Green algae as a substrate for biogas production - cultivation and biogas potentials

Yang Liu

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Supervisor: Anna Karlsson

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Abstract

Algae is regarded as a good potential substrate for biogas production, due to high cells productivity, low cellulose and zero lignin content. Two parts were included in this study: first, cultivations of micro-algae (*Chlorella sorokiniana* and *Tetraselmis suecica*) at two different nitrate concentrations, also the effect of addition of CO₂ on algae grow was investigated in this first part. Second, batch fermentations of the cultivated micro-algae as well as a powder *Chlorella* (obtained from Raw Food Shop) and a dry mix filamentous algae (collected in the pounds in the park at the back of the Tema-building and then dried) were performed. In this part also effects of thermo-lime pretreatment (room temperature, 80°C, 105°C and 120°C) on the algae biogas potentials was investigated.

Both strains of micro-algae cultured at low nitrate gave more CH₄ yield: 319 (±26) mL and 258 (±12) mL CH₄ per added gVS was obtained during the degradation of *Chlorella sorokiniana* grown at 0.4mM-N and 2mM-N level, respectively. For *Tetraselmis suecica* 337 (±37) mL and 236 (±20) mL CH₄ per added gVS was obtained at 2.4mM-N and 12mM-N level, respectively. Powder *Chlorella* gave the highest biogas production (719 ±53 mL/added gVS) and CH₄ yields (392 ±14 mL/added gVS), followed by the dry filamentous algae (661 ±20 mL biogas and 295 ±9 mL CH₄ per added gVS) and *Tetraselmis suecica* (12 mM-N; 584 ±7 mL biogas and 295 ±9 mL CH₄ per added gVS).

A negative effect of lime treatment at room temperature on CH₄ yield of algal biomass was obtained. Lime treatment at 120°C showed the fastest degradation rate for *Tetraselmis suecica* and powder *Chlorella* during the initial 5 days of incubation.

*Chlorella sorokiniana* and *Tetraselmis suecica* cultures flushed with biogas containing 70% and also CO₂ enriched air (5% CO₂) did not increase cells growth (measured as OD₆₀₀) if compared to references grown under air. On the contrary, a clearly inhibition effect on the algal cells growth was observed in some cultures.

Key words: Anaerobic digestion, micro-algae cultivations, N limiting, thermo-lime pretreatment.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AD</td>
<td>anaerobic digestion</td>
</tr>
<tr>
<td>C.S.</td>
<td><em>Chlorella sorokiniana</em></td>
</tr>
<tr>
<td>T.Su</td>
<td><em>Tetraselmis suecica</em></td>
</tr>
<tr>
<td>GC-FID</td>
<td>gas chromatography-flame ionization detector</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute. 1 rpm = $2\pi \text{ radian min}^{-1} = 2\pi/60 \text{ radian s}^{-1}$</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>TS</td>
<td>total solids</td>
</tr>
<tr>
<td>VS</td>
<td>volatile solids</td>
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</table>
Chapter 1. Introduction

Global warming is recognized as one of the main environmental issues of our time and it is attributed primarily to the increase of the carbon dioxide (CO₂) level in the atmosphere (Kondili & Kaldellis, 2007). The combustion of fossil fuels is the primarily reason for the accumulation of CO₂. The Kyoto Protocol (1997), with the objective of greenhouse gas reductions, started to push research and development aiming at CO₂ mitigation and thus various strategies have been investigated (Gutierrez et al., 2008).

One way to decrease the release of fossil CO₂ can be to increase the use of biofuels. Biomass acts as raw material to produce biofuels. The cycle of biomass converted to biofuels is regarded as CO₂ neutral as not net CO₂ is emitted into the atmosphere in this process. (Schenk et al., 2008) Among various biofuels, biogas formed by anaerobic degradation seems to be one of the best biofuels, as its production consumes relatively small amounts of energy, it also have relatively low construction costs and space requirement, etc (Reith et al., 2003).

Substrates utilized for biogas production includes solid waste (such as agricultural and household wastes, etc), waste water and sludge, as well as manure or liquid manure. (Reith et al., 2003). Besides, new substrates are also being investigated, like algae. Anaerobic digestion based on algae can be a possible future substrate for the production of biogas as a renewable fuel. They lack lignin and have low cellulose content, which make them a good substrate for anaerobic digestion (Vergara-Fernández et al., 2008). Besides, microalgae have an efficiency of 10 to 50 times greater than that of terrestrial plants when capturing CO₂ and solar energy (Li et al., 2008; Usui & Ikenouchi, 1997). Thus, CO₂ mitigation culturing micro-algae combined with biogas production seems to be a part of a good strategy for reaching the goal of the Kyoto Protocol.

In spite of that the research of anaerobic digestion of micro-algae biomass started already in 1956, only a few studies have been reported (Dong et al., 2006; Golueke et al., 1956; Nair et al., 1983; Samson & Duy, 1982; Sialve et al., 2009; Xu et al., 2009). There are also some studies on biogas production from macro-algae (Habig et al., 1984; Morand & Briand, 1998; Vergara-Fernández et al., 2008; Viglia, 1985). A lot of researches have however been carried out on the oil extraction for biodiesel production, due to micro-algae with potential high lipid content and the lipid content has also been shown to increase when micro-algae are cultivated under nitrogen (N) limiting conditions (Takagi et al., 2000; Yamaberri et al., 1998; Li et al., 2008a).

Algae cells are protected by rigid cell walls which are composed of cellulose or other polymers (Graham & Wilcox, 2000ab). Pretreatment could be an effectively way to break down the rigid cells walls and improve the digestibility of algae biomass. Considering low cost and high efficiency in terms of biodegradability (Hoon, 2004; Heo
et al., 2003; Saiki et al., 1999), a thermo-lime treatment was chosen for this study.

Based on the above, I chose to investigate the methane production potential of one marine and one fresh water micro-algae grown at high and low N-level. *Chlorella sorokiniana* (Tebouxiophyceae, Chlorella) and *Tetraselmis suecica* (Prasinophyceae, Tetraselmis) were chosen as the research objects for micro-algae. The biogas potential of a mix filamentous macro-algae was also investigated. The effect of pretreatment (thermo-lime) of the algae on their methane potential with was also investigated.

This thesis can be divided into two parts including cultivation and degradation: in the first part *C. sorokiniana* was cultivated at 0.4 mM-N and 2 mM-N concentrations and *T. suecica* was cultivated at 2.4 mM-N and 12 mM-N concentrations. The optical density (OD) of the cultures was monitored over time. The cultures where normally flushed with air but also a mix of gas air/CO₂ (95:5) and biogas (70% CO₂) from an acid reactor was tested. The biogas and methane production potentials for both macro- and micro-algae were then investigated in the second part. Additionally, pretreatment of algal biomass with lime at four temperatures (room temperature-RT, 80°C, 105°C and 120°C; over night) was applied in order to study if pretreatment with lime and or heat could improve the methane yield from the algal biomass.

### Aim and Hypothesis

The main aim of this thesis is to investigate the biogas and methane potential on three kinds of green algae. The influence of N concentrations during algae growth as well as thermo-lime pretreatment on the algae biomass before digestions will be investigated. In addition, possible increases in micro-algae growth rates/cell density by supplying CO₂ during cultivation of algae will be investigated.

Hypothesis behind my study are:

- The micro-algae *C. sorokiniana* and *T. suecica* grown at low N-level can give a higher fat content in the algae and thus a higher methane production than that of algae grown at high N-level.

