modified Gompertz model was able to determine ultimate methane yields very similar to experimental values. Moreover, this model also gave lag time and maximum methane production rates, so that, it helped to give a better discussion on the nature of anaerobic digestion.

In the case of *Scenedesmus sp.* results showed that the hydrolysis rates were 0.038 day^{-1} for mesophilic digestion and 0.044day^{-1} untreated biomass, and these rates were improved by enzymatic pretreatment. The results of modified Gompertz model suggested a lag phase of 5.21 for untreated biomass and this value was lowered to 3.43 with enzymatic pretreatment in mesophilic digestion. It was also concluded that enzymatic pretreatment increased maximum methane production rates. The results of modified Gompertz model for thermophilic digestion indicated that after enzymatic pretreatment the lag phase was decreased to 1.0 days, showing a significant effect on lag phase. It was also determined that maximum methane production rates in thermophilic digestion were increased after enzymaticpretreatment.

In the case of *P. cruentum,* as *Scenedesmus sp.,* hydrolysis rates for untreated biomass were improved by enzymatic pretreatment. Modified Gompertz Model results showed that lag phase difference was observed and reduced after enzymatic pretreatment at both mesophilic and thermophilic digestions. The model results also showed that maximum methane production rates were increased after enzymatic pretreatment in both mesophilic and thermophilic digestion.

As two microalgae were compared in terms of kinetic models, the hydrolysis rates were found higher for *Scenedesmus sp.* in mesophilic digestion and found higher for *P. cruentum* thermophilic digestion. This difference was due to different biomass characteristics of microalgae. After enzymatic pretreatment, hydrolysis rates were improved. However, here it is important to notice that due to different effect of enzymes on biomass, the hydrolysis rate constants were not directly related to methane production yields. For instance, hydrolysis rate of carbohydrates is higher than proteins. As enzymes behave differently from each other, it is not appropriate to compare hydrolysis rates of enzymatic pretreatment. Moreover, first order rate kinetic model was not able tofit the data very well. So, in case of enzymatically pretreated microalgae, it

was better to apply modified Gompertz model. This model provided lag phase, and maximum methane production rate (mL CH_4/g VS). According to the results, *P. cruentum* showed lower lag phase for untreated biomass which could be attributed to easiness of cell wall disruption and carbohydrate content. For both microalgae, the lag phases for untreated biomass were 4.14 and 5.21 days. These value were lowered after enzymatic pretreatment and maximum methane productions were improved. In order to decrease lag phase further, use of acclimatized inoculum to microalgae biomass could be an effective solution.

4.4.4. Comparison of Energy Assessment Results

Energy requirement is the main tackle for applicability of pretreatment of microalgae. The ratio of energy input (energy requirement for pretreatment) and energy output (methane production improvement by anaerobic digestion) should be less than 1 to provide a feasible pretreatment process. The energy assessment results of this study showed that energy ratios for pretreated *Scenedesmus* biomass were between 0.68 and 1.41 for mesophilic digestion and thermophilic digestion the values were more than 1. Lowest energy ratios were belonging to enzyme mix and protease pretreated biomass for both mesophilic and thermophilic digestion. For pretreated P. *cruentum* biomass, the ratios were 0.69-1.05 and 0.54-1.04 for mesophilic digestion and thermophilic digestion, respectively and the lowest energy ratios were belonging to protease pretreated biomass (0.5 mL enzyme mix).

There is some research regarding energy assessment of enzymatic pretreatment of microalgae. Ometto et al. [104] found out that energy ratio for enzymatic pretreatment was between 0.06-0.07 for *Scenedesmus obliquus sp.* In the same study, ultrasound pretreatment yielded an energy ratio between 1.21 and 27.85 and thermal pretreatment yielded an energy ratio between 1.33 and 3.09. The ratio was between 0.75-2.51 for hydrothermal pretreatment. He also found out similar values other microalgae (*Chlorella sorokiana* and *Arthrospira maxima*). Passos et al. [109] found out that after ultrasound pretreatment and anaerobic digestion of *Chlorella vulgaris* the energy ratio was between 32.0 and 43.8. These comparisons showed that enzymatic pretreatment has a low energy demand among other pretreatment methods and it was evident with this study.

5. CONCLUSION

Anaerobic digestion is a straightforward way to produced gaseous energy to tackle global energy problem and microalgae can be utilized in anaerobic digestion. There are some factors affecting biogas production from microalgae like macromolecular composition and cell wall characteristics and operational parameters such as pH, temperature, mixing, reactor design etc. Among these, cell wall degradability of microalgae is considered as one of the most important characteristics of microalgae that may influence biogas yields. Microalgae having rigid cell wall characteristics including cellulose, hemicellulose, or algeanan are very resistant to degradation and it reduces the availability of organic matter in the cell to the hydrolytic bacteria during anaerobic digestion. So, prior to anaerobic digestion, cell disruption of microalgae by various pretreatment methods provides enhanced contact of biomass with anaerobic archea and thus higher methane yields.

This thesis aimed to focus on the effect of enzymatic pretreatment on biomass solubilization and methane yields of two different microalgae. Moreover, it was also aimed to evaluate the effect of different digestion temperatures on methane production potentials. The conclusions derived from this thesis were given as below:

Results of enzymatic pretreatment

- Due to rigid multilayer cell wall characteristics of *Scenedesmus* sp., only thermal pretreatment increased solubilization only 6.8%. This can be attributed exopolymer release from cell wall. On the other hand, for *P. cruentum*, even with thermal pretreatment cell wall disruption was achieved and biomass solubilization was observed as 12.9%.

- Enzymatic pretreatment of *Scenedesmus sp.* with cellulase at 55°C increased solubility by 8.8-12.3%. At 40°C, the highest increase in solubility was achieved as 7.9% with 0.5 mL cellulase pretreatment. This can be attributed to cellulosic cell wall degradation and release of intercellular compounds from *Scenedesmus sp.* On the contrary, cellulase pretreatment did not have an effect due to lack of cellulose in *P. cruentum.* At 55°C, the solubilization increases were observed as 14.5-19.3% for most of the solubilization was due to temperature itself. At lower

temperatures, very low solubilization increases were obtained which were not significant.

- Protease pretreatment of *Scenedesmus sp.* at 55 °C increased solubility by 14.7-16% after 9 hours depending on the protease dose. At lower temperatures, solubility increase was less than 7% for 40ºC, and less than 2% for 25ºC. Cell wall of *Scenedesmus sp.* includes glycoproteins, so; with the help of protease they could be disrupted. Protease pretreatment of *P. cruentum* biomass at 55°C, showed solubility increases between 14.9-32.2%. Protease pretreatment at 40°C and 25°C showed lower solubilization increase. Protease pretreatment were effective on both microalgae and 55°C was found to be most effective temperature for biomass solubilization due to higher protease activity. Both microalgae have high content of protein in their cells and cell wall, so; protease pretreatment could be effective for hydrolysis of both intercellular cell components and cell wall components.

- Enzymatic pretreatment of *Scenedesmus sp.* with viscozyme resulted in 8.2-16% solubility increase at 55ºC. At 40°C, solubilization increase was observed as between 3.9-10.3% and 25°C, they were between 1-6.3%. Viscozyme pretreatment of *P. cruentum* biomass at 55°C showed solubilization increase as between 27% and 32.5%. At lower temperatures (25-40°C), sCOD release values were lower. The solubility increase was an indicator for carbohydrate degradation from cell wall and intercellular components of *Scenedesmus sp.* and *P. cruentum.*

- *Scenedesmus sp.* biomass pretreated with enzyme mix had a solubilization efficiency between 22.5-37.8%. In case of *P. cruentum,* the solubilization increases were reached as 16.1%-30%. In case of *Scenedesmus sp.,* Enzyme mix pretreatment resulted in highest sCOD release among other pretreatment methods. This can be explained by synergistic effect of enzymes. Viscozyme and protease together provided disruption of both exterior and interior cell wall components and more solubilization of biomass was observed. On the other hand,enzyme mix pretreatmen to *P.cruentum* showed similar solubility.

increases with single enzyme pretreatment. It is mainly due to more fragile cell wall characteristics of microalgae. Temperature itself provided cell wall disruption and addition of enzymes whether as single or mixture did not affect the total sCOD release after pretreatment. However, it is important to notice that the hydrolysis of cell components was better with enzymatic pretreatment.

- The enzymatic pretreatment results showed that in general, at lower temperatures the solubilization increases were lower. The possible reasons for that 55ºC pretreatment provided exopolymer release to some extent. Moreover, activity of enzymes is generally higher at high temperatures than at low temperatures. Although low temperature pretreatment requires less energy for heating, the efficiency of the process was found to be unfeasible due to low solubility. Another conclusion derived from enzymatic pretreatment experiments was that for most of the cases, after 9 hours, solubilization increases reached to the maximum level. This result was very important in terms of selecting time of pretreatment since at high temperatures the cost of pretreatment process gradually increases with time. These results were also proved with optimization studies applied by Response Surface Methodology.

- In terms of *Scenedesmus sp.*, single enzyme pretreatment resulted in similar solubilization increase (%), however; enzyme mix pretreatment was found to be most efficient. These results were the evident for synergistic effect of enzyme mixtures. *Scenedesmus sp.* has a complex wall contain cellulose, hemicelluloses, and trilaminar structure. The application of enzyme mix including carboydrase mix and protease to *Scenedesmus sp.* provided disruption of cell wall and solubilization of complex cell components. In terms of *P. cruentum,* temperature is the most effective parameter for organic matter solubilization and it had a synergistic effect with the enzymes (except for cellulase) at high temperatures resulting in higher biomass solubilization.

