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Biogas Production from Solvent Pretreated Orange Peel

Master of Science Thesis in the Master's Program MPISC

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CHALMERS UNIVERSITY OF TECHNOLOGY
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Abstract

Orange peel has been demonstrated to be a potential source for biogas production. However, D-limonene present in the peel is known as an anti-microbial agent which can decrease biogas production. In this work, biogas production from orange peel was improved by solvent pretreatment. A simple pretreatment procedure following solid-liquid technique for the removal of D-limonene was designed. In addition, experimental design has been employed as an important tool for conducting experiments efficiently and analyzing experimental results in a correct statistical manner. The results showed that biogas production at 2% Volatile Solid concentration increased from 0.061 m^3 methane/kg VS to 0.217 m^3 methane/kg VS if the chopped peel was treated using n-hexane as solvent at the condition of 20°C, 10 minutes and a hexane/peel ratio (volume/weight) of 12. D-limonene in orange peel was partly removed and the amount varied depending on pretreatment conditions. The research also revealed that the improvement of biogas production was not only a result of the D-limonene removal, but also caused by other factors related to the pretreatment step prior to digestion stage. However, the results obtained from this research showed an interested way to improve biogas production from orange peel.

Key words: Orange peel, biogas, solvent pretreatment, D-limonene.

Acknowledgment

I would like to express my deepest gratitude to my examiner, Professor Claes Niklasson for his conscientious support during my thesis work. Without his support, I cannot know how far I can reach on the way of my thesis. I would like to thank my supervisor, Professor Mohammad Taherzadeh and Dr. Ria Millati for their guidance and research ideas.

I would like to give my thanks to Linda Hellström (Chalmers University of Technology) and Wikan, my friend in Boras University who kindly helped me with practical issues.

Last but not least, I would like to give my warm thanks to Duy, my dear friend who is always next to me during my thesis work. Thanks also for his research ideas which helped me find the way to perform my study more scientifically.

Contents

Abbreviation

TS: Total Solid

VS: Volatile Solid

GC: Gas Chromatography

GC –MS: Gas Chromatography – Mass Spectrometry

FID: Flame Ionization Detector

TCD: Thermal Conductivity Detector

ANOVA: Analysis of Variance

Nomenclature

A: the area of the solid-liquid interface (m^2)

b: the effective thickness of liquid film surrounding the solid particles (m)

c: the concentration of the solute in the bulk of solution at the time t (mol m^{-3})

c_s: the solute concentration of the saturated solution contacting with the solid particles (mol m^{-3})

M: Mass of the solute transferred in the time t (mol)

k': the diffusion coefficient (m^2 s^{-1})

K_L: mass transfer coefficient (m s^{-1})

V: Total volume of solution (m^3)

P: Atmospheric pressure (101325 Pa)

R: Gas constant (8.314 J mol^{-1} K)

T: Temperature at ideal condition (K)

$V_{standard}$: Volume of injected gas standard (ml)

1. Introduction

World orange production in 2007 was about 64 million tons of which 70% was used for producing juice or marmalade[1]. A large amount of the processed fruit ends up as orange peel waste, composing of peel, seed, membrane residue[1] which is required to be processed further to avoid environmental problems. Currently, the main part of this waste is used for cattle feed production and the rest is burnt [1]. Therefore, more effective and sustainable alternatives for using orange peel are highly desirable.

One of the promising ways to use orange peel waste is making it as feedstock for biogas production which will give benefits in terms of both energy recovery and environmental concerns. Actually, orange peel has been demonstrated as a potential source for biogas production [1, 2]. However, essential oil extracted from orange peel contains approximately 90% D-limonene [2, 3] which is known as anti-microbial agent [4]. As a result, D-limonene can cause decreasing of the biogas yield or even total cause failure of the anaerobic digestion if untreated orange peel is used as feedstock. D-limonene has been reported to be highly toxic to anaerobic digestion by many researches; however, just a few researches in which detailed effect of D-limonene on biogas production from orange peel has been done. Therefore, investigating effect of D-limonene on anaerobic digestion of orange peel in more detail is necessary. In addition, it was demonstrated that biogas production performed better if pretreatment of orange peel to reduce the amount of D-limonene was carried out [2, 4, 5]. Thus, looking for an effective pretreatment method which is employed to detoxify i.e. reduce D-limonene content and improving biogas production from orange peel are objectives of this research.

2. Literature Review

In this section, some general literature related to the thesis subject will be presented.

2.1 Anaerobic Digestion

2.1.1 Overview

Anaerobic digestion to produce biogas is an efficient treatment of organic waste sources in which waste will be decomposed in oxygen-free environment [6]. Variety of feed-stocks can be used for anaerobic digestion including waste water, food industry waste and biomass [6]. The expected product of the anaerobic digestion is biogas which is composed of 60-65% methane, 35-40% carbon dioxide by volume[7]. This source of energy can be used for on-site heating and electricity production [4] or will be upgraded to increase methane content so that it can be used as vehicle fuel. Anaerobic digestion gives some specific advantages in comparison with other methods of waste treatments such as decreasing odor emission, producing a source of carbon neutral energy in form of biogas [6] as well as low nutrient requirements, high efficiency, high methane production [4].