- Algae treated with lime at 120°C will give the highest methane production.

- *C. sorokiniana* and *T. suecica* cultured under flushing with CO₂ enriched gas can increase algal biomass productivity.
To prove the hypotheses, the following question was addressed:

1. What are the methane yield per added g VS of \textit{C. sorokiniana} grown at 0.4 mM-N and 2 mM-N level and \textit{T. suecica} grown at 2.4 mM-N and 12 mM-N level?

2. What are the biogas and methane yield per added g VS of lime/heat pretreated and untreated micro- and macro-algae?

3. What are the values of optical density (OD) of \textit{C. sorokiniana} and \textit{T. suecica} grown under CO$_2$ enriched gas?
Chapter 2. Background

2.1 Micro-algal CO₂ bio-mitigation

Anthropogenic emissions of CO₂ are estimated to $2 \times 10^{10}$ tons per year, which are primarily due to the combustion of fossil fuels. Additionally, an increasing global population further speeds up annual energy consumption and atmospheric CO₂ accumulation, which aggravates the effects of global warming. (Zeiler et al., 1995

The Kyoto Protocol (1997) promoted by the United Nations aims for CO₂ mitigation (Gutierrez et al., 2008). Methods of capturing CO₂, such as absorption, adsorption and chemical fixation, have thus been investigated. However, these methods are relatively costly and energy consuming if to be used in a large-scale (Wang et al., 2008). A method called micro-algal CO₂-fixation has in this context been given attention as a less expensive and energy consuming alternative (Karube et al., 1992).

CO₂-fixation can be carried out by plants photosynthesis utilizing solar energy to produce carbohydrates. (Kondili & Kadellis, 2007; Ragauskas et al., 2006; de Morais & Costa, 2007). However, it is estimated that plants in agriculture are responsible for only 3-6% of fossil fuel emissions in terms of CO₂ capture, which is primarily due to the conventional terrestrial plants with low growth rates (Skjanes et al., 2007). Micro-algae, with the advantage of being fast-growing are however considered to be good alternative for CO₂ mitigation. The micro-algae have an efficiency of 10 to 50 times greater than that of terrestrial plants in terms of capturing CO₂ and solar energy (Li et al., 2008; Usui & Ikenouchi, 1997). Therefore, CO₂ bio-mitigation culturing microalgae might be one of good ways to reach the goal of the Kyoto Protocol, especially if combined with biofuels production.

Atmosphere and industrial flue gases provide “free” or low cost sources for CO₂ capture using micro-algae. Compared to low atmospheric CO₂ concentration (0.03-0.06%) (Chelf et al., 1993), industrial exhausted gases containing hundreds times higher CO₂ can provide a CO₂-rich source for micro-algae cultivation. Since industrial flue gases are responsible for more than 7% of the total world CO₂ emissions (Sakai et al., 1995), micro-algal CO₂ capturing from these gases can achieve the CO₂ abatement and contribute algal biomass to be used for biofuels production.

2.2 Effects of micro-algal growth at different N-levels

Currently, there are many researches on biodiesel production from micro-algae with
high lipid contents. The micro-algae lipid content has been shown to depend on N availability as large quantities of lipids seems to be accumulated in cells when algae is grown at limiting N-conditions. (Liu et al., 2008; Xiong et al., 2008; Takagi et al., 2006; Bigogno et al., 2002; Li & Qin, 2005). Early as in the 1940s, N limited *Chlorocella pyrenoidosa* was shown to accumulate up to 85% lipid in their cells. (Spoehr & Milner, 1949).

The reason for this accumulation seems to be a redirection of carbon flows from protein synthesis to lipid or carbohydrate. Rodolfi et al. (2009) also suggested that the increase in lipid content of micro-algae cultured at limiting N-level are mainly obtained at the expense of other components, particularly proteins. Unfortunately, these high lipid cell contents are achieved under N limitation growth, which is often associated with low biomass productivity (Li et al., 2008a).

### 2.3 Biogas

Biogas comprises primarily CO₂ and the inflammable methane (CH₄), but also trace amounts of nitrogen, hydrogen, hydrogen sulfide and oxygen can be present. Biogas is a renewable energy carrier which is formed from biological breakdown of organic matter in oxygen-free environments (Raven & Gregersen, 2004). Normally, biogas contains 55-75% CH₄ and 25-45% CO₂, the proportions are largely determined by what type of raw materials that is degraded. According to the theoretical equations of digestion, fat gives a higher CH₄ content of the produced gas than both proteins and carbohydrates, thus making substrates with a high fat content attractive. (Berglund & Börjesson, 2006)

Biogas is mainly used for heat and electricity production, but it can also be used as a vehicle fuels or be injected into the natural gas grid (Börjesson & Mattiasson, 2008). Unlike fossil fuels the combustion of biogas gives no net release of CO₂ into the atmosphere, biogas can thus play a significant role in a future, environmentally sustainable, society.

### 2.4 The Biogas process

#### 2.4.1 Anaerobic digestion (AD)

CH₄ and CO₂ are produced through a series of degradation processes involving several species of microorganisms and enzyme’s activities. The process can roughly be divided into: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1)
Hydrolysis is the first phase in the process of anaerobic digestion. Fermentative microorganisms are active in this phase, breaking down complex polymers (proteins, carbohydrates and lipids) into soluble organic compounds ($C_{10}$-$C_{100}$, e.g. glucose, fatty acids and amino acids). Due to the size of the polymers, fermentative bacteria cannot perform intracellular metabolism directly, and thus extracellular enzymes are used in the degradation (Kaseng et al., 1992).

Acidogenesis is the phase following hydrolysis. Fermentative bacteria also perform the acidogenesis, where they metabolize organic compounds from the hydrolysis. Fermentation products are volatile fatty acids such as acetate, propionate and butyrate, but also alcohols, lactate, and $CO_2 + H_2$ (Gujer & Zehnder, 1983).
In the third phase (acetogenesis), intermediary compounds (fatty acids and alcohols, etc) resulting from acidogenesis are converted to acetate, H$_2$ and CO$_2$ by proton reducing acetogenic bacteria. A low partial pressure of H$_2$ is favorable in this process. (Zinder, 1984)

Methanogenesis is the last phase of anaerobic digestion. Methanogenesis occurs mainly by two pathways: one is called acetotrophic where acetate is converted to CH$_4$ by aceticlastic methanogens, the other is called hydrogenotrophic where H$_2$ and CO$_2$ is used to produce CH$_4$ by CO$_2$-reducing methanogens (Zinder, 1984).

### 2.4.2 Pretreatment

The process of anaerobic digestion is often limited by the rate of the first phase-hydrolysis, the rate of hydrolysis of the organic materials (Tiehm et al., 2001). Pretreatment methods can be roughly categorized into: (Kumar et al., 2009)

- Physical pretreatment, such as milling and grinding
- Physicochemical pretreatment, such as steam, thermal, hydrothermolysis, and wet oxidation.
- Chemical pretreatment, such as alkali, acid and oxidizing agents.
- Biological and Electrical pretreatment.
- Or a combination of some of the above.