Results of methane production potentials

- At mesophilic conditions, untreated (raw) *Scenedesmus sp.* biomassyielded 105.9 mL CH_4/gVS. Thermal pretreated *Scenedesmus sp.* biomass had similar methane production potential with untreated biomass. The results of enzymatically pretreated *Scenedesmus sp.* showed that pretreated biomass showed better methane production potential than untreated biomass. Cellulase pretreated biomass showed 17-67% methane yield improvement and there was a positive correlation between enzyme dose and methane yield. Methane potential of protease pretreated *Scenedesmus sp.* biomass was also higher than untreated biomass. Highest improvement was achieved by 0.5 mL protease pretreatment as 83% increase. Viscozyme pretreated *Scenedesmus sp.* biomass showed methane yield increased between 4% and 42%. Enzyme mix (viscozyme and protease) pretreated biomass increased methane production by 22- 109%. As pretreated *Scenedesmus sp.* was compared, the methane yield was significantly higher when enzyme mix was used in the pretreatment at mesophilicdigestion.

- At thermophilic conditions, untreated *Scenedesmus sp.* biomass had a methane production potential as 170.1 mL CH_4/ gVS. Thermally pretreated (55°C) biomass showed a small increase in methane production as 5%. Cellulase pretreated biomass had methane improvements between 16.1-39.3%. Protease pretreated biomass also had a positive effect on methane production potential from *Scenedesmus sp.* biomass at thermophilic digestion as 25.5- 71.4% improvement. Viscozyme pretreated biomass showed higher methane production as compared to untreated biomass with 7.8-29.7% increase. Mixed enzyme pretreated biomass after thermophilic digestion increased methane production by 15.3-70.2 %. Highest improvement was observed with enzyme mix and protease pretreatment at thermophilicdigestion.

- At mesophilic conditions, untreated *P. cruentum* biomass had a net cumulative methane yield of 130.3 mLCH$_4$/ gVS. Thermally pretreated biomass showed 10.4 % improvement in net methane yield. Cellulase pretreated *P. cruentum* biomass showed methane improvements as between 3.9% and 15.2%. Protease pretreated biomass showed better methane yields with an increase of 26.1-71.7% when compared to untreated biomass. Viscozyme pretreated biomass had the highest methane yield for 0.5 mL viscozyme dose with a net methane yield

increase of 29.2 %. Enzyme mix pretreatment was able to increase net methane yields from *P. cruentum* biomass up to 62.3 % improvement. It was observed that enzyme mix pretreatment yielded better results than single viscozyme pretreatment, however; as compared to protease pretreatment, the improvement was lower.

- At mesophilic conditions, untreated *P. cruentum* biomass had a net cumulative methane yield of 135.2 mL CH_4/ gVS. Cellulase pretreated biomass resulted in methane yields between 152-156 mL CH_4/ gVS. Pretreated biomass showed highest increase of 100% with 0.5 mL protease and net cumulative methane yield was found to be 270 mL CH_4/ gVS. Viscozyme pretreated biomass, on the other hand, produced less amount of methane than protease pretreated biomass. The methane yields for viscozyme pretreated biomass were between158.1-229.8 mL CH_4/ gVS (16.9-70% improvement). Enzyme mix pretreatment also increased effectively methane yield to 256.8 mL CH_4/ gVS (90% increase). It was clear that after application of enzyme mix to *P. cruentum* biomass methane yields were improved gave comparatively better results than viscozyme pretreatment itself. These findings showed that, application of protease/ enzyme mix helped degradation of proteins in the intracellular components and cell wall components of *P. cruentum* and increased methane production.

- The most efficient enzymatic pretreatment was found to be enzyme mix and protease pretreatment for both microalgae. They provided release of both exocellular and intercellular components of microalgae biomass Especially, protease can be considered as a key enzyme for protein solubilization. During anaerobic digestion, the hardest macromolecular structure is protein to be degraded and it takes longer times (days) than carbohydrates (hours). So, it was concluded that degradation of proteins prior to anaerobic digestion provided better methane yields and degradation of carbohydrates did not have a significant effect as proteins.

- There was a correlation between solubilization and methane production. Generally, increase in biomass solubilization increased methane yields as expected. As the dose of the enzymes increased, the methane production was also increased. However, as the types of enzymes are compared, it was observed that, although the solubilization efficiencies were similar, the methane production potential did not have similar trend. In general, protease pretreatment. showed better methane production potential for both microalgae. In contrast, for viscozyme pretreatment, it was observed that methane yields did not increase methane yields as expected for *Scenedesmus sp.* This result could be related to higher degradability of carbohydrates giving lower methane productions than proteins during anaerobic digestion.

- First order kinetic model was able to predict hydrolysis rates and it showed that showed that hydrolysis rates (1/d) during anaerobic digestion were improved after enzymatic pretreatment for both microalgae. However, hydrolysis rates for each enzymatic pretreatment showed different values independent on the methane yield. This can be due to the difference in the amount of carbohydrates and proteins released after enzymatic pretreatment.

- Modified Gompertz model were able to fit better than first order kinetic model since it can predict natural behavior of anaerobic digestion. For both microalgae, the lag phases for untreated biomass were 4.14 (*P. cruentum)* and 5.21 (*Scenedesmus sp.)* days. However, these value were lowered after enzymatic pretreatment showing the positive effect on hydrolysis rates.

- The energy assessment results of this study showed that energy ratios for pretreated *Scenedesmus* biomass were between 0.68 and 1.41 for mesophilic digestion and thermophilic digestion the values were more than 1. Lowest energy ratios were belonging to enzyme mix and protease pretreated biomass for both mesophilic and thermophilic digestion. For pretreated P. *cruentum* biomass, the ratios were 0.69-1.05 and 0.54-1.04 for mesophilic digestion and thermophilic digestion,respectively.

Future work

Microalgae have a great potential in terms of methane production and with enzymatic pretreatment higher methane yields were achieved throughout this study. However, there is still research needed to fulfill the gaps in literature and improve the knowledge about pretreatment and biogas production from microalgae. In order to further improve methane production from microalgae some future work is essential which was out of scope of this study. Future works should include:

- Continuous studies can be tested with the most promising enzyme and microalgae at mesophilic/thermophilic temperatures.
- Although enzymatic pretreatment is energy efficient, the cost of enzymes could be a concern for total cost of the process. Use of immobilized enzymes that provides consecutive use of enzymes can be investigated for its feasibility.
- Acclimatized anaerobic inoculums can be used to reduce lag phase of the digestion and increase hydrolysis rate.
- Different substrate concentrations can be investigated for both enzymatic pretreatment and methane production. Moreover, different substrate to inoculum ratios can be studied to improve methane production.
- Co-digestion can be applied to pretreated microalgae to increase C: N ratio for preventing ammonia inhibition at high loading rates.