Anaerobic digestion from biomass has attracted a lot of interest. It is reported that different kinds of biomass give different biogas production potential. In fact, biogas yield and quality varies according to compositions of biomass waste sources [8]. Fruit and vegetable wastes often have low total solid (TS), high volatile solid (VS) and can be decomposed easily in anaerobic digestion [6].

2.1.2 Stages of Anaerobic Digestion

Anaerobic digestion includes three main stages: hydrolysis, acid-forming (including acetogenesis) and methanogenesis. Different groups of bacteria will dominate different the stages of digestion and products of one group will serve as feed for another group [7]. Description of three stages of biogas production is as follows:

- **Hydrolysis**: Insoluble complex substrates will be degraded by the large community of hydrolytic bacteria, producing simple substrates such as simple sugars, amino acids and fatty acids.
- **Acid - forming**: Substrates which are the products of the first stage will be degraded, producing carbon dioxide, hydrogen, alcohols, organic acids, a few organic-nitrogen compounds and organic-sulfur compounds. One important product of this stage is acetic acid which is the principal organic acid digested by methane-forming bacteria. Acetic acid is produced through the fermentation of soluble organic compounds and through acetogenesis and is the predominant acid produced from the acid-forming stage. In fact, acetogenesis can be considered as a sub-stage of the acid-forming stage in which many acids and alcohols produced during the acid-forming stage can be degraded to acetic acid.
- **Methanogenesis**: The products from the acid – forming stage will be consumed by methane-forming bacteria, producing methane. Actually, methane production occurs in three different ways, as follows [7]:

Acetoclasticmethanogenesis Acetic acid ⟶ Methane + Carbon dioxide

Hydogenotrophicmethanogenesis Hydrogen + Carbon dioxide ⟶ Methane

Methyltrophicmethanogenesis Methanol ⟶ Methane + Water

Stages of biogas production process are summarized in figure below [7].

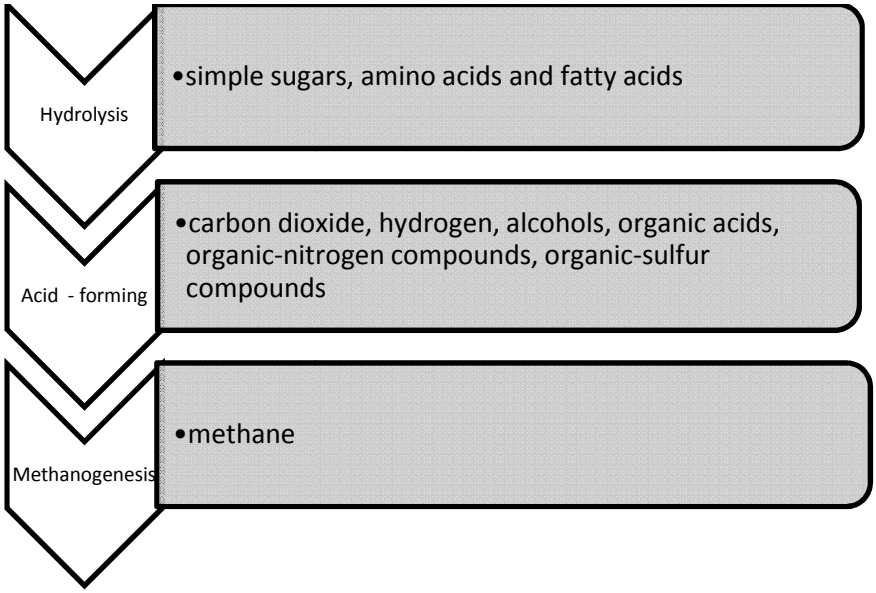

Figure 1. Three main stages of anaerobic digestion

2.1.3 Operational Conditions

There are some factors that can cause significant effect on anaerobic digestion, such as temperature, pH, alkalinity [7], inhibitory substances (ammonia, sulfide, heavy metals, alcohol, carboxylic acid, etc.) [6]. However, only the factors of temperature, pH and alkalinity which are considered as the most important ones since methane-forming bacteria are sensitive to temperature and pH will be discussed in detail in this section.

- **Temperature**

Temperature is one of the most important factor that have significant effect on microbial activity [7]. In fact, acetic acid – forming bacteria and methane – forming bacteria are very sensitive to temperature. Therefore, methane production is strongly dependent on temperature and fluctuation in temperature can cause significant difference in digestion performance [7].

Anaerobic digestion is often carried out at two temperature ranges, including the mesophilic range from 30°C to 35°C and the thermophilic range from 50°C to 60°C since methane – forming bacteria are active at these temperatures. Optimal temperatures for mesophilic condition and thermophilic condition are 35°C and 55°C, respectively [4, 6]**.** The microbial communities at two optimal temperatures are different, i.e. a convertibility from mesophilic condition to thermophilic condition or vice versa can cause a significant decrease in biogas production until the number of specialized bacteria have increased [9].