Every pretreatment method has their advantages and limitations, which are shown in table 1 of the appendix (lignocellulose biomass is used as an example). A combination of thermal and alkali treatment was applied in this study, due to the low cost and high efficiency on biodegradability shown for substrates such as corn stover and municipal organic wastes (Hoon, 2004; Heo et al., 2003). Also the thermo-alkali pretreatment is the best known method for enhancing the biodegradation of complex materials (Haug et al., 1983; Millet et al., 1975; Pavlostathis & Gossett 1985; Patel et al., 1993).
Chapter 3. Methods and Materials

3.1 Description of substrates

The potential of green micro- and macro-algae as substrates for biogas production was investigated. For micro-algae, pure startup cultures of fresh water C. sorokiniana and sea water T. suecica were obtained from CCAP (Culture Collection of Algae and Protozoa--Oban, UK). C. sorokiniana (0.4 mM-N and 2 mM-N) and T. suecica (2.4 mM-N and 12 mM-N) were obtained by cultivating in an artificial medium at different N-levels. Since enough C. sorokiniana (2 mM-N) could not be obtained for the batch 2 experiment, powder Chlorella (figure 2a), obtained from Raw Food Shop, was used instead. The macro-alga was a mix of dry filamentous algae (figure 2b) collected in the ponds in the park at the back of the Tema-building during July, 2009 and then dried.

![Figure 2a Dry powder Chlorella](image)

![Figure 2b Dry filamentous algae](image)

3.2 Cultivation of micro-algae

3.2.1 Medium preparation

The fresh water medium for C. sorokiniana had the following ingredients (per liter): MgSO₄, 9.8 mg; K₂HPO₄, 20 mg; KNO₃, 0.2 g and proteose peptone, 1.0 g. The sea water medium for T. suecica had the following ingredients (per liter): MgSO₄, 1.19 g; KCl, 0.6 g; NaNO₃, 1.0 g; CaCl₂, 0.23 g; KH₂PO₄, 0.05 g; NH₄Cl, 26.7 mg; Na₂EDTA, 4.5 mg; FeCl₃·6H₂O, 582 µg; MnCl₂·4H₂O, 246 µg; CoCl₂·6H₂O, 12 µg; ZnSO₄·2H₂O, 16.8 µg; Na₂MoO₄·2H₂O, 24 µg; Tris, 1.0 g; NaCl, 30.0 g; and soil ex-
tract (see below), 30 mL; the pH of the sea water medium was adjusted to 8.0 using 2.5 M HCl. Both the fresh water and sea water media were autoclaved at 120°C for 20 minutes. Filter sterilized solution of vitamin B1 (1.2 mg/L medium) and vitamin B12 (10 µg/L medium) were added into the sea water medium after autoclavage.

The same media as above but with low N (KNO₃ and NaNO₃ at 0.04 g and 0.2 g respectively) was also prepared for the fresh and sea water medium respectively.

Frozen soil samples were used for the preparation of soil extract. After fine sieving, twice the soil volume of Milli-Q water was mixed with the soil. The mix was autoclaved at 1 atmosphere overpressure for 30 minutes and left to cool, the supernatant was then decanted and filtered through Munktell filter paper (S-79020 Grycksbo, Sweden). The extract was transferred to a 500 mL glass bottle and stored at +4°C until used.

3.2.2 Cultivations

100 mL of cultures of micro-algae was started in 250 mL Erlenmeyer flasks. The flasks were incubated in a climate room at 20°C and on reciprocal shaking (12 rpm) for one week. Media with both high and low N-level were used. The light intensity of the room was set to 2000 lux using florescent lamps.

Large-scale culture was preformed for the batch fermentations of algal biomass. The 100 mL seed cultures of *C. sorokiniana* (0.4 mM-N and 2 mM-N) and *T. suecica* (2.4 mM-N and 12 mM-N) was individually transferred into fresh and sea medium (total volume of 2 L and 6 L medium with high and low N-level, respectively). The same temperature and light conditions as above was applied while both shaking and air bubbling using aquarium pumps were used. See figure 3

![Figure 3 Large-scale cultivation of micro-algae, air bubbling using aquarium pumps and reciprocal shaking.](image-url)
Small-scale culture was used testing algal cells effect of different CO₂ concentrations flushing. Seed cultures of *C. sorokiniana* (2 mM-N) and *T. suecica* (12 mM-N) was individual transferred into 100 mL of fresh or sea water medium with high N-levels. Two cultivation experiments were performed while bubbling the cultures with gases containing 70% and 5% CO₂, respectively. The 70% CO₂ gas was obtained from an acid reactor while the 5% CO₂ enriched air was a commercial gas mix. The gas containing 70% CO₂ was applied at a rate of about 60 mL/hour. The cells were grown in room temperature, using a 60 W lamp as light source. A magnetic stirrer was applied for mixing (figure 4a). The algae cultivated under 5% CO₂ (flow rate was about 230 mL/min) was again inoculated at 20°C with reciprocal shaking (12rpm) and the light intensity was 1000 lux using fluorescent lamps (figure 4b). In both experiments control cultures without CO₂-addition was included.

![Figure 4 Small-scale cultivation of microalgae, testing the influence of two CO₂ concentrations on growth of *C. sorokiniana* and *T. suecica.*](image-url)
Table 1 Design for cultivations experiments

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Large - scale culture</th>
<th>Small - scale culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental period</td>
<td>Jan, 2010 – Mar, 2010</td>
<td>April, 2010 – May, 2010</td>
</tr>
<tr>
<td>Incubation duration</td>
<td>56 days(^1)</td>
<td>9 days</td>
</tr>
<tr>
<td>Algae strain</td>
<td>C. sorokiniana</td>
<td>C. sorokiniana</td>
</tr>
<tr>
<td>T. suecica</td>
<td></td>
<td>T. suecica</td>
</tr>
<tr>
<td>Cultivation conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>Medium(^{a1}) 20°C</td>
<td>Medium(^{a1}) 20°C</td>
</tr>
<tr>
<td>Light intensity</td>
<td>Medium(^{a2}) 2000lux</td>
<td>Medium(^{b1}) 20°C</td>
</tr>
<tr>
<td>CO(_2) sources</td>
<td>Air</td>
<td>Air</td>
</tr>
<tr>
<td>2000lux Air</td>
<td>Air</td>
<td>Air</td>
</tr>
<tr>
<td>Medium(^{a2}) 20℃ 2000lux Air</td>
<td>60W lamp 70% CO(_2) of biogas</td>
<td>60W lamp 70% CO(_2) of biogas</td>
</tr>
<tr>
<td>Medium(^{b2}) 20℃ 2000lux Air</td>
<td>60W lamp 70% CO(_2) of biogas</td>
<td>60W lamp 70% CO(_2) of biogas</td>
</tr>
<tr>
<td>Medium(^{a1}) RT Air</td>
<td>1000lux 5% CO(_2) enriched air</td>
<td>1000lux 5% CO(_2) enriched air</td>
</tr>
<tr>
<td>Medium(^{b1}) RT Air</td>
<td>1000lux 5% CO(_2) enriched air</td>
<td>1000lux 5% CO(_2) enriched air</td>
</tr>
<tr>
<td>Medium(^{a1}) RT Air</td>
<td>1000lux 5% CO(_2) enriched air</td>
<td>1000lux 5% CO(_2) enriched air</td>
</tr>
<tr>
<td>Medium(^{b1}) RT Air</td>
<td>1000lux 5% CO(_2) enriched air</td>
<td>1000lux 5% CO(_2) enriched air</td>
</tr>
</tbody>
</table>

\(^{a1}\)Medium and \(^{a2}\)Medium, the difference between them is the concentration of nitrate. 0.04g KNO\(_3\) (L\(^{-1}\) medium) was added for \(^{a2}\)Medium (0.4mM-N ), while 0.2g KNO\(_3\) (L\(^{-1}\) medium) was added for \(^{a1}\)Medium (2mM-N ).