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APPENDIX

APPENDIX 1- RESULTS OF THE KINETICS OF METHANE PRODUCTION

1.1. First Order Kinetic Model for *Scenedesmus sp.* (MesophilicDigestion)
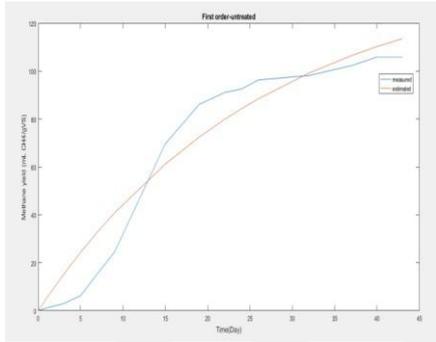
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Thermal pretreatment (55°C)
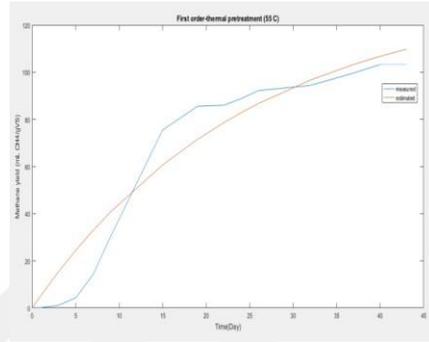
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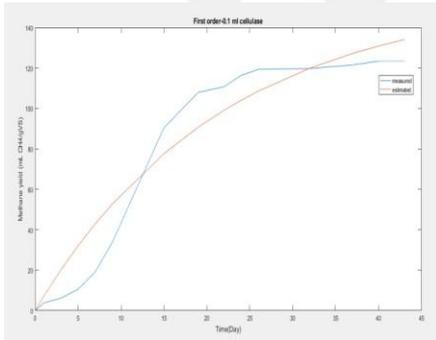
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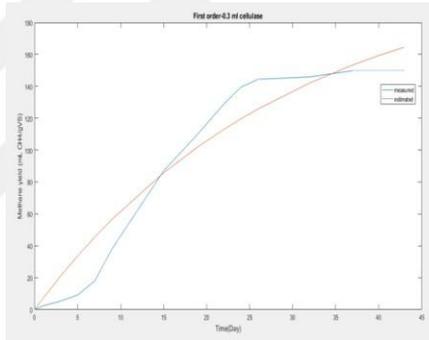
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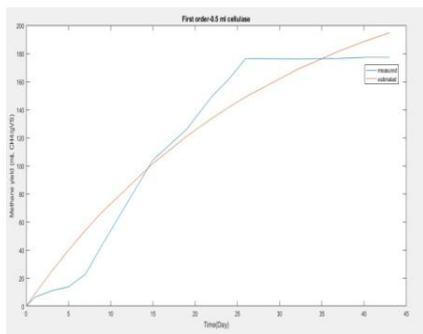
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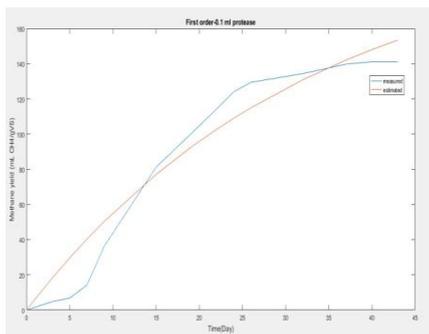
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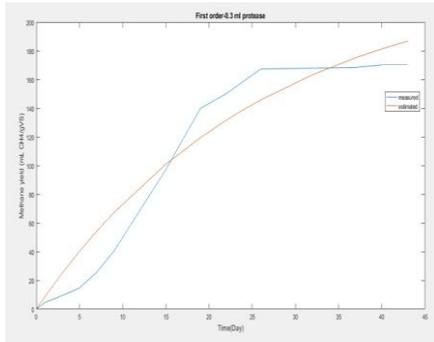
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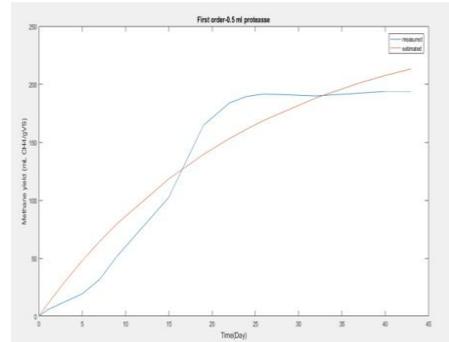
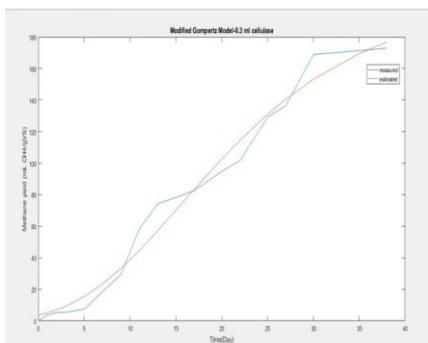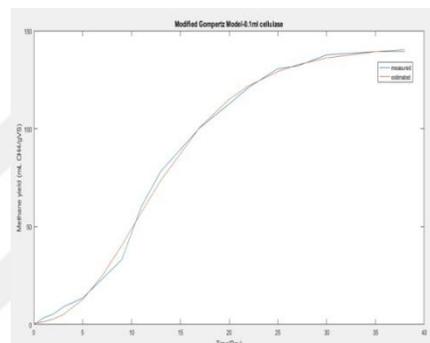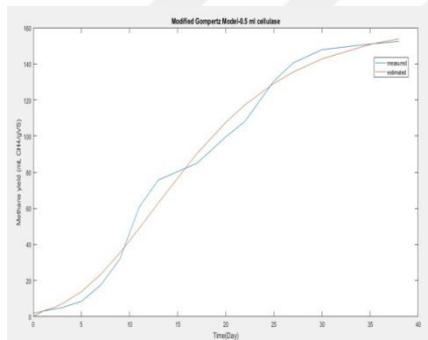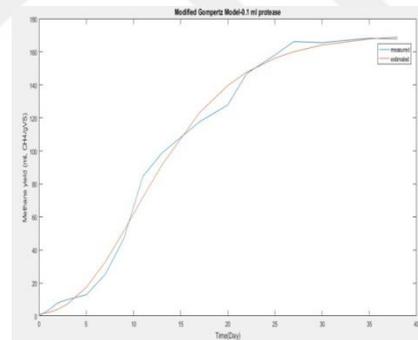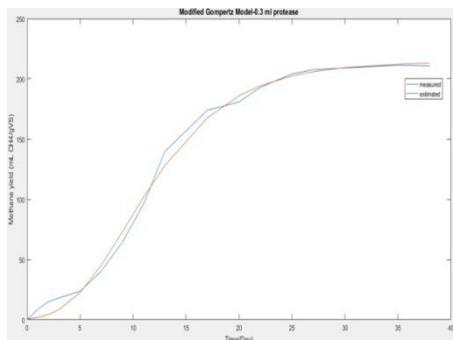## Viscozyme pretreatment (0.1 mL)
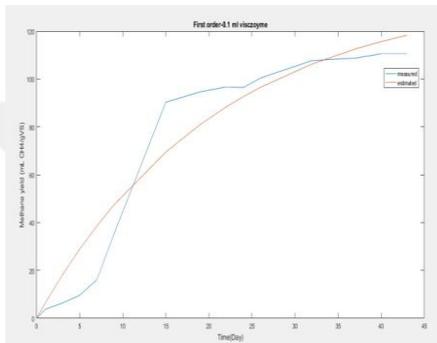
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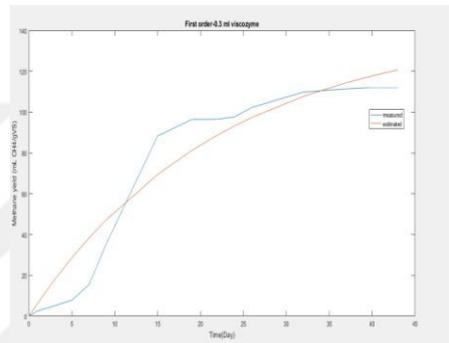
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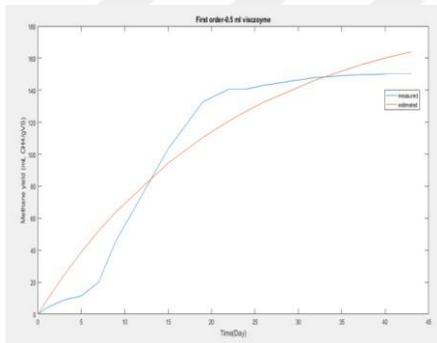
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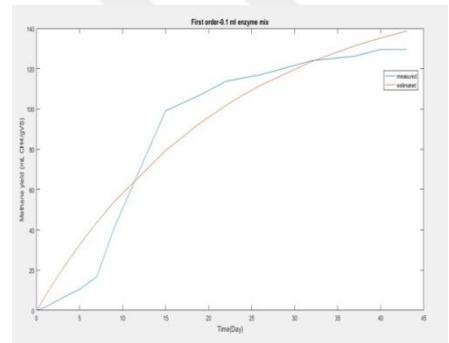
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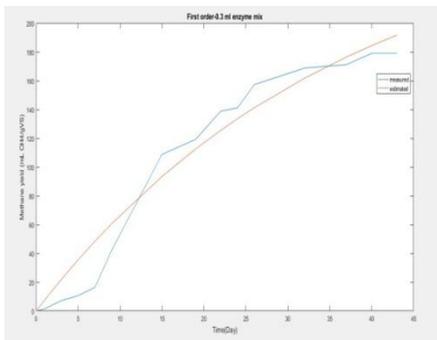
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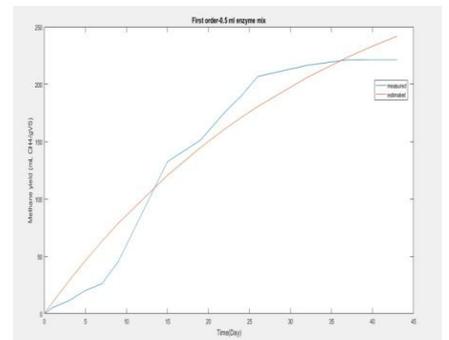
1.2. First Order Kinetic Model for *Scenedesmus sp.* (ThermophilicDigestion)
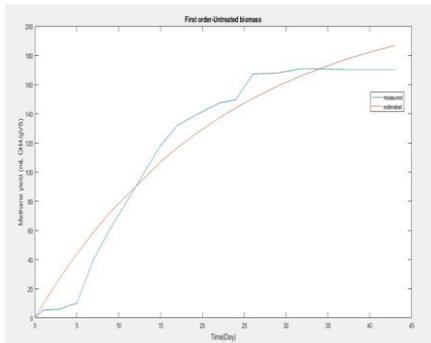
Untreated

Thermal pretreatment (55°C)
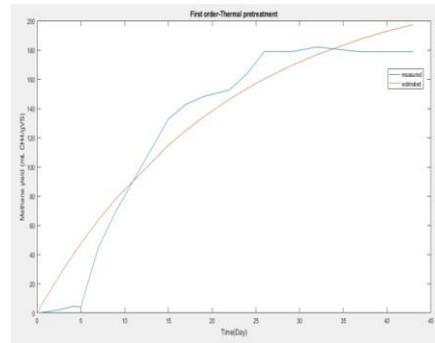
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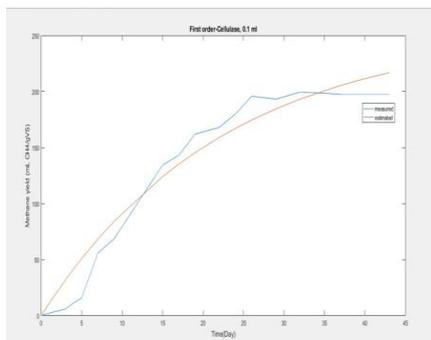
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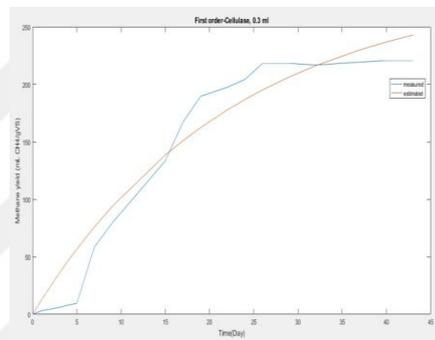
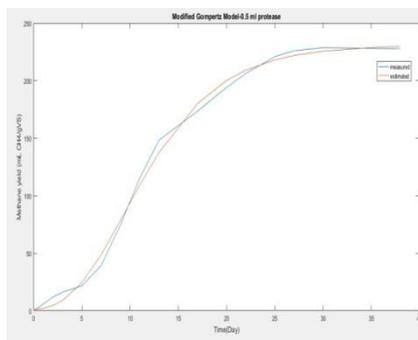

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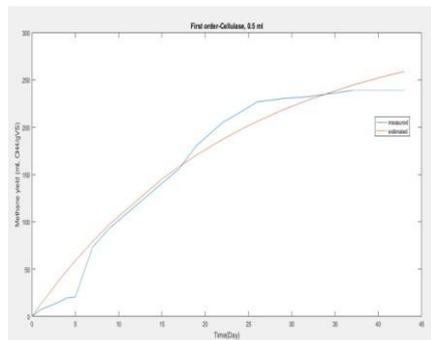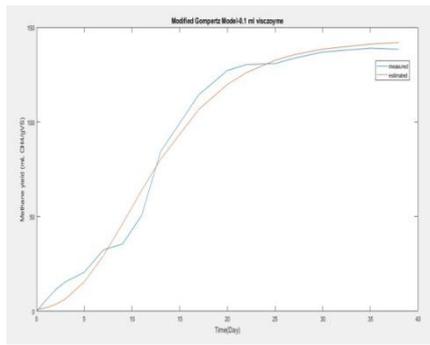