Mesophilic condition has some advantages compared to thermophilic condition. Actually, more different bacterium species can be found in nature under mesophilic than thermophilic conditions [9]. Moreover, mesophilic condition requires less energy for the biogas production process. Otherwise, thermophilic condition gives high methane production rate, high loading potential and shorter retention time [7]. Moreover, that thermophilic bacteria can digest substrates that are not biodegradable under mesophilic condition and the hydrolysis rate can increase 6 times at thermophilic temperature [4]. A comparison regarding to some typical features of anaerobic digestion at mesophilic condition and thermophilic condition is presented in Table 1.

Table 1. Comparison of mesophilic and thermophilic conditions

Feature	Mesophilic condition	Thermophilic condition
Loading rate	Low	High
Retention time	High	Low
Methane production rate	Low	High
Energy consumption	Low	High
Operational cost	Low	High

- **pH and Alkalinity**

The ideal pH for anaerobic digestion ranges from 6.8 to 7.2. A significant decrease in growth rate of methane – forming bacteria occurs if the value of pH is below 6.6. Furthermore, high alkaline pH can cause disintegration of microbial granules and consequently, failure of anaerobic digestion [6].

In the second step, volatile fatty acids are produced and will be consumed by methane – forming bacteria. However, if methane – forming bacteria cannot degrade volatile fatty acids, these acids will accumulate, causing a decrease in pH and finally digestion process can fail. In addition, the degradation of organic compounds can release carbon dioxide which also contribute to the reduction of pH [9].

Alkalinity is a buffer that prevents the decrease in pH. Alkalinity is primarily in the form of bicarbonates with are in equilibrium with carbon dioxide at given pH [7]. It is known that reducing organic loading rate and addition of strong bases or carbonate salts are methods that can be

employed to remove carbon dioxide, result in increase of pH [6]. If amino acids and proteins are degraded, ammonia will be released and can also serves as a source of alkalinity [7].

2.2 Biogas Production from Orange Peel

Orange peel has been considered as a potential source for biogas production [1, 2]. Actually, citrus peel contains a large amount of soluble and insoluble carbohydrates, therefore, citrus peel can become feedstock for production of biogas and bioethanol as well [1, 10]. The soluble carbohydrates include simple sugars such as glucose, fructose, sucrose and the insoluble carbohydrates are cell-wall carbohydrates such as pectin, cellulose, hemicelluloses [10]. However, D-limonene abundant in orange peel is known as an anti-microbial agent [4]. In other words, bacteria will be inhibited by D-limonene, resulting in the failure of anaerobic digestion. Therefore, effect of D-limonene will become one important factor for anaerobic digestion if orange peel is used as feedstock. In order to control the inhibitory effect caused by D-limonene, the limiting load of D-limonene to anaerobic digestion should be estimated. From the obtained value, the loading rate of orange peel can be calculated so that the loading of D-limonene amount does not exceed the threshold inhibitive amount. E. Mizuki et al. [2] used citrus unshu peel as feedstock for anaerobic digestion and found that the limiting load of D-limonene was 58.5μl/liter inoculum per day. Since this value is quite small, the feeding rate will be very low if untreated orange peel is digested, leading to ineffective digestion process. One way to overcome this problem is that untreated orange peel mixed with municipal waste is used as feedstock for anaerobic digestion [11]. Another effective way to deal with the problem caused by D-limonene which is also an objective of our study is that orange peel will be pretreated to decrease the D-limonene content or essential oil content prior to carrying out anaerobic digestion.

As above-mentioned, temperature is one of the most important factors for anaerobic digestion and definitely, this is also the case for anaerobic digestion of orange peel. In fact, a research performed by M.A.Martín et al. [4] stated that thermophilic temperature was considered as the most suitable temperature for anaerobic digestion of orange peel waste in a pilot scale. Thermophilic condition showed some significant conveniences compared to mesophilic condition i.e. methane production rate and biodegradability were higher at thermophilic condition. In fact, the methane yield coefficients at standard temperature and pressure conditions (STP) for thermophilic and mesophilic conditions were 332 ± 17 mL_{STP} CH_4/g added VS and 230±16 mL_{STP} CH_4/g added VS, respectively. Moreover, stability of the biogas digestion which was evaluated by monitoring the change in pH, alkalinity, volatile acidity, volatile acidity/alkalinity ratio and volatile fatty acids profile during the process showed that thermophilic condition is more suitable for biogas digestion of pretreated orange peel [4].