\(^{b1}\)Medium and \(^{b2}\)Medium, the difference between them is the concentration of nitrate. 0.2g NaNO\(_3\) (L\(^{-1}\) medium) was added for \(^{b2}\)Medium (2.4mM-N ), while 1g NaNO\(_3\) (L\(^{-1}\) medium) was added for \(^{b1}\)Medium (12mM-N ).

\(^{1}\)56 days means the all large-scale culture period, including seed culture and large-scale algae culture for later two batch experiments.
3.3 Preparation of substrates for AD

3.3.1 Preparation of micro-algae biomass

The cultivated micro-algae solutions were concentrated by centrifugation at 5000 rpm for 10 minutes using a Beckman centrifuge (model J2 – 21M, USA; figure 5a). The supernatant (growth medium) was then removed and its volume determined. The pellet (algae paste; figure 5b) was dissolved in tap water with a volume corresponding to 10% of the removed growth medium. The cells grown in sea water was washed once with the same amount of tap water as the growth medium taken away. The prepared algae were kept at +4°C until required. TS and VS of *C. Sorokiniana* (0.4 mM-N and 2 mM-N) and *T. suecica* (2.4 mM-N and 12 mM-N) and dry powder *Chlorella* used for two batch experiments are given in tables 2 & 3.

3.3.2 Preparation of macro-algae biomass

The dried filamentous green algae were cut into 1cm long pieces using a scissor. The prepared algae was kept in room temperature until required. The TS and VS of this alga were measured to be 91.5% and 54.7% of TS, respectively.

Figure 5 Centrifugation of microalgae
Table 2 Design for Batch 1 digestion of microalgae grown at different N levels

<table>
<thead>
<tr>
<th>Experimental period</th>
<th>Mar, 4 – April, 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation duration</td>
<td>56 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test substrate</th>
<th>C. sorokiniana = C.S.</th>
<th>T. suecica = T.Su.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.S. 0.4mM-N</td>
<td>C.S. 2mM-N</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>Lime+RT</td>
<td>None</td>
</tr>
<tr>
<td>Substrate digested (ml)</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>TS(%)</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>VS(%TS)</td>
<td>87.5</td>
<td>87.5</td>
</tr>
<tr>
<td>Liquid volume (ml)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>OLR(gVS/100ml)</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Note: Three replications were performed for each substrate in each and every batch experiments, in order to minimize the experimental errors.

The substrate for digestion was not in gram but in volume (ml), centrifuged algae paste was dissolved using tap water with a volume corresponding to 10% of removed grown medium. The value of TS was also measured based on the dissolved algae.

OLR should be 0.25 according a normative digestion system; the low values of OLR are due to low harvest of algae.
Table 3 Design for Batch 2 digestion of macro- and microalgae

<table>
<thead>
<tr>
<th>Experimental period</th>
<th>April, 1 – May, 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation duration</td>
<td>42 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test substrate</th>
<th>Green Micro-algae</th>
<th>Green Macro-algae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T. Su (12mM)</td>
<td>T. Su (12mM)</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>Lime + 80°C</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Lime + 105°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lime + 120°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid volume (ml)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>OLR(gVS/100ml)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>TS(%)</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>VS(%TS)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Substrate digested</td>
<td>37ml</td>
<td>37ml</td>
</tr>
</tbody>
</table>

Note: The substrate of Tetraselmis (Tetra. S) digestion was in volume (ml) but in gram, centrifuged algae paste was dissolved using tap water with a volume corresponding to 10% of removed growth medium. The value of TS was measured based on the dissolved Tetraselmis. While, The digestion of (Chlorella and Filamentous algae) without any pretreatment were in gram form, but substrates digested were in volume form regarding treated Chlorella and Filamentous algae because of lime solution addition. The value of TS was measured based on the powder or dry algae.

<sup>a</sup>Chlorella is in form of powder, which was collected from Scandinavianbiogas lab (brought from Raw Food Shop, made in Japan).

<sup>b</sup>Filamentous algae is dry one, which was collected in the ponds in the park at the back of the Tema-building and then dried.


3.3.3 Lime pretreatment

Lime (0.1 g Ca(OH)\(_2\)/gTS) was applied according to Chang et al. (1997). The lime was combined with four temperature treatments (RT, 80\(^\circ\)C, 105\(^\circ\)C and 120\(^\circ\)C) over night. For the liquid micro-algae suspensions, 0.1 g lime powder/g TS was added while a lime solution (16 mM) was applied to the dried algae. Details of the treatments and the amount of lime added are given in table 3.4. Before batch startup, pH of the treated algae was adjusted to a pH at 7 using CO\(_2\) flushing (for batch 1) or 2.5 M HCl (for batch 2).

<table>
<thead>
<tr>
<th>No.</th>
<th>Batch</th>
<th>Algae</th>
<th>Lime loading Powder (0.1g/g-TS)</th>
<th>Solution (16mM)</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>C.S.(0.4mM-N)</td>
<td>0.012g</td>
<td>---</td>
<td>RT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C.S.(2mM-N)</td>
<td>0.013g</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T.Su.(2.4mM-N)</td>
<td>0.013g</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>T.Su.(12mM-N)</td>
<td>0.075g</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>T.Su.(12mM-N) powder Chlorella dry filamentous</td>
<td>---</td>
<td>37ml</td>
<td>80,105,120(^\circ)C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23ml</td>
<td>RT,80,105,120(^\circ)C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>38ml</td>
<td>RT,80,105,120(^\circ)C</td>
</tr>
</tbody>
</table>

3.4 Anaerobic digestion

The study on biogas potential of algal biomass followed the general procedure of batch degradation which has been described by Shelton and Tiedje (1984). Two individual series of experiments (batch 1 and batch 2) were performed.

- Batch 1 was to determine methane potential of *C. sorokiniana* (0.4 mM-N and 2 mM-N); *T. suecica* (2.4 mM-N and 12 mM-N) and effects of lime pretreatment at RT overnight.
- Batch 2 was to determine the effect of over-night lime pretreatment at RT, 80\(^\circ\)C, 105\(^\circ\)C and 120\(^\circ\)C of micro-algae: *T. suecica* (12 mM-N), powder *Chlorella* and the filamentous green macro-algae.

2.5 g VS/L of algae biomass was transferred into 330 mL experimental serum bottles. 2mL salt solution (W: NH\(_4\)Cl (0.45 M), NaCl (0.41 M), CaCl\(_2\) *2H\(_2\)O (0.06 M) and
MgCl₂·6H₂O (0.04 M) was added together with boiled Milli-Q water (in the range of 40-78 mL; to make a finally volume of 100 mL). Finally 15 mL (for batch 1) or 20 mL (for batch 2) inocula was added, which was a 50:50 mix from lab scale biogas reactor at DWES lab and waste water treatment sludge from Ryaverken in Gothenburg. N₂ was applied to flush bottles continuously during the preparation of the serum bottles. The bottles were then sealed with rubber stoppers and aluminum screw caps. The gas phase of the bottles was changed to N₂:CO₂ (80:20), by filling and evacuating at least 9 times. The overpressure of bottles was then released before addition of 0.3 mL of 100 mM Na₂S (W7). The bottles were incubated in the dark at 37°C for 56 days (for batch 1) and 42 days (for batch 2).