Protease pretreatment (0.1 mL)
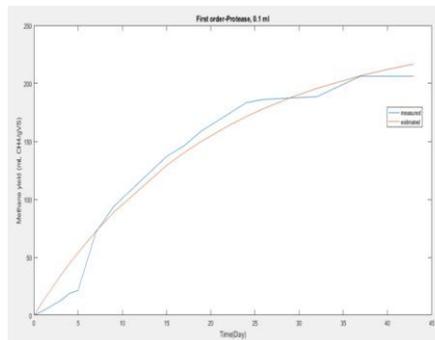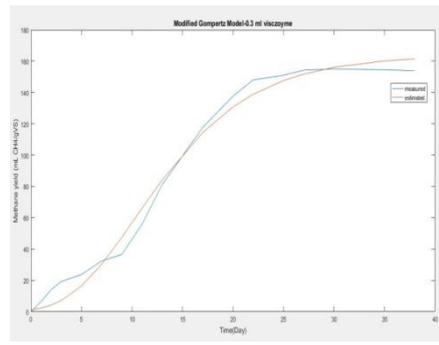

Protease pretreatment (0.3 mL)
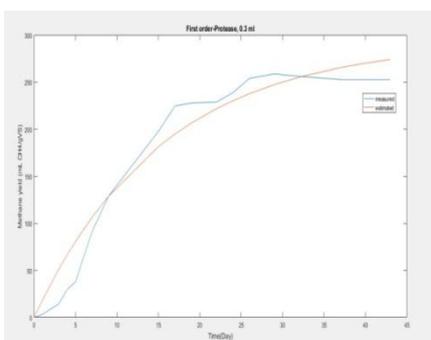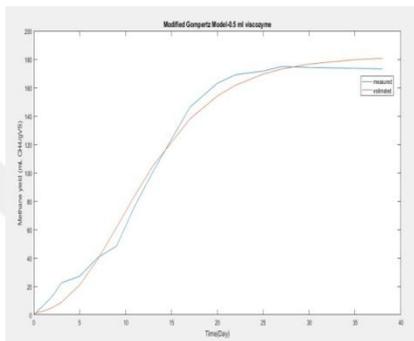

Protease pretreatment (0.5 mL)
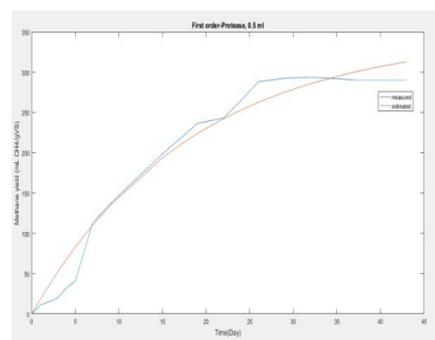
Viscozyme pretreatment (0.1 mL)   Viscozyme pretreatment (0.3 mL)
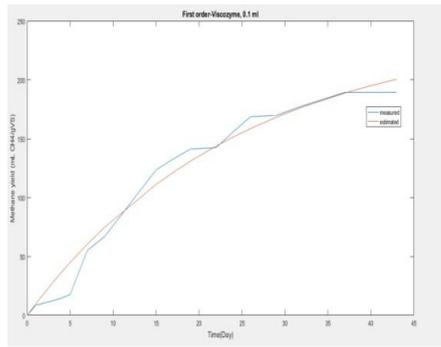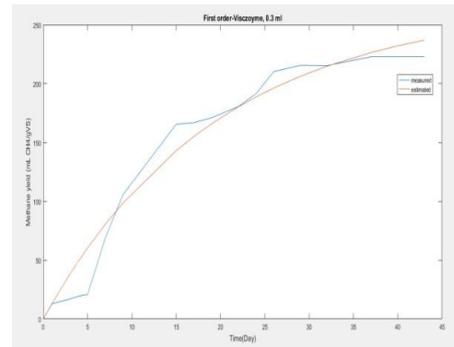
Viscozyme pretreatment (0.5 mL)   Enzyme mix pretreatment (0.1 mL)
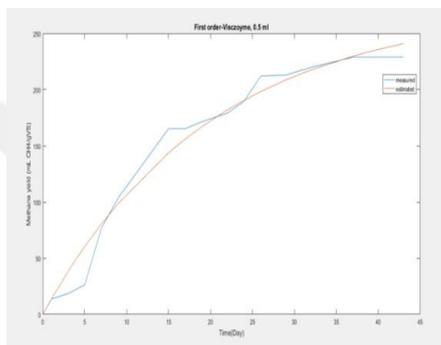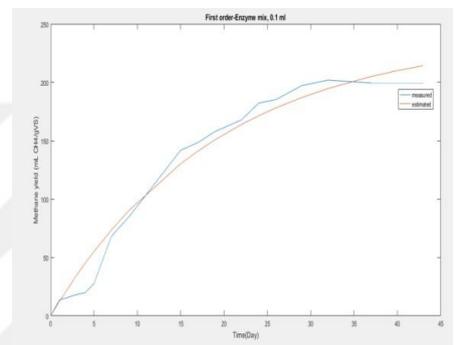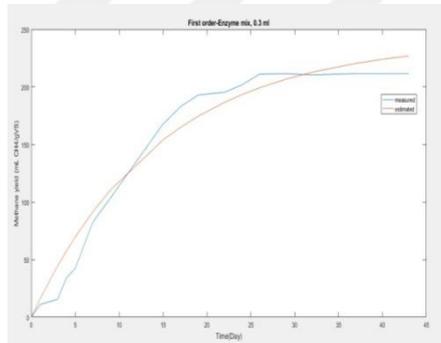
Enzyme mix pretreatment (0.3 mL)   Enzyme mix pretreatment (0.5 mL)
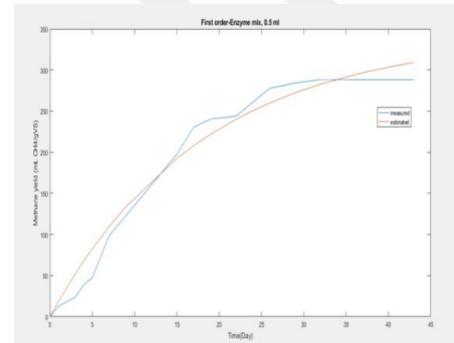
1.3. Modified Gompertz Model for *Scenedesmus sp.* (MesophilicDigestion)