2.3 D-limonene

The chemical name and chemical structure of D-limonene are as follows:
Chemical name: 4-isopropenyl-1-methyl-cyclohexene[12]
Chemical structure:

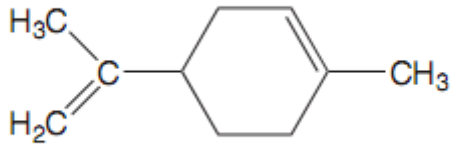

Figure 2. Chemical structure of D-limonene[12]

D- limonene is the main odorous components of citrus oil[4]. Generally, 1000 kg of orange gives an average amount of 5.4 kg oil of which D- limonene amount is approximately 90% [2, 4]. D-limonene is present not only in essential oil from orange but also in other natural oils from lemon, grapefruit, peppermint, caraway, etc. D-limonene is a valuable product with annual production of 50 to 75 thousand tons [13] and has a wide variety of applications in manufacture of foods, medicines, cosmetics, household products [4].

2.4 Possible Pretreatment Methods for Orange Peel

As above-mentioned, D-limonene is an anti-microbial substance and will hinder the anaerobic digestion. Therefore, it is highly desirable to reduce D-limonene content e.g. remove or convert D-limonene into non-toxic substances before carrying out biogas production of orange peel. There are three suggestions to reduce the amount content of D-limonene in orange peel. In fact, D-limonene can be recovered in form of essential oil from orange peel in the pretreatment step. If this is the case, essential oil will be obtained and can be used for other purposes since it is a high value product. The other suggestion is that essential oil in orange peel could be vaporized since it is volatile. The last suggestion is that D-limonene can be converted into other compounds which are expected not to be toxic to anaerobic digestion. Based on these ideas, various pretreatment methods for orange peel have been considered, including pressing, steam distillation, steam explosion, acid/alkaline treatment, solid-liquid extraction, supercritical CO_2 extraction, aeration, thermal treatment and ozone treatment. Some methods are well-developed since they have been employed to produce essential oil from citrus for a long time. Other methods have been studied and the rest are just under-suggestion since they seem feasible. In fact, pressing method, steam distillation, steam explosion, acid/alkaline treatment, solid-liquid extraction, and supercritical CO_2 extraction can be considered as recovery methods in which essential oil will be obtained to be used for other purposes. Thermal treatment and aeration are methods that take advantage of the volatility of orange essential oil. Ozone treatment is the method in which D-limonene is expected to be converted into other compounds. However, in the thesis, these all methods will not be discussed in detail, except for solid-liquid extraction which is the focus in the thesis.

2.5 Solid-Liquid Extraction

In solid-liquid extraction, also called leaching operation, some interested components in the solid will be dissolved into a solvent that has contact with the solid sample. In other words, there is a diffusivity of the solute from the solid into the surrounding solvent when solid-liquid extraction is carried out. Solid-liquid extraction seems to be difficult operation in practice. In fact, diffusion in the solid is slow and some solvent often remains inside the solid after completion of the extraction process. Extraction of organic compounds can be conducted by using Soxhlet extraction, extraction using mechanical shaking and extraction under sonication [3].

For solid-liquid extraction, it is very important to choose appropriate solvents with respect to the organic compounds of interest in the solid. In general, n-hexane and some alcohols such as methanol and ethanol are often used. Solid-liquid extraction is one of many interesting methods used to extract organic compounds from natural materials where these compounds present at low concentration.

Brief description of solid-liquid extraction procedure is presented in the Fig. 3. The solid feed consists of insoluble A and solute B. The solvent C can dissolve B. The overflow is solid-free liquid of solvent C and dissolved B. The underflow composes of solid A and retained liquid phase. It should be noticed that the composition of the retained liquid phase in the underflow is identical to the composition of the liquid overflow [14].

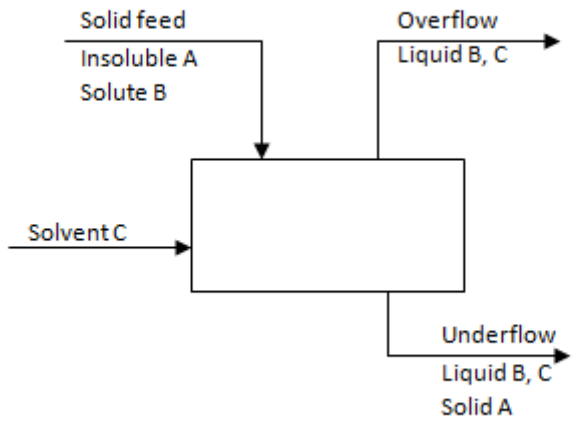

Figure 3. Leaching stage [14]

The extraction process can be divided into three stages [15]:

- The change of phase of the solute when it dissolves into surrounding solvent.
- The diffusion of solute through the solvent from the pores of the solid to surface of the particles.
- The transfer of the solute from the solution contacting with the particles to the main bulk of the solution [15]

In solid-liquid operation, mass transfer can be written using the thin film concept as the resistance to the transfer as follows:

$$\frac{dM}{dt} = \frac{k' A(c_s - c)}{b}$$

with $k'/b = K_L$ (Mass transfer coefficient)