In each batch experiment, three controls were included: 0.5 g VS cellulose paper (Whatman filter paper; positive control) and only inocula (15 mL or 20 mL) were run to evaluate if microorganisms function good or not. An external methane standard was also prepared: 15 mL pure CH₄ was injected to sealed serum bottle only filled with 100 mL boiled Milli-Q. Three controls were run in order to evaluate if the batch experiment performs stable or not. A standard pattern of biogas production from a substrate and controls is shown in figure 6.

Three replications of every substrate and control treatment were run.

![Figure 6 A standard curve of accumulated biogas production, ○ positive control, ● substrate, × Inocula control, CH₄ control.](image-url)
3.5 Analysis

3.5.1 Optical density (OD) and pH

A spectrophotometer (Ultrospec 2100 Pro; figure 7) was used to measure OD of the algae cultures. 1mL algae sample was transferred into cuvettes and the OD$_{600}$ was measured.

![Figure 7 The spectrophotometer](image)

A pH meter (model WTW Inolab pH 730, Copenhagen) was used when testing the growth medium of micro-algae flushed by CO$_2$ enriched gas and adjusting pH after lime treatments.

3.5.2 Total Solids (TS) and Volatile Solids (VS)

TS is used to determine the dry fraction of algal biomass and VS to determine its organic fraction. The TS and VS determination of test samples was measured according to “Laboratory Analytical Procedure (LAP-001), Standard Method for Determination of Total Solid in biomass”\(^1\) and “Laboratory Analytical Procedure (LAP-005), and Standard Method for Determination of Ash in Biomass”\(^2\), respectively. Test samples were poured in crucibles and then dried in an oven at 105°C for 20 hours. The dried substrates were then burnt in a furnace at 550°C for 2 hours. TS is determined by the difference in mass before and after the drying process, while VS of TS is determined

---

1 Standard Method for Determination of Total Solid in Biomass, Chemical Analysis and Testing Task, Laboratory Analytical Procedure (LAP-001)
by the difference in mass before and after the ignition of dried substrates.

3.5.3 Overpressure measurement

The biogas produced from the substrates was determined by measuring overpressure of experimental bottles in the climate room (37°C). A 5 mL glass syringe equipped with a blue needle of 0.6×25 mm (Sterican 23 G×1, B.Braun Melsungen AG) was used to do this measurement. Detailed procedure has been described in chapter 3.4.5 of Biswas (2009). After the measurement of gas pressure, 1 mL gas sample was transferred into a 13.4 mL dilution bottle, capped with a rubber stopper, using a 1 mL plastic syringe equipped with a blue needle. The diluted gas sample was used to determine the CH₄ concentration of the formed biogas. After that, the overpressure was released till pressure equalization, inserting a 5 mL syringes filled approximately 2 mL of Milli-Q water.

3.5.4 Gas chromatography

CH₄ concentration in the biogas was analyzed using a GC-FID equipment (HP 5880A). 0.3 mL of sample was taken out from the dilution bottles and injected into the septum of the GC using a 1 mL plastic syringe with orange needle (0.4×25 mm; Sterican 27 G×1½, Braun Melsungen AG). Three injections into the GC were performed for every sample. CH₄ concentration was then calculated using standard curve method from the peak areas obtained. Detailed preparation procedure of standard samples has been described in chapter 3.4.6.1 of Biswas (2009). Three sealed 123 mL bottles with different CH₄ concentrations (0.066%, 0.63% and 1.71%) were prepared every other week (ca 14 days). Five injections of each standard sample were done at each analysis occasion.
Chapter 4. Results

4.1 Cultivations

4.1.1 Different N-levels

The OD$_{600}$ values of *C. sorokiniana* and *T. suecica* cells grown at different N-levels are shown in figure 8a&b. *C. sorokiniana* cells showed a significant increase between the first two measurements, with an average rate of 0.22 OD/day from day 1 to day 4. After that the OD of *C. sorokiniana* cells varied depending on different N in the medium. The OD of cells of *C. sorokiniana* grown at high N-level showed an increase of 0.76 (±0.03) to 1.37 (±0.06) from day 4 to day 15, while the OD of same alga grown at low N-level showed an increase of 0.79 (±0.05) to 1.17 (±0.1) from day 4 to day 10 and then the OD values were stable at around 1.2 (±0.06) during the left incubation days (figure 8a).

As shown in figure 8b, the OD of *T. suecica* increased slowly during the first 5 days, a faster rate was then obtained, with the most rapid increase for the cultures grown at high N-level. The OD of cells of *T. suecica* grown at high N-level increased at an average rate of 0.12 OD/day from day 5 to day 10, varying from 0.12 (±0.11) to 1.31 (±0.1), while the OD of same alga grown at low N-level showed a slower increase (average rate: 0.04 OD/day, varying from 0.15 (±0.12) to 0.56 (±0.13)) in the same period. High N-level had more than doubled the cell density of *T. suecica*. 
Figure 8a Mean OD₆₀₀ values of a *C. sorokiniana* grown in medium with $\blacklozenge$ 0.4 mM-N and $\blacksquare$ 2 mM-N. Error bars show the standard deviation of 3 samples.

Figure 8b Mean OD₆₀₀ values of a *T. suecica* grown in medium with $\blacklozenge$ 2.4 mM-N and $\blacksquare$ 12 mM-N. Error bars show the standard deviation of 3 samples.
4.1.2 Enriched CO₂ flushing

The effects of growth of micro-algae cultivated under a high partial CO₂ (70%; average flow rate: 60 mL/hour) are shown in figure 9a&b. For both algae an inhibitory effect of 70% CO₂ was obtained if compared to references grown under air.

As shown in figure 9a, the OD of *C. sorokiniana* showed a considerable decrease from day 4, the OD value at day 9 (the finally day of incubation) was about half of the reference grown under air. The OD measured for *T. suecica* was shown to be approximately five times lower than that of reference grown under air at day 9 (figure 9b). The pH of growth medium was measured at day 9 and values were 7.2 and 8.5 for *C. sorokiniana* and reference, while the corresponding values for *T. suecica* were 6.6 and 8.4.

![Graph](image)

Figure 9a OD₆₀₀ values of a *C. sorokiniana* (2 mM-N) grown under biogas from an acid reactor (70% CO₂; ◆), the reference grown under air is also shown (■). Only one sample was run for each treatment.
Figure 9b OD$_{600}$ values of a *T. suecica* (12 mM-N) grown under biogas from an acid reactor (70% CO$_2$; ——), the reference grown under air is also shown (——). Only one sample was run for each treatment.

The effects on micro-algae grown under 5% CO$_2$ (average flow rate: 230 mL/min) are shown in figure 10a&b. The OD of *C. sorokiniana* did not show positive effect based on the 5% CO$_2$, the OD value at day 8 was at the same level as the start of the experiment. The OD values of *T. suecica* did not give a clearly positive effect of the 5% CO$_2$, although it gave slightly a lower OD value (0.65) compared to OD value (0.66) of reference at day 8. The pH of the growth medium was measured at day 8 and values were 6.9 and 8.2 for *C. sorokiniana* and reference, while the corresponding values for *T. suecica* were 6.8 and 8.3.
Figure 10a OD$\text{600}$ values of a *C. sorokiniana* (2 mM-N) grown under CO$_2$ enriched air (5% CO$_2$; ◆◆◆), the reference grown under air is also shown (■■■). Only one sample was run for each treatment.