Untreated   Thermal pretreatment (55°C)
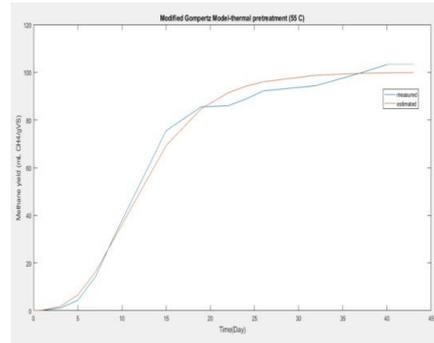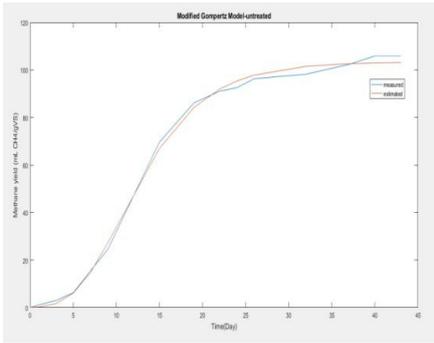
Cellulase pretreatment (0.1 mL)　　　　　Cellulase pretreatment (0.3 mL)
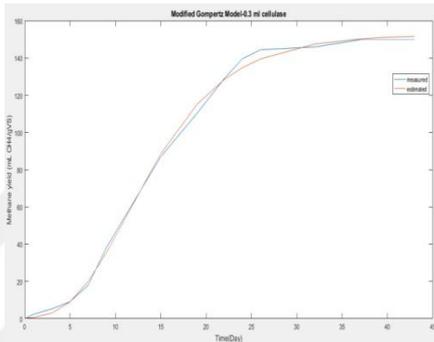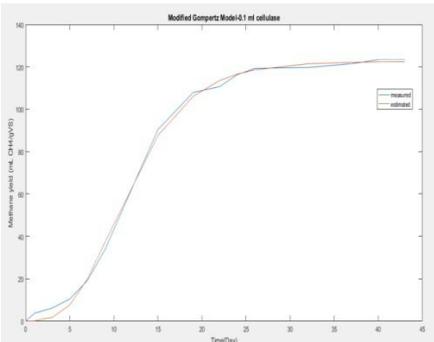
Cellulase pretreatment (0.5 mL)　　　　　Protease pretreatment (0.1 mL)
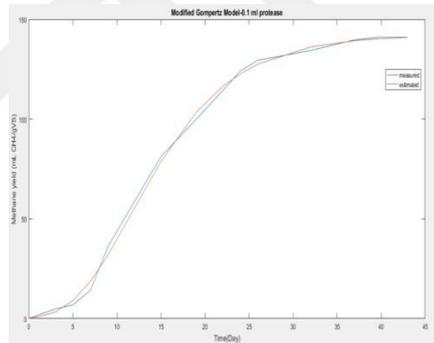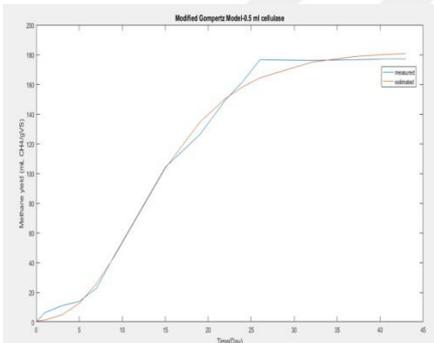
Protease pretreatment (0.3 mL)　　　　　Protease pretreatment (0.5 mL)
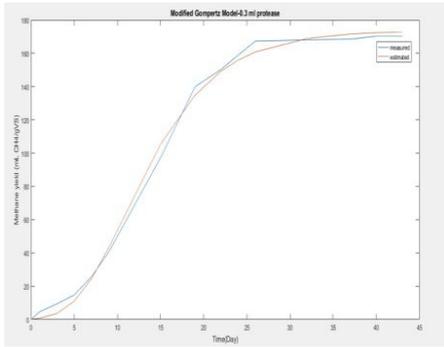
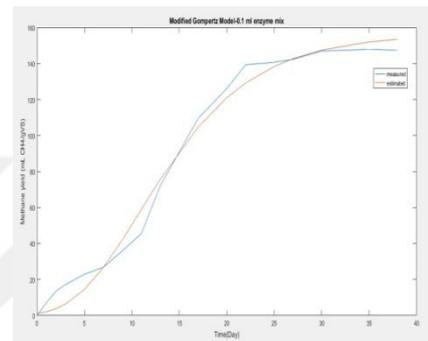Viscozyme pretreatment (0.1 mL)
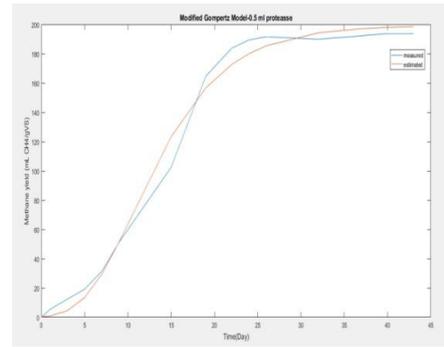
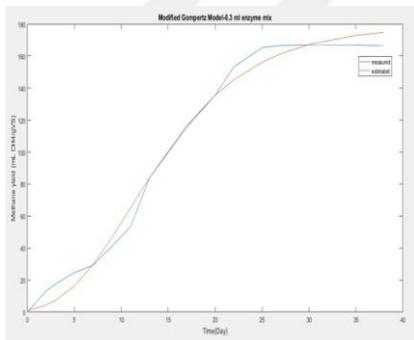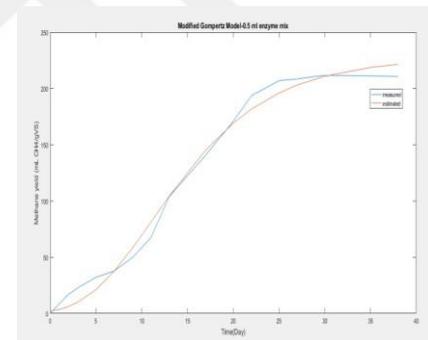Viscozyme pretreatment (0.3 mL)
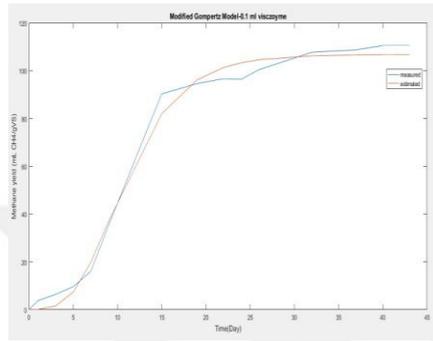
Viscozyme pretreatment (0.5 mL)
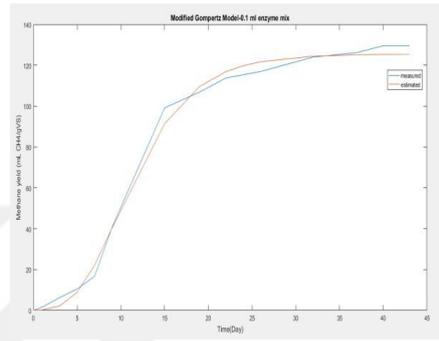
Enzyme mix pretreatment (0.1 mL)
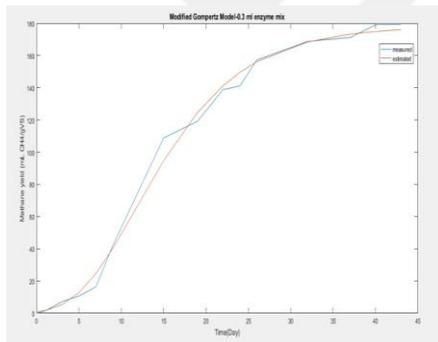
Enzyme mix pretreatment (0.3 mL)
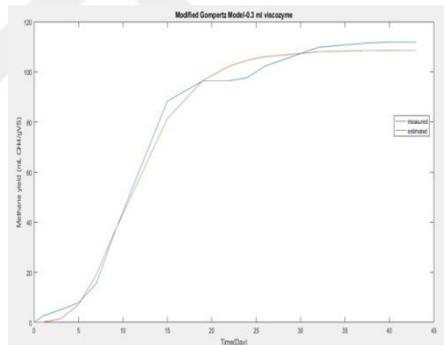
Enzyme mix pretreatment (0.5 mL)
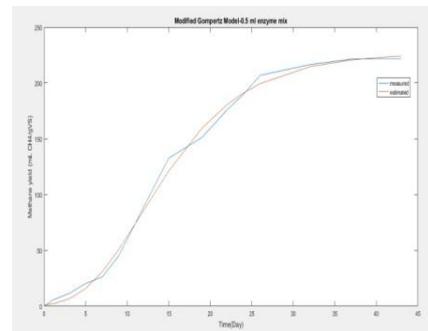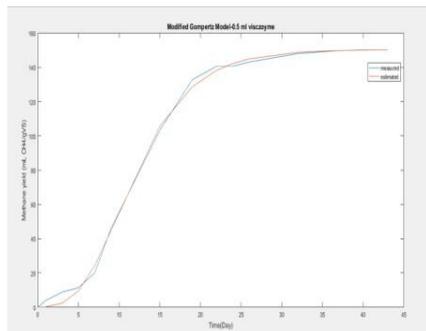
1.4. Modified Gompertz Model for *Scenedesmus sp.* (ThermophilicDigestion)
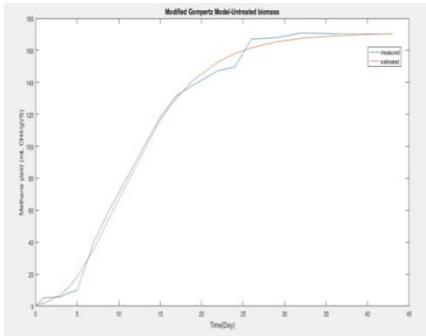
Untreated

Thermal pretreatment (55°C)
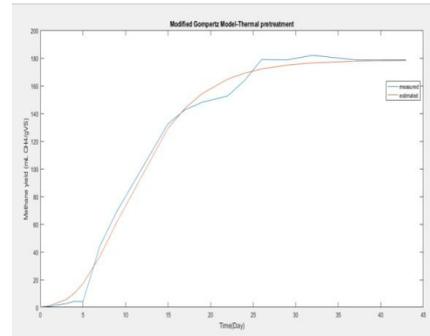
Cellulase pretreatment (0.1 mL)
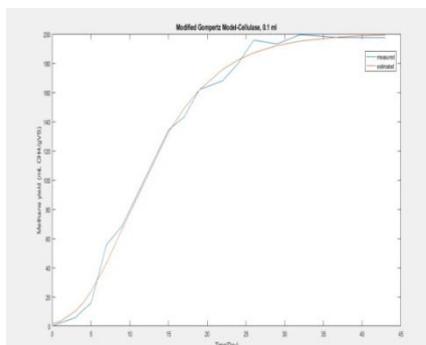
Cellulase pretreatment (0.3 mL)
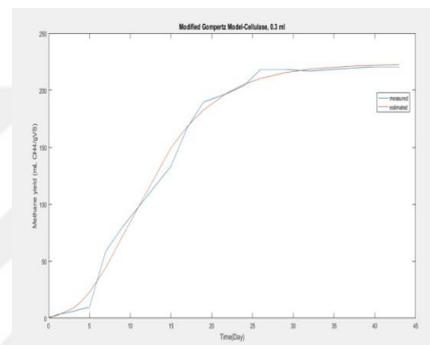
Cellulase pretreatment (0.5 mL)
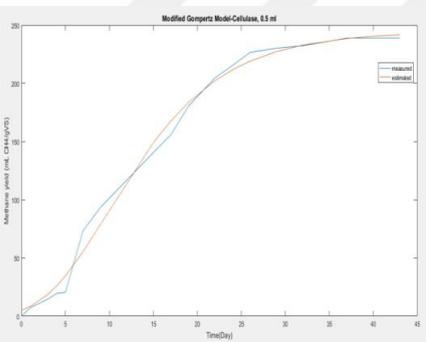
Protease pretreatment (0.1 mL)
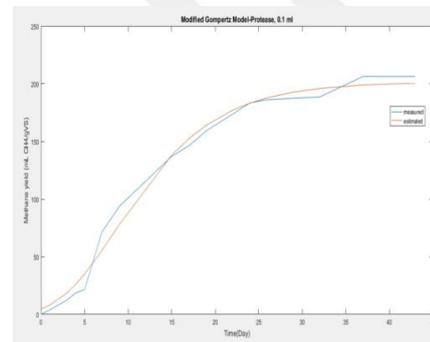
Protease pretreatment (0.3 mL)
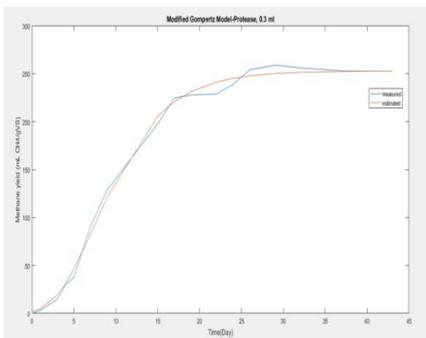
Protease pretreatment (0.5 mL)
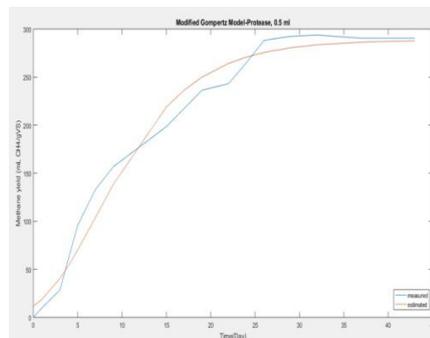
Viscozyme pretreatment (0.1 mL)