For the batch process, the total volume of solution can be assumed as constant, the following equation will be defined:

$$dM = Vdc$$

Then $\frac{dc}{dt} = \frac{k' A(c_s - c)}{bV}$

The solute concentration c will increase from the initial value c_0 to the final value c_s. Taking integration with the assumption that b and A remain constant, then the following equation will be deduced:

$$ln\frac{c_s - c_0}{c_s - c} = \frac{k' A}{Vb}t$$

Assuming that the pure solvent is used initially (c_0=0), then:

$$c = c_s(1 - e^{-\left(\frac{k' A}{bV}\right)t})$$

From the equation of mass transfer, it can be seen that the extraction rate is affected by a number of factors and there are four important factors that should be considered, as follows:

- Particle size: The smaller the size of the solid particles is, the greater the specific interface between the solid and liquid will be obtained, resulting in the higher mass transfer rate and the smaller distance the solute will diffuse within the solid phase. Thus, higher extraction rate will be achieved. However, a very fine solid seems to give negative effect to some extent if the solvent is preferred to be recycled. This is because the separation of the liquid from the solid and the drainage of the residuals are more difficult.
- Solvent: The extraction solvent should have good selectivity towards the solute. During the extraction process, the concentration of the solute will increase gradually and the extraction rate will decrease. However, the pure solvent should be used at the beginning since the driving force for extraction process, concentration gradient, will be progressively reduced.
- Temperature: The temperature affects the extraction rate in numbers of ways. Firstly, the solubility of material that is extracted will increase with the rising of the temperature, resulting in the higher extraction rate. Secondly, as the temperature increases, the diffusion coefficient will increase which also give higher rate of extraction.
- Agitation: Agitation improves the mass transfer rate of the solute from the surface of the solid particle to the bulk of the solution to a greater extent [15].

In our case, it can be recognized that the overflow stream is the solvent containing D-limonene and the underflow stream is extracted peel with a little solution of solvent containing D-limonene as the same composition compared to the overflow stream. The fact that some organic solvent still remains inside pretreated orange peel may cause negative effects on anaerobic digestion process. Therefore, selection of solvents should be performed carefully so that these solvents will be less toxic to further anaerobic digestion.

2.6 Experimental Design

Experimental design methods have played important roles in this thesis work since they were used to plan, conduct experiments, analyze and evaluate experimental results. In fact, nested design and factorial design were chosen for planning experiments and Analysis of Variance (ANOVA) was used for evaluating experimental results. Minitab and Design-Expert are soft-wares that have been chosen to assist the experimental design.

2.6.1 Two-Level Factorial Design

Factorial design is often applied in experiments where several factors are involved and interactions between these factors also need to be taken into account so that their effects on the responses can be estimated. The most important case of this special design is that of k factors at two levels which are quantitative and qualitative. A complete replicate of such a design requires 2^k observations and called 2^k factorial design. One significant advantages of the kinds of design is the fact that it provides the smallest number of runs in which k factors can be investigated. Therefore, factorial designs are often used in factor screening experiments [16].

2.6.2 Nested Design

In experiments involved multi-factors, the levels of one factor (e.g. factor B) are similar but not identical for different levels on another factors (e.g. factor A). This kind of design is called Nested design in which the levels of factor B are nested under the levels of factor A. A two-stage nested design with three observations is shown in Fig.4.

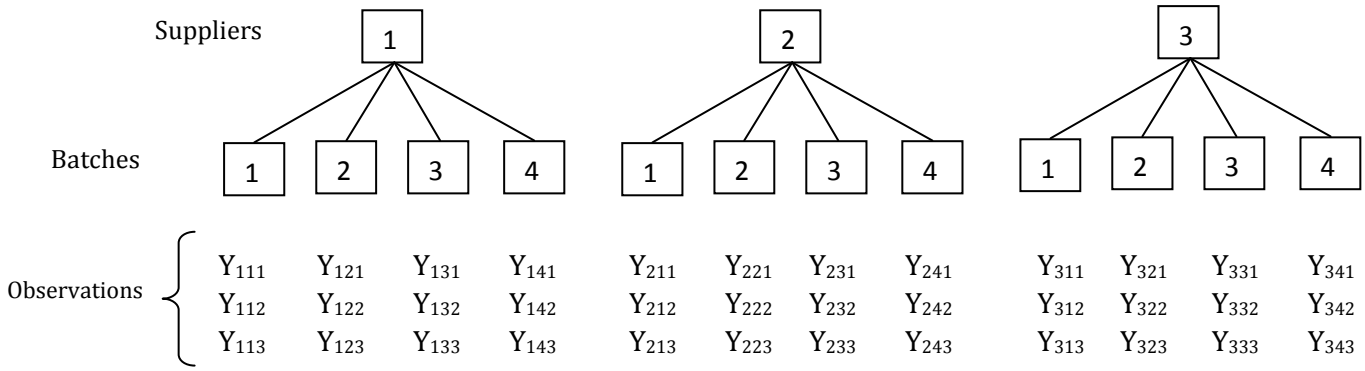

Figure 4. A two-stage nested design [16]