Figure 10b OD$\text{600}$ values of a *T. suecica* (12 mM-N) grown under CO$_2$ enriched air (5% CO$_2$; ◆◆◆), the reference grown under air is also shown (■■■). Only one sample was run for each treatment.

The dry biomass content (TS and VS) of the both micro-algae grown under 5% CO$_2$ was performed (shown in table 5) but not for 70% CO$_2$. I assumed that the dry biomass of micro-algae cultivated under 70% CO$_2$ could be approximately 0 due to the quite low OD values obtained (shown in figure 9a&b).
Table 5 Dry biomass content of 100 mL *C. sorokiniana* and *T. suecica* grown under 5% CO$_2$ enriched air

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>C. sorokiniana</em></th>
<th><em>T. suecica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>TS(g)</td>
<td>0.026</td>
<td>0.038</td>
</tr>
<tr>
<td>VS(g)</td>
<td>0.026</td>
<td>0.038</td>
</tr>
<tr>
<td>Test (5% CO$_2$)</td>
<td></td>
<td>Test (5% CO$_2$)</td>
</tr>
<tr>
<td>Reference</td>
<td>0.067</td>
<td>0.091</td>
</tr>
</tbody>
</table>

4.2 Evaluation of Batch 1 and Batch 2 experiments

The patterns of accumulated biogas production of untreated substrates and controls of the batch 1 and batch 2 experiments are shown in figure 11a&b. If we compare the graphs we can see large difference; Positive control in batch 1 showed significant increase of biogas production from the 2$^{nd}$ day and 37$^{th}$ day (figure 11a), while the corresponding of batch 2 showed a significant increase of biogas production from the 2$^{nd}$ day (figure 11b). Inocula control in batch 1 showed a least 3 times smaller gap of biogas production from inocula control and algal substrates (figure 11a) if compared to the corresponding gap of inocula control and algal substrates showed in batch 2 (figure 11b). For CH$_4$ control both batch experiments showed a similar pattern.

Figure 11a Mean accumulated biogas production of positive control (−○−), *Chlorella* grown at 2mM-N (−◆−), *C. sorokiniana* grown at 0.4mM-N (−■−), *T. suecica* grown
at 12mM-N (▲), T. suecica grown at 2.4mM-N (※), Inocula (×), CH₄ control (●), in bottles of the batch 1 experiment. Error bars show the standard deviation (n=3).

Figure 11b Mean accumulated biogas production of waterman paper (○), powder Chlorella (◆), T. suecica grown at 12mM-N (▲), dry filamentous (●), Inocula (×), CH₄ control (●), in bottles of the batch 2 experiment. Error bars show the standard deviation (n=3).

4.3 Biogas and CH₄ potentials of untreated algae

4.3.1 C. sorokiniana and T. suecica grown at different N-levels

Total biogas and CH₄ yields of C. sorokiniana grown at 0.4 mM-N and 2 mM-N levels and T. suecica grown at 2.4 mM-N and 12 mM-N levels are shown in figure 12a&b. The total biogas yield of C. sorokiniana grown at 0.4mM-N level was 479 (±66) mL/added gVS, which was about the same as that of C. sorokiniana grown at 2 mM-N level (440 ±46 mL/added gVS). The total biogas yields of T. suecica grown at 2.4 mM-N and 12 mM-N levels were similar, producing about 520 (±70) mL/added gVS. (figure 12a)

As shown in figure 12b, C. sorokiniana and T. suecica grown at low N-level had a higher CH₄ per g VS than that of the same algae grown at high N-level. 319 (±26) mL and 258 (±12) mL CH₄/added gVS was obtained during the degradation of C. sorokiniana grown at 0.4 mM-N and 2 mM-N level, respectively. While 337 (±37) mL and 236 (±20) mL CH₄/added gVS was obtained for T. suecica grown at 2.4 mM-N and 12 mM-N level, respectively.
Figure 12a Mean accumulated biogas yields of *C. sorokiniana* grown at 2 mM-N (◆), and 0.4 mM-N (■), and *T. suecica* grown at 12 mM-N (▲), and 2.4 mM-N (※), in the batch 1 experiment. Error bars show the standard deviation (n=3). The amount of biogas formed in the inocula control bottles has been withdrawn.

Figure 12b Mean accumulated CH₄ yields of *C. sorokiniana* grown at 2 mM-N (◆), and 0.4 mM-N (■), and *T. suecica* grown at 12 mM-N (▲), and 2.4 mM-N (※), in the batch 1 experiment. Error bars show the standard deviation (n=3). The amount of CH₄ formed in the inocula control bottles had been withdrawn.
4.3.2 Powder *Chlorella, T. suecica* and dry filamentous algae

The total biogas and CH$_4$ yields of *T. suecica* grown at 12 mM-N level, powder *Chlorella* and dry filamentous algae are shown in figure 13a&b. As shown in figure 13a, powder *Chlorella* produced most biogas (719 ±53 mL/added gVS), while 661 (±20) mL and 584 (±7) mL biogas/added gVS was obtained for the dry filamentous algae and *T. suecica*, respectively. Powder *Chlorella* also gave the highest CH$_4$ yield (392 ±14 mL/added gVS). *T. suecica* (12 mM-N) and dry filamentous algae produced quite similar CH$_4$ yields (about 295 (±9) mL/added gVS). See figure 13b

Compared to the total biogas and CH$_4$ of *C. sorokiniana* and *T. suecica* in batch 1 experiment (figure 12a&b), powder *Chlorella* run in batch 2 experiment gave higher biogas and CH$_4$ yields than that of *C. sorokiniana* grown in the lab at WES. The total biogas yields of *T. suecica* grown at 12mM-N level were about same in the two batch experiments.

![Figure 13a](image_url)

Figure 13a Mean accumulated biogas yields of powder *Chlorella* (−●−), *T. suecica* grown at 12 mM-N (−▲−), dry filamentous (−●−), in the batch 2 experiment. Error bars show the standard deviation (n=3). The amount of biogas formed in the inocula control bottles had been withdrawn.
Figure 13b Mean accumulated CH$_4$ yields of powder *Chlorella* (−◆−), *T. suecica* grown at 12mM-N (−▲−), dry filamentous (−●−), in the batch 2 experiment. Error bars show the standard deviation (n=3). The amount of CH$_4$ formed in the inocula control bottles had been withdrawn.

4.4 CH$_4$ potentials of treated algae

4.4.1 *C. sorokiniana*, powder *chlorella* and *T. suecica*

The effects of CH$_4$ yields of *C. sorokiniana*, powder *Chlorella* and *T. suecica* treated with lime at RT, 80°C, 105°C, and 120°C are shown in figure 14a, b&c.

*C. sorokiniana* treated with lime at RT did not give a higher CH$_4$ yield; on the contrary, less CH$_4$ was formed than that of untreated *C. sorokiniana* (figure 14a). As shown in figure 14a&b, *T. suecica* treated with lime at RT and 80°C did not show positive effects on CH$_4$ production, but it treated with lime at 105°C and 120°C showed high degradation rates during the first 5 days of incubation, in which *T. suecica* treated with lime at 120°C had the fastest degradation rate and the highest CH$_4$ yield (315 ±7 mL/added gVS).