Viscozyme pretreatment (0.3 mL)
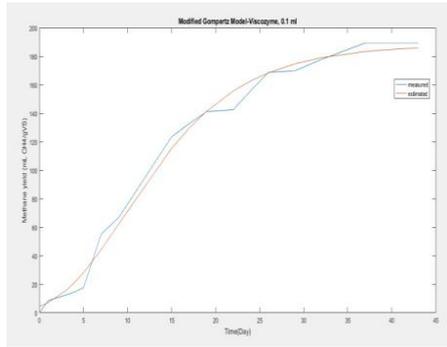
Viscozyme pretreatment (0.5 mL)
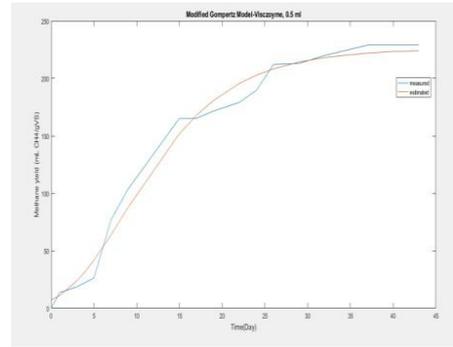
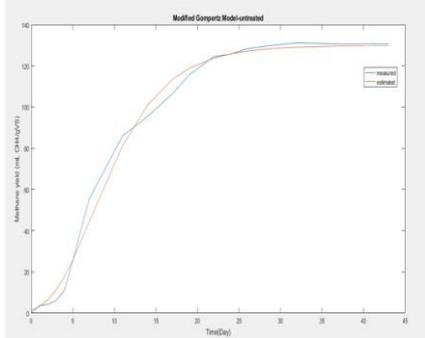Enzyme mix pretreatment (0.1 mL)
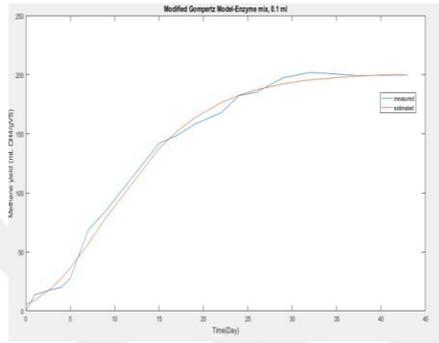
Enzyme mix pretreatment (0.3 mL)
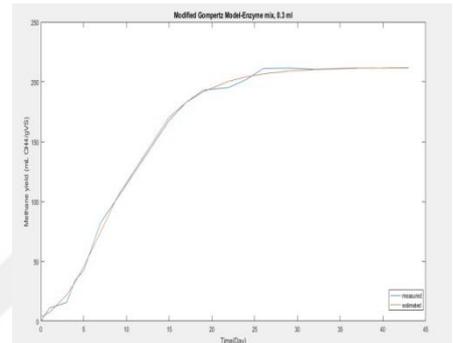
Enzyme mix pretreatment (0.5 mL)
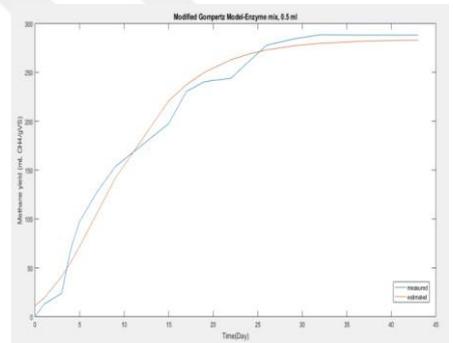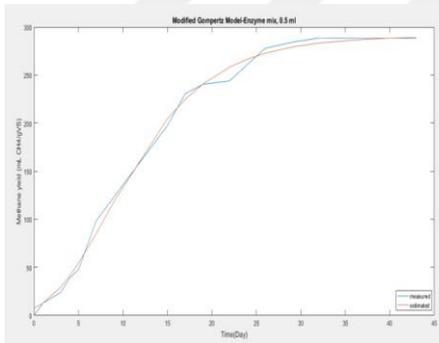
## 1.5. First Order Kinetic Model for *P.cruentum* (MesophilicDigestion)
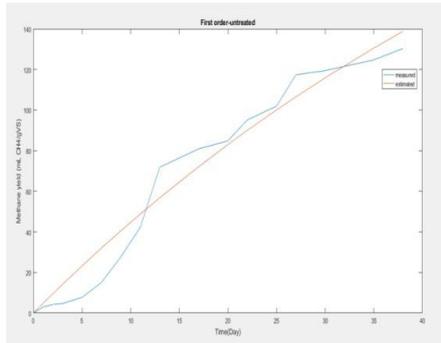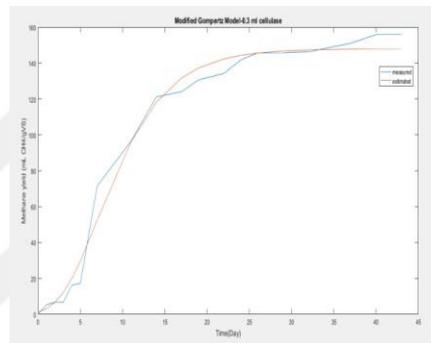
Untreated