3. Objectives and Methodology

Orange peel has been demonstrated as one of potential sources for biogas production [4, 6]. However, essential oil extracted from orange peel contains approximately 90% D-limonene [4, 6] which is known as anti-microbial agent [4]. D-limonene can cause a substantial problem if orange peel, without pretreatment, is used as a substrate for biogas production. Therefore, choosing an effective method to reduce D-limonene in order to improve biogas digestion performance of orange peel are the main aims of this study. As above-mentioned, solid-liquid extraction is an technique of interest for extraction of natural compounds. Moreover, to the best of our knowledge this technique has not been employed to pretreat orange peel used for biogas production. Therefore, it is highly desirable to examine solid-liquid technique in our study for D-limonene removal purpose. After carrying out pretreatment step, peel was digested under thermophilic condition to produce biogas. GC (FID) and GC-MS analysis were used to determine amount of D-limonene extracted and GC (TCD) was used to determine the composition of the biogas. Moreover, experimental design played an important role during the thesis where it was used for planning the experiments, analyzing, evaluating experimental results and constructing statistical models. Nested Design and Factorial Design were chosen for planning experiments and Analysis Of Variance (ANOVA) was employed for evaluating experimental results. Minitab 16 and Design-Expert 8 packages are softwares that were chosen to assist the experimental design.

This work is divided into two parts. In the first part, the main aims was to design an initial pretreatment procedure and look for an appropriate solvent for solid-liquid extraction process with regard to extraction efficiency and their effect on biogas production as well. The solid material was chopped orange peel and four different solvents, including n-hexane, diethyl ether, dichloromethane and ethyl acetate were investigated. After completion of extraction process, extracts were analyzed to determine extracted D-limonene content. Different kinds of peel treated by different solvents as well as untreated peel were digested to produce biogas. Biogas production was carried out with different VS concentration (%). Biogas production was monitored during the digestion time. Nested design was used for planning experiments with the response of interest was methane amount in produced biogas. ANOVA was carried out to evaluate obtained data. Consequently, the appropriate solvent, n-hexane was chosen and the pretreatment procedure was also designed.

With the obtained results so far from the first stage of the thesis, experiments for further study were planned. The main purpose of the second stage was to redesign the pretreatment procedure as well as improve conditions for extraction process using n-hexane so that biogas will be produced with the highest yield from pretreated orange peel and of course, be improved compared to that of

untreated orange peel. Four factors, including the size of orange peel, extraction temperature, extraction time, n-hexane/peel ratio have been considered the most important ones causing effects on extraction process; therefore, they were studied. Two-level factorial design with four above-mentioned factors was chosen for planning the experiments. The biogas yield was chosen as the factor response and completely factorial design with four variables was conducted. Furthermore, additional experiments at the center points were carried out to estimate the pure error since the factorial design is single replicate. After being treated by n-hexane, all types of treated orange peel were digested under thermophilic condition to produce biogas. From the results of biogas production, normal probability method and ANOVA were performed to check the significance of variables and that of the model. Likewise, the appropriate extraction conditions would be chosen.

4. Screening Selection of Solvents and Application of Two-Stage Nested Design

4.1 Introduction

This is the first part of the thesis where the main aim was to initially design a pretreatment procedure and look for an appropriate solvent as well. A simple pretreatment procedure was designed and four different solvents were employed. After pretreatment step, different treated peel types were digested at different VS concentration (%) to produce biogas. The two-stage nested design was chosen to conduct experiments and ANOVA was used to analyze results. Moreover, Minitab software was employed to assist experimental design. Experiments and results will be presented in detail in this section.

4.2 Materials and Instrumentations

- Chemicals including n-hexane, diethyl ether, ethyl acetate, dichloromethane and sodium sulfate were used as received.
- Orange peel waste was collected from Brämhults Juice AB in Borås, Sweden.
- Active inoculum was collected from Sobacken, thermophilic biogas plant in Boras, Sweden. Since the temperature of inoculum dropped during the delivery period, the collected inoculum was kept at the thermophilic condition (55°C) for 3 days before it was used for digestion process.
- GC analyses for biogas composition were performed using Varian 450-GC equipped with the packed column (J&W Scientific GS-GasPro, 30 m x 0.320 mm) and a thermal conductivity detector (TCD). The carrier gas was nitrogen with the flow-rate of 2 ml/min. The temperature profile for GC analyses is shown in Table 2.
- GC analyses with FID detector were performed using Clarus 400, Perkin Elmer equipped with a capillary column (ZB-WAX-Plus, 30 m x 0.25 mm x 0.25 μm). The carrier gas was nitrogen with the flow-rate of 1 ml/min. The detector gas was hydrogen with the flow-rate of 1 ml/min. The temperature program for GC (FID) analyses was held at 80°C for 3 minutes, heated from 80°C to 140°C at 15°C /minute, from 140°C to 275°C at 45°C/minute.
- Minitab 16 package was employed to assist experimental design.