As shown in figure 14c, powder *Chlorella* treated with lime at 80°C, 105°C and 120°C showed high degradation rates during the first 5 days of incubation, the highest obtained with 120°C-pretreatment gave a lower finally CH$_4$ yield (355 ± 17 mL/added gVS) than that of untreated powder *Chlorella* (392 ± 14 mL/added gVS).
Figure 14a Mean accumulated CH$_4$ yields of *C. sorokiniana* treated with lime at RT (◇) and untreated *C. sorokiniana* (◆); and *T. suecica* treated with lime at RT (Δ) and untreated *T. suecica* (▲), in the batch 1 experiment. Error bars show the standard deviation (n=3). The amount of CH$_4$ formed in the inocula control bottles had been withdrawn.

Figure 14b Mean accumulated CH$_4$ yields of *T. suecica* treated with lime at 80°C (○), 105°C (□), 120°C (■) and untreated *T. suecica* (▲), in the batch 2 experiment. Error bars show the standard deviation (n=3). The amount of CH$_4$ formed in inocula control bottles had been withdrawn.
Figure 14c Mean accumulated CH₄ yields of powder *Chlorella* treated with lime at RT (−◇−), 80°C (−○−), 105°C (−□−), 120°C (−■−) and untreated powder *Chlorella* (−◆−), in the batch 2 experiment. Error bars show the standard deviation (n=3). The amount of CH₄ formed in inocula control bottles had been withdrawn.

### 4.4.2 Filamentous algae

The effect of CH₄ yields of the dry filamentous algae treated with lime at RT, 80°C, 105°C, and 120°C is shown in figure 15. Also for the dry filamentous algae the highest CH₄ yield was obtained from untreated algae (293 ± 10 mL CH₄/added gVS). The dry filamentous algae treated with lime at 105°C and 120°C did not give any higher initial degradation rates, and it treated with lime at 105°C gave the lowest CH₄ yield (199 ± 19 mL /added gVS)
Figure 15 Mean accumulated CH₄ yields of dry filamentous algae treated with lime at RT (−◇−), 80°C (−○−), 105°C (−□−), 120°C (−■−) and untreated filamentous (−●−), in the batch 2 experiment. Error bars show the standard deviation (n=3). The amount of CH₄ formed in the inocula control bottles had been withdrawn.
Chapter 5. Discussion

5.1 Influence of different N feedings on cell growth

As shown in Figure 8a&b, the growth of sea water T. suecica seems to be limited by N level at 2.4 mM-N. Due to low N (0.4 mM-N) nutrient supply, the fresh water C. sorokiniana cells seemed to stop to grow at day 10. Also Takagi et al. (2000) investigated the effect the N-levels had on microalgae growth. These authors studied the growth of Nannochloris sp in medium with 6 different nitrate concentrations (9.9, 9.0, 6.0, 4.0, 2.0 and 0.9 mM). The results (table 2 of the appendix) showed that the medium containing the highest N (9.9 mM) concentration gave the most cells biomass (2.9 g/L), while 0.24 g cells/L was obtained for medium containing 0.9 mM-N. In the present study, 0.4 mM-N and 2 mM-N levels were used to grow C. sorokiniana, but I could not draw that the high N-level (2 mM-N) would give a higher cells growth for C. sorokiniana because that there was no clearly difference of OD values of C. sorokiniana cultivated at the two N-levels (figure 8a). While 2.4 mM-N and 12 mM-N levels were used for T. suecica culture and the higher OD values was obviously observed for the T. suecica grown at 12 mM-N level (figure 8b).

5.2 Influence of enriched CO2 flushing on cell growth

The results from cultivation of micro-algae in biogas (70% CO2) are shown in figure 9a&b, showing inhibitory to the micro-algae cells growth. A greatly decrease of C. sorokiniana cells occurred 4 days after startup (figure 9a), and also a suppression of cells growth of T. suecica was shown (Figure 9b). The high CO2 concentration of the gas bubbled through the growth medium likely decreased its pH and this likely caused the inhibition. The growth medium should have a pH of 8.0, but in the biogas bubbled cultures pH-values of 7.2 and 6.6 was measured for C. sorokiniana and T. suecica, respectively. Excess CO2 was likely dissolved in the growth medium, thus acidifying it. According to similar studies (Hanagata et al., 1992; Zeiler et al., 1995), cultivation at high concentrations of CO2 can cause negative effects on cells growth of micro-algae. Besides, H2S, NH4 and reduced sulfur compounds might be contained in the biogas and they could have an inhibitory effect on algal cells growth.

The trial with cultivation of algae under 5% CO2 enriched air is shown in figure 10a&b. I can not see any positive effects of 5% CO2 on the C. sorokiniana cells growth (figure 10a). For T. suecica no clear inhibition was observed (figure 10b) but neither a clear positive effect compared to that of reference flushed by air. Lower dry biomass content (table 5) of C. sorokiniana and T. suecica was obtained in this ex-
periment and again this is likely due to the measured decrease in pH compared to the reference reactors. Two previously studies (Takagi et al., 2000; Yamaberi et al., 1997) on micro-alga (Nannochloris sp.) showed that a positive result on the growth rate could be obtained with 3% CO₂ enriched air at a 500 mL/min while 5% CO₂ with a flushing rate at 230 mL/min used in my study might be too much, resulting in an increased acidity of growth medium: lower pH was measured to be 6.9 for C. sorokiniana and 6.8 for T. suecica.

5.3 CH₄/biogas potentials of micro-algae grown at different N levels

According to the CH₄ yield of the C. sorokiniana and T. suecica cultivated at different N-levels (figure 12b), both strains gave at low N feeding a higher CH₄ yield compared to that of strains under high N feeding. The differences in CH₄ yields could be due to differences in lipid content as N deprivation can bring about a substantial increase of lipid production in micro-algae (Takagi et al., 2000). The Takagi et al. – study showed that the micro-algae grown at N concentration of 0.9 mM had the highest lipid content (40%; table 1 in the appendix). A previously study of Roessler (1990) also showed that N-limiting conditions gave algae with increased fat content. Material with a high lipid content can provide higher CH₄ yield per g VS that material rich in carbohydrate or protein (Berglund & Börjesson, 2006).

However, the N limiting showed a clearly inhibition on T. suecica cells growth, resulting in lower cells biomass production (figure 8b). Thus, this culture at low N-level seems to be not good to supply much biomass for biogas production. However, some studies had investigated on some special strains of micro-algae with both characters of high biomass productivity and high lipid content. The results of Rodolfi et al. (2009) showed that the strains with the best lipid producers can give the best combination of biomass productivity and lipid content, the authors also investigated a marine genus Nannochloropsis sp. F&M-M24 and two freshwater, Chlorella sp. F&M-M48 and Scenedesmus sp. DM were among the best producers in terms of biomass and lipid.