Thermal pretreatment (55°C)
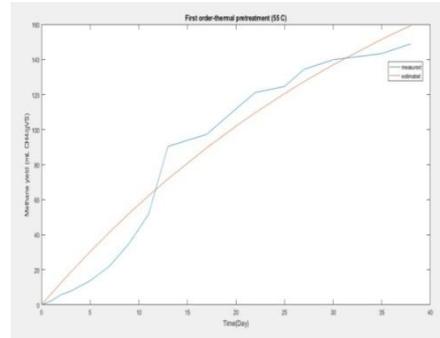
Cellulase pretreatment (0.1 mL)
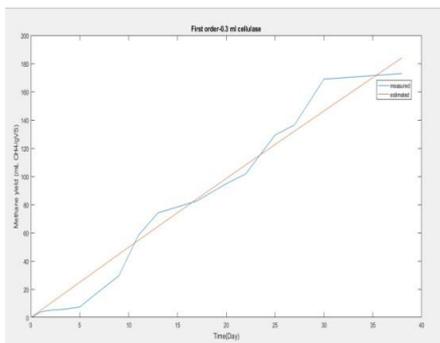
Cellulase pretreatment (0.3 mL)
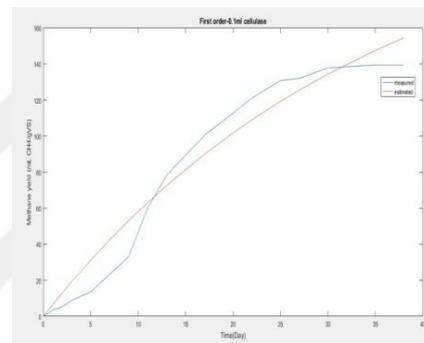
Cellulase pretreatment (0.5 mL)
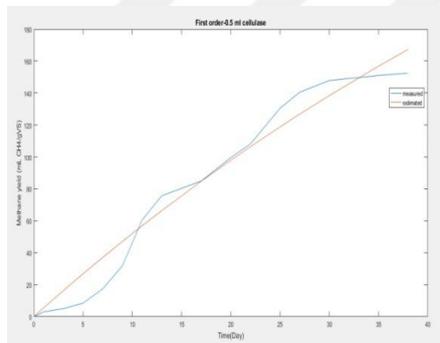
Protease pretreatment (0.1 mL)
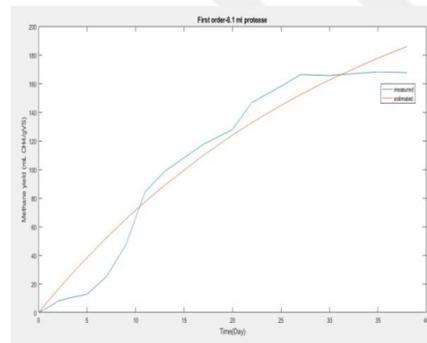
Protease pretreatment (0.3 mL)
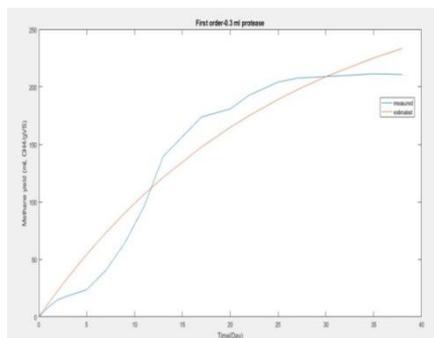
Protease pretreatment (0.5 mL)
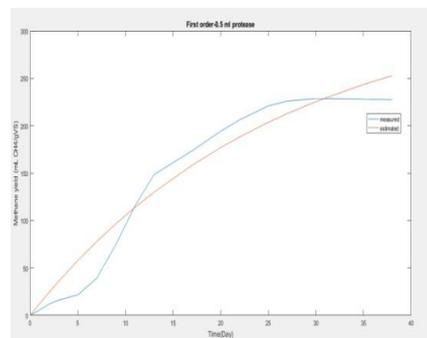
Viscozyme pretreatment (0.1 mL)
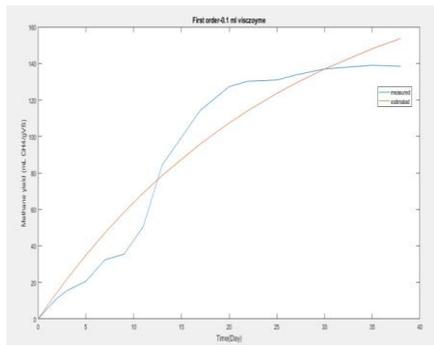
Viscozyme pretreatment (0.3 mL)
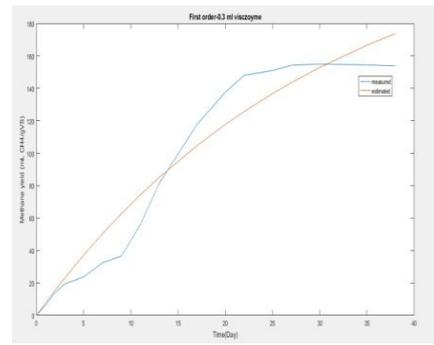
Viscozyme pretreatment (0.5 mL)
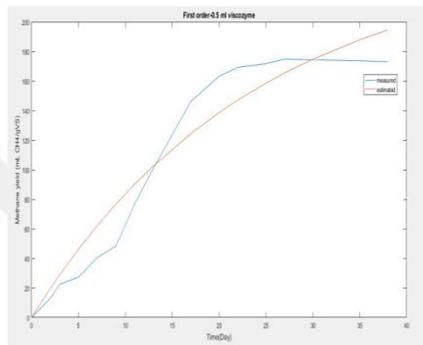
Enzyme mix pretreatment (0.1 mL)
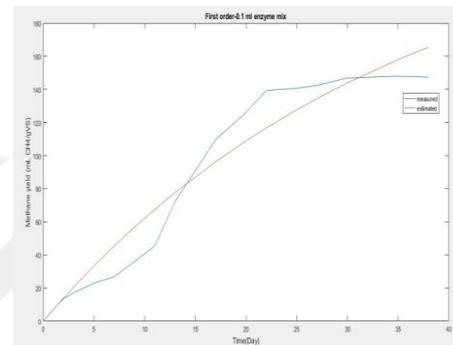
Enzyme mix pretreatment (0.3 mL)
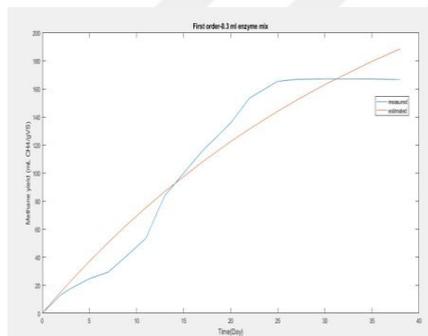
Enzyme mix pretreatment (0.5 mL)
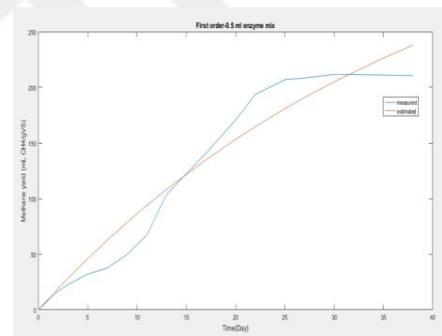
1.6. First Order Kinetic Model for *P. cruentum* (ThermophilicDigestion)
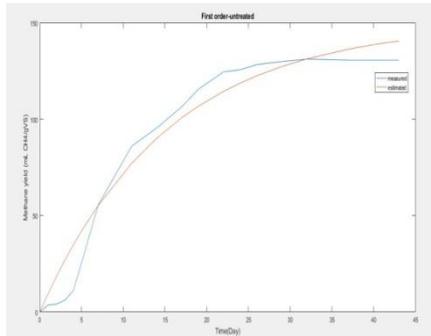
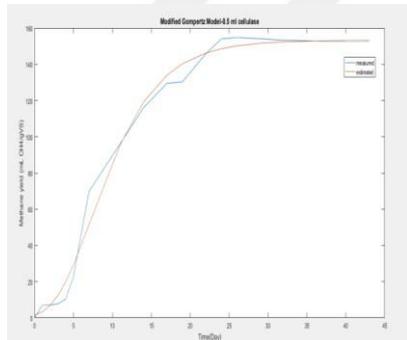Untreated

Thermal pretreatment (55°C)
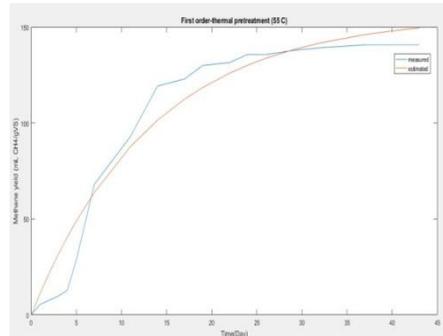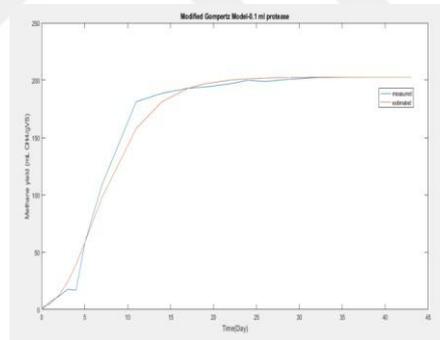

Cellulase pretreatment (0.1 mL)
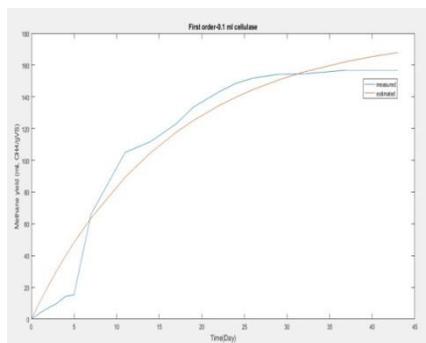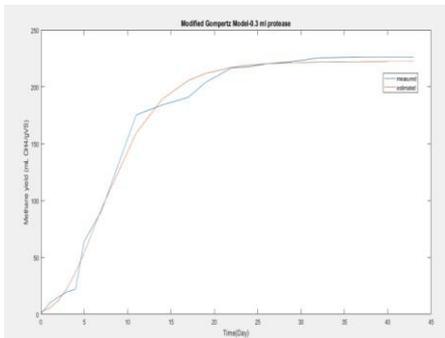