Table 2. Temperature profile for GC (TCD) analysis

Position	Temperature (°C)
Inject	75
Oven	100
Detector	120

4.3 Experimental

4.3.1 Pretreatment of Orange Peel by Solid-Liquid Extraction Technique

Orange peel was chopped into small pieces. The chopped orange peel (40 g) was placed into an Erlenmeyer flask containing a known volume of solvent (150 ml). Four different solvents, including n-hexane, diethyl ether, dichloromethane and ethyl acetate were added. The Erlenmeyer flasks were shaken vigorously for 10 minutes and then kept for an additional period of time (20 minutes). After completing the extraction time, extracts were removed from residuals by vacuum filtration. Extracts were stored and purified for further analysis by GC (FID) to determine D-limonene content.

After carrying out treatment step, all these kinds of treated peel were determined TS and VS content and then digested at different VS concentration (%), including 0.5, 1, 1.5, 2 %. Two-stage nested design was chosen as the method for conducting experiments. Our purpose was to confirm whether biogas production is really affected by different kinds of treated orange peel and/or different VS concentrations (%) or not. Brief schematic representation of experimental design is shown in Fig. 6. Notice that this design was applied for all kinds of peel including untreated peel, peel treated by n-hexane, peel treated by diethyl ether, peel treated by dichloromethane and peel treated by ethyl acetate, resulting in totally 60 experimental setups.

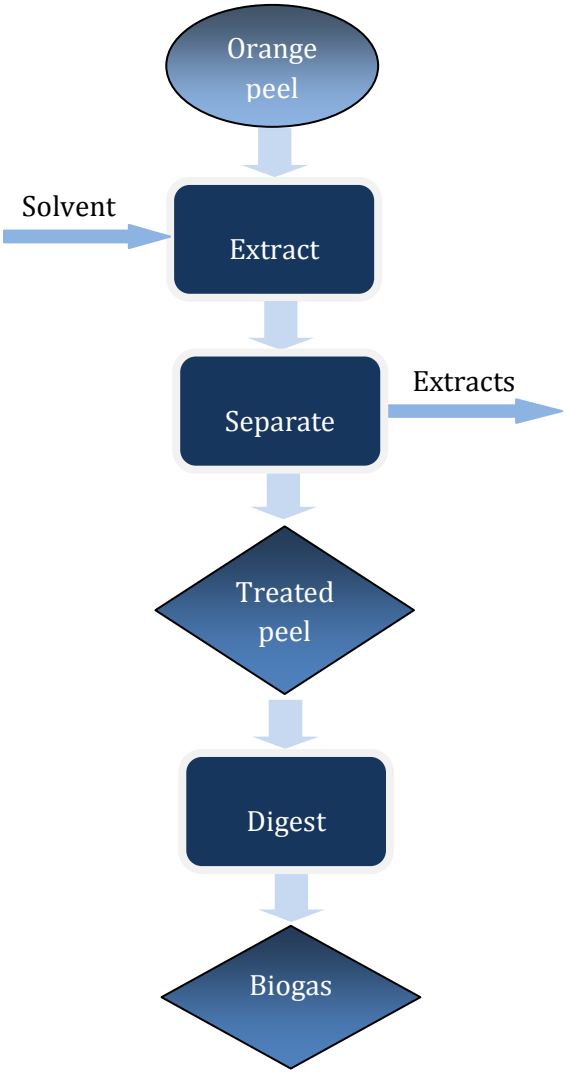

Figure 5. Block diagram of pretreatment and anaerobic digestion process

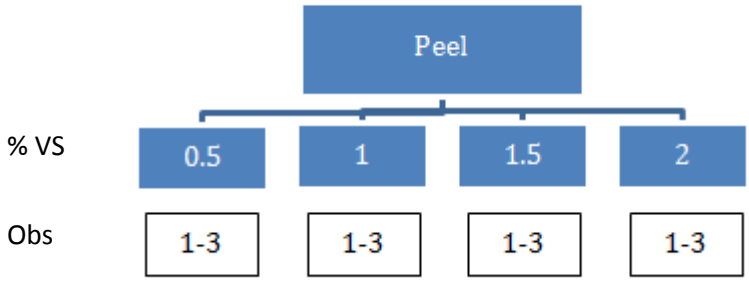

Figure 6. A brief schematic representation of the two-stage nested design with three observations

4.3.2 Determination of TS and VS

TS and VS were determined for untreated orange peel, treated orange peel as well as for inoculum. TS are all solids in the sample and can be measured by a known amount of a sample after drying at 105°C in an oven. Firstly, an empty evaporation crucible was put into the oven and dried overnight at 105°C. It was then cooled down in desiccator and the weight was measured. A known amount of samples was placed into the dried crucible and kept in the oven at 105°C for 24 hours. After completion of drying time, the crucible containing remaining solid was cooled down in the desiccator and then the weight was measured. The TS content was calculated accordingly:

$$TS(\%) = \frac{x_3 - x_1}{x_2} \times 100\%$$

Where

x_1: weight of the dried crucible (g)

x_2: weight of sample (g)

x_3: weight of the crucible plus the remaining solids after drying at 105°C (g)