5.4 Biogas potentials of different algae

In this study, Powder Chlorella gave the highest biogas (719 ±53 mL/added gVS), followed by dry filamentous algae (661 ±20 mL biogas/added gVS) and T. suecica (12 mM-N; 584 ±7 mL biogas/added gVS) running a batch experiment in 42 days of incubation. Similar researches on biogas potentials of micro- and macro-algae have been reported by Dong et al. (2006) and Biswas (2009). Dong et al. (2006) obtained an average biogas yield of 491 mL/gVS from fresh blue algae (Cyanobacteria; micro-algae) running a batch experiment in 66 days of incubation; and Biswas (2009) obtained an average biogas yield of 250 mL/gVS from a Polysiphonia (macro-algae) running a batch experiment in 35 days of incubation.
5.5 CH$_4$ potentials of algae treated with thermo-lime

According to pretreatment experiments of batch 1 and batch 2, lime treatment at RT did not show positive effects on CH$_4$ yields of micro- and macro-algae (figure 14a&c and figure 15). 0.1g/gTS lime loading at RT did not give fast degradation rate and high CH$_4$ yield, this might be due to inhibition of the degrading microorganisms at 0.1g/gTS lime addition. No articles were found to report the same inhibition, but high concentrations of calcium cations had been reported to be inhibitory or toxic to microorganisms in some papers (Bachir & Matin, 2004; Kugelman & McCarty, 1964; Yu et al., 2001). High temperature seemed to be the most efficient way to treat $T$. suecica and powder *Chlorella* but not for dry filamentous algae in this study. The higher the temperature, the higher the degradation rate as 120$^\circ$C treatment gave the fastest degradation rate in the first 5 days of incubation (figure 14b&c). Also Biswas (2009) investigated a macro-alga (*Polysiphonia*) treated with high temperature (autoclave at 120$^\circ$C for 30 mins), the results showed an increased degradation rate within the initial 5 days of incubation and a higher CH$_4$ amount formed compared to that of untreated algae (figure 1 in the appendix). Besides, thermal pretreatment has been reported to accelerate substrates hydrolysis and improve the overall digestion process, thus reducing anaerobic digester retention time and enhancing CH$_4$ production rates (Müller, 2000).

However, the CH$_4$ yield of untreated powder *Chlorella* had exceeded that of powder *Chlorella* treated at 120$^\circ$C after 36 days incubation (figure 14c). For dry filamentous algae lime treatment at 120$^\circ$C and 105$^\circ$C caused a clearly decrease in the CH$_4$ yield compared to that of untreated filamentous algae, and lime pretreatment at 105$^\circ$C gave the lowest CH$_4$ yield. The finally lower CH$_4$ yield of algae treated under high temperature pretreatment maybe be explained by that substrates were partly boiled away during the overnight high temperature treatment. Similar result was shown in Kashani (2009), the author investigated CH$_4$ production of chicken feather treated with 0.2g/gTS lime at 120$^\circ$C for 1 and 2 hours, the results showed the treated feather for 2 hours gave a lower CH$_4$ yield than that of treated feather for 1 hour (figure 2 in the appendix).
Chapter 6. Conclusion

The questions of this study are answered as follows:

319 (±26) mL and 258 (±12) mL CH₄/added gVS was obtained during the degradation of *C. sorokiniana* grown at 0.4mM-N and 2mM-N level, respectively, while 337 (±37) mL and 236 (±20) mL CH₄/added gVS was obtained for *T. suecica* grown at 2.4mM-N and 12mM-N level, respectively. Both strains of micro-algae cultured at low N gave more CH₄ per g VS, which could be due to a high lipid accumulated under N stress condition.

Powder *Chlorella* gave the highest biogas (719 ±53 mL/added gVS) and CH₄ yield (392 ±14 mL/added gVS), followed by the dry filamentous algae (661 ±20 mL biogas and 295 ±9 mL CH₄/added gVS) and *T. suecica* (12 mM-N; 584 ±7 mL biogas and 295 ±9 mL CH₄/added gVS).

A negative effect of lime treatment at room temperature on CH₄ yield of algal biomass was observed. Lime treatment at 120°C showed the fastest degradation rate for *T. suecica* and powder *Chlorella* during the initial 5 days of incubation, but not for the dry filamentous algae. An initial 269 (±9) mL CH₄/added gVS produced within 5 days of incubation and a finally 355 (±17) mL CH₄/added gVS was obtained for powder *Chlorella*, while the corresponding a lower initial CH₄ (171±7 mL/added gVS) and a higher finally CH₄ yield (392 ±14 mL/added gVS) was obtained for untreated powder *Chlorella*.

The algae culture flushed with biogas containing 70% and a 5% CO₂ enriched air did not give higher OD values or cells growth if compared to that reference grown under air.
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Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert,


# Appendix

Table 1. Summary of Various process used for pretreatment of lignocellulosic biomass
(Kumar et al. 2009)

<table>
<thead>
<tr>
<th>pretreatment process</th>
<th>advantages</th>
<th>limitations and disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical comminution</td>
<td>reduces cellulose crystallinity</td>
<td>power consumption usually higher than inherent biomass energy</td>
</tr>
<tr>
<td>Steam explosion</td>
<td>causes hemicellulose degradation and lignin transformation; cost-effective</td>
<td>destruction of a portion of the xylan fraction; incomplete disruption of the lignin-carbohydrate matrix; generation of compounds inhibitory to microorganisms</td>
</tr>
<tr>
<td>AFEX</td>
<td>increases accessible surface area, removes lignin and hemicellulose to an extent; does not produce inhibitors for downstream processes</td>
<td>not efficient for biomass with high lignin content</td>
</tr>
<tr>
<td>CO₂ explosion</td>
<td>increases accessible surface area; cost-effective; does not cause formation of inhibitory compounds</td>
<td>does not modify lignin or hemicelluloses</td>
</tr>
<tr>
<td>Oxidation</td>
<td>reduces lignin content; does not produce toxic residues</td>
<td>large amount of oxos required; expensive</td>
</tr>
<tr>
<td>Acid hydrolysis</td>
<td>hydrolyzes hemicellulose to xylose and other sugars; alters lignin structure</td>
<td>high cost; equipment corrosion; formation of toxic substances</td>
</tr>
<tr>
<td>Alkaline hydrolysis</td>
<td>removes hemicelluloses and lignin; increases accessible surface area</td>
<td>long residence times required; irreversible cells formed and incorporated into biomass</td>
</tr>
<tr>
<td>Organosolv</td>
<td>hydrolyzes lignin and hemicelluloses</td>
<td>advents need to be drained from the reactor, evaporated, condensed, and recycled; high cost</td>
</tr>
<tr>
<td>Pyrolysis</td>
<td>produces gas and liquid products</td>
<td>high temperature; ask production</td>
</tr>
<tr>
<td>Pulsed electrical field</td>
<td>ambient conditions; disrupts plant cells; simple equipment</td>
<td>process needs more research</td>
</tr>
<tr>
<td>Biological</td>
<td>degrades lignin and hemicelluloses; low energy requirements</td>
<td>rate of hydrolysis is very low</td>
</tr>
</tbody>
</table>
Table 2. Effect of initial nitrate concentration on the cells growth and lipid contents of Nannochloris sp. UTEX LBI999 (Takagi et al., 2000)

<table>
<thead>
<tr>
<th>Initial nitrate conc. (mM)</th>
<th>Cell (g/l)</th>
<th>Lipids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.9</td>
<td>2.86</td>
<td>31</td>
</tr>
<tr>
<td>8.0</td>
<td>2.18</td>
<td>32</td>
</tr>
<tr>
<td>6.0</td>
<td>1.73</td>
<td>30</td>
</tr>
<tr>
<td>4.0</td>
<td>1.44</td>
<td>30</td>
</tr>
<tr>
<td>2.0</td>
<td>0.79</td>
<td>30</td>
</tr>
<tr>
<td>0.9</td>
<td>0.24</td>
<td>40</td>
</tr>
</tbody>
</table>

Figure 1. Accumulated specific methane yields (i.e., methane produced from inoculum was excluded) in the first batch experiment investigating biogas potential of Algae (old) [■], Algae (old) pretreated in autoclave [▲] and Whatman paper [◆]. Error bars show the standard deviation and yields are corrected at STP. (Biswas, 2009)
Figure 2. Average methane production curves for triplicate lime treated samples during 50 days of incubation (Kashani, 2009).