Cellulase pretreatment (0.3 mL)
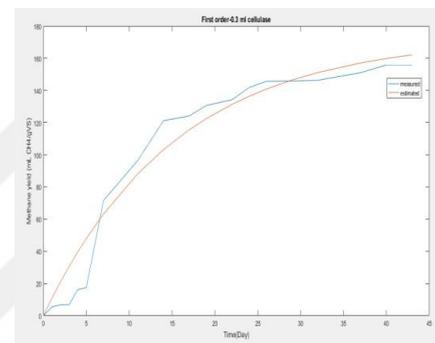
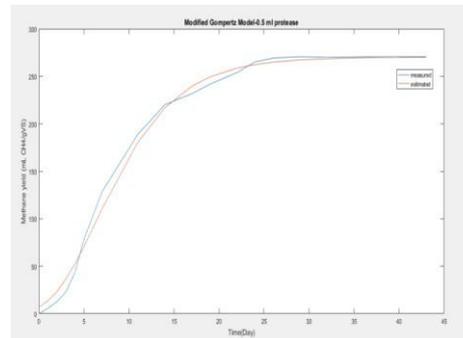Cellulase pretreatment (0.5 mL)
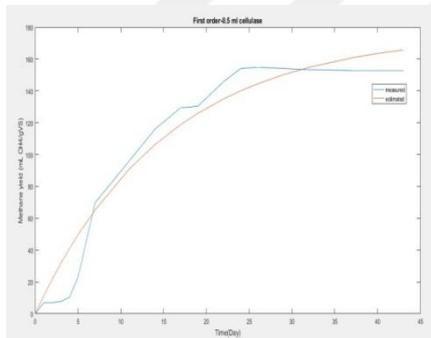
Protease pretreatment (0.1 mL)
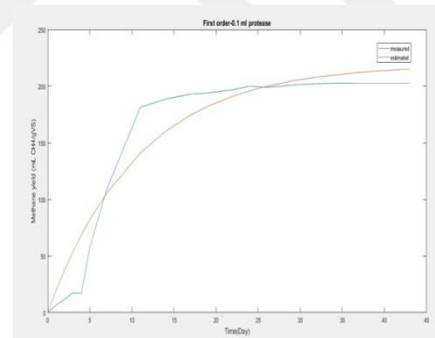
Protease pretreatment (0.3 mL)
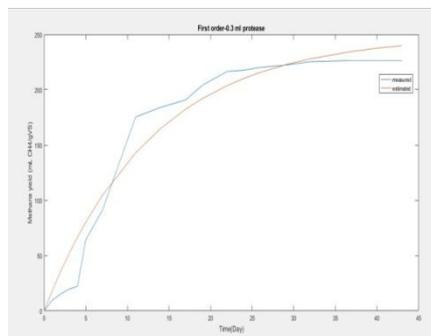
Protease pretreatment (0.5 mL)
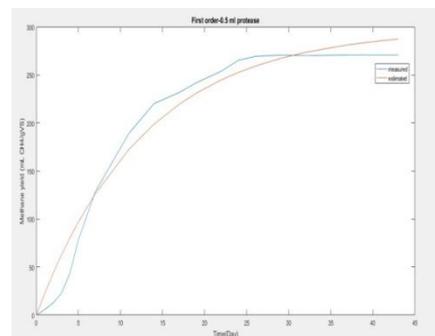
Viscozyme pretreatment (0.1 mL)   Viscozyme pretreatment (0.3 mL)
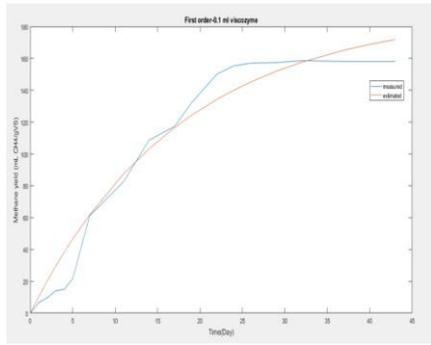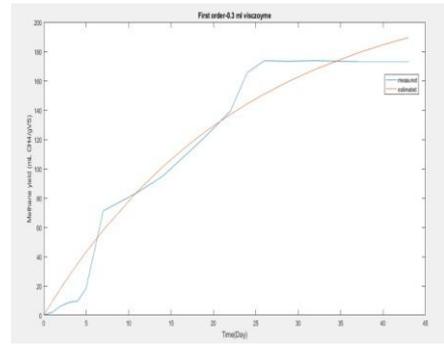
Viscozyme pretreatment (0.5 mL)   Enzyme mix pretreatment (0.1 mL)
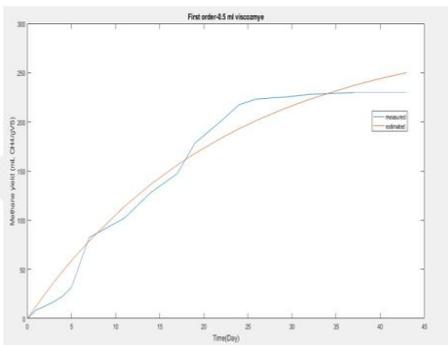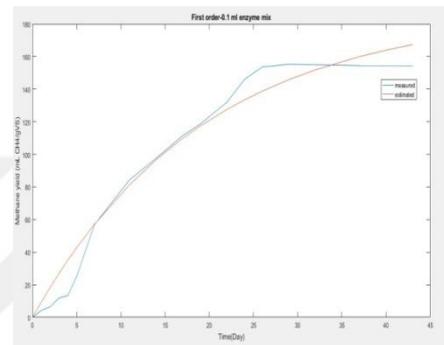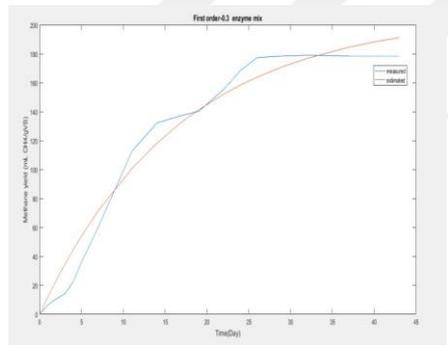
Enzyme mix pretreatment (0.3 mL)   Enzyme mix pretreatment (0.5 mL)
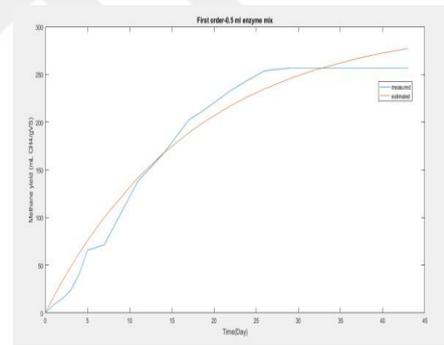
1.7. Modified Gompertz Model for *P.cruentum*(MesophilicDigestion)
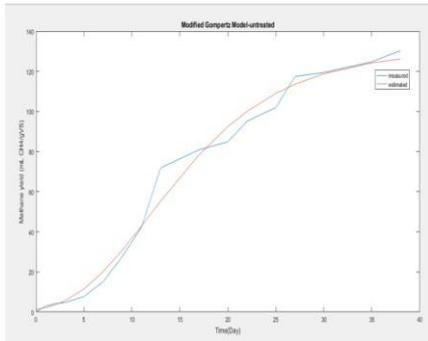
Untreated

Thermal pretreatment (55°C)
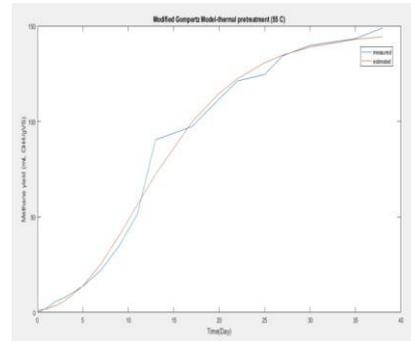
Cellulase pretreatment (0.1 mL)

Cellulase pretreatment (0.3 mL)

Cellulase pretreatment (0.5 mL)

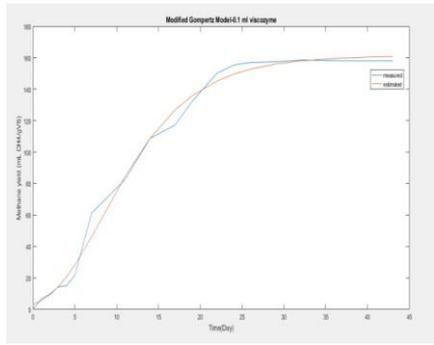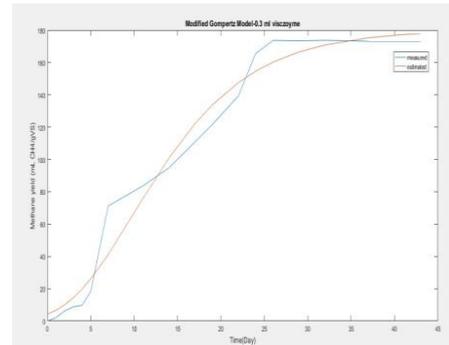Protease pretreatment (0.1 mL)

Protease pretreatment (0.3 mL)

Protease pretreatment (0.5 mL)

Viscozyme pretreatment (0.1 mL)

Enzyme mix pretreatment (0.1 mL)

Viscozyme pretreatment (0.3 mL)

Viscozyme pretreatment (0.5 mL)

Enzyme mix pretreatment (0.3 mL)

Enzyme mix pretreatment (0.5 mL)

1.8. Modified Gompertz Model for *P.cruentum*(ThermophilicDigestion)

Untreated

Thermal pretreatment (55°C)
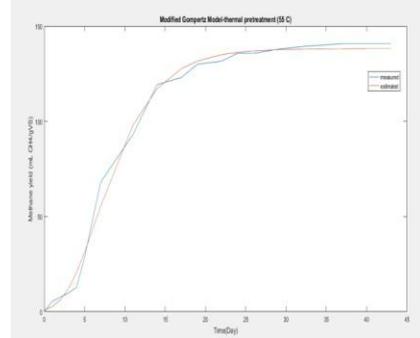
Cellulase pretreatment (0.1 mL)
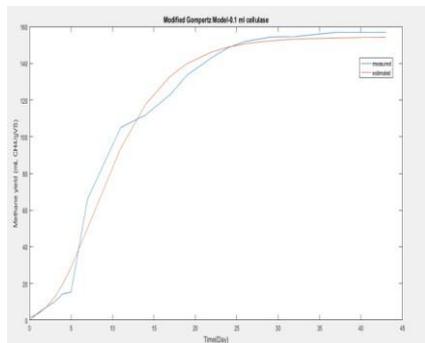
Cellulase pretreatment (0.3 mL)

Cellulase pretreatment (0.5 mL)

Protease pretreatment (0.1 mL)

Protease pretreatment (0.3 mL)

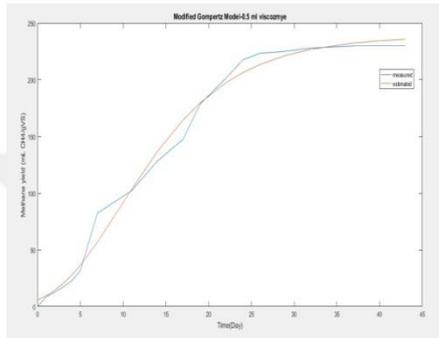Protease pretreatment (0.5 mL)
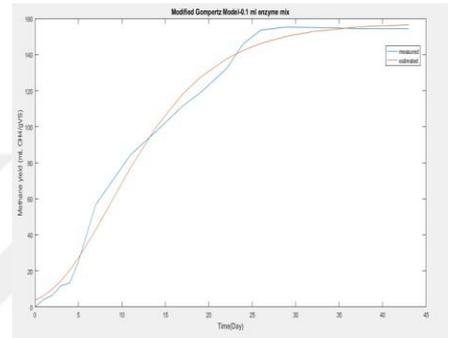
Viscozyme pretreatment (0.1 mL)
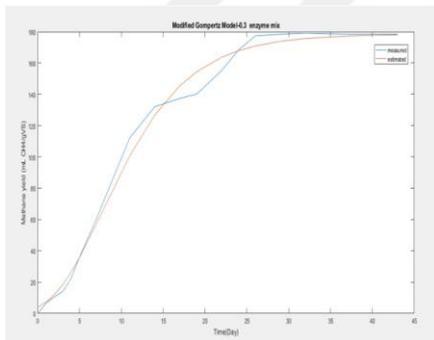

Viscozyme pretreatment (0.3 mL)

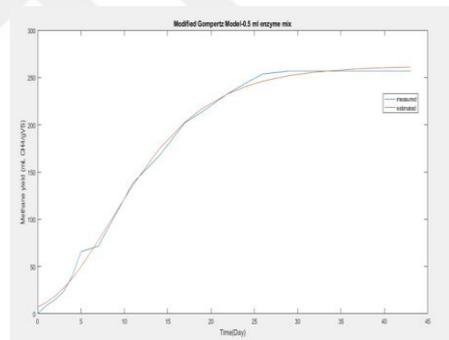Viscozyme pretreatment (0.5 mL)

Enzyme mix pretreatment (0.1 mL)

Enzyme mix pretreatment (0.3 mL)

Enzyme mix pretreatment (0.5 mL)

HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF SCIENCE AND ENGINEERING
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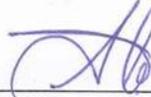I respectfully submit this for approval.

26. 07. 2019

Date and Signature

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