VS are solids that can be removed by burning the sample at 550°C in a muffle furnace. After determining the TS, the crucible containing the remaining solid was placed into the furnace at 550°C for 1 hour. The crucible containing the remaining ash was then cooled down in the desiccator and the weight was measured. The VS content was calculated as follows:

$$VS(\%) = \frac{(x_3 - x_1) - (x_4 - x_1)}{x_2} \times 100\%$$

Where

x_1- x_3: see above

x_4: weight of the crucible plus the remaining solids after burning at 550°C (g)

4.3.3 Batch Digestion, Monitoring and Data Treatment

Batch biogas digestion was carried out in 120 ml-glass bottles as reactors under thermophilic condition (55°C). The total sample-volume was 25ml, including 20 ml of inoculum and the rest was orange peel and added water. The reactors were then flushed for about 2 minutes with a mixed gas containing 80% of N_2 and 20% of CO_2 in order to ensure the anaerobic condition inside the reactors. The reactors were then placed into the incubator at 55°C. During the digestion process, the reactors were shaken everyday to compensate for variations in temperature inside the reactor. Each experimental setup was conducted in triplicates since the method is biological test. Biogas production with different VS concentrations (%) of substrate, including 0.5%, 1%, 1.5%, 2% were studied for both untreated and treated orange peel waste.

Three blanks with only water and inoculum were carried out to measure biogas production originating from the inoculum so that the methane potential of orange peel can be determined more accurately to some extent.

Biogas production was monitored during the digestion time using GC (TCD). Gas samples of 100 µl were withdrawn from the headspace of the reactors using a 250 µl pressure tight-syringe (VICI, Precision Sampling Inc., USA). This type of syringe was used so that the fixed volume of samples at the actual pressure inside the reactor can be taken. The samples were then injected directly into the gas chromatograph to measure the mass of methane in biogas produced. Pure methane and carbon dioxide with known volume, temperature and pressure were used as standards for each time of measurement occasion. Since the fixed volume of samples was used, the measured mass of methane in samples can be determined with reference to standard methane and carbon dioxide. The results, assuming ideal gas and using the ideal gas law, methane and carbon dioxide content in the reactor headspace, can be calculated without measuring the actual pressure in the reactors. One more thing should be noticed is that the headspace of the reactor can be calculated by subtracting the added amount of inoculum, water and samples (25 ml in total) from the volume of the reactors.

Assuming ideal gas, the number of moles of standard sample can be calculated as follows:

$$n_{standard} = \frac{PV_{standard}}{RT}$$

The GC analysis provides the peak area of methane (carbon dioxide) standard and the peak area of that in samples taken from the reactors. As the result, the mole number of methane (carbon dioxide) produced in each reactor can be calculated using the following equation:

$$n = \frac{A_{sample}}{A_{standard}} \times n_{standard} \times \frac{V_{headspace}}{V_{sample}}$$

Where:

n: number of moles (methane/carbon dioxide) in the reactor

A_{sample}: peak area of methane/carbon dioxide of the sample

$A_{standard}$: peak area of methane/carbon dioxide of the standard

$n_{standard}$: number of moles (methane/carbon dioxide) of the standard

$V_{headspace}$: volume of headspace of the reactor = 95 ml

V_{sample}: volume of sample injected with = 0.1 ml

Methane production is performed as volume gas (ml) as function of time. The gas in the reactor needs to be released to avoid too high pressure in the reactor leading to leakage of gas. Significant pressure build up can be easily recognized from the shape of the rubber septum. It should be emphasized that after releasing, the produced biogas has to be accumulated. pH value of digested mixture in each reactor was measured at the end of each experiment if needed.

4.3.4 Determination of Total D-limonene Content by Soxhlet Extraction

Homogenized peel (50 g) was used as solid material for the Soxhlet extraction. The solvent was ethanol with an amount of 200 ml. The extraction process was carried out for 4 hours. After completion of extraction process, the obtained extraction was purified and then analyzed by GC-MS for the determination of the D-limonene content.

4.4 Results and Discussion

Three samples from each type of substrate were prepared to determine the TS and VS content. The average values are shown in Table 3.

Table 3. Summarized results of TS and VS measurement

	Inoculum	Untreated peel	Peel treated by hexane	Peel treated by diethyl ether	Peel treated by dichloromethane	Peel treated by ethyl acetate
TS (%)	2.57 ± 0.13	21.26 ± 0.36	21.77 ± 0.95	19.37 ± 1.05	20.19 ± 0.98	19.08 ± 0.90
VS (%)	1.50 ± 0.09	16.07 ± 0.65	15.68 ± 0.99	16.53 ± 0.96	15.05 ± 0.68	16.49 ± 0.57

The total amount of D-limonene content presented in orange peel was determined employing Soxhlet extraction technique with ethanol as solvent extraction. The analysis results showed that the composition of D-limonene in the original peel was 0.9% of total solid. By comparing results of D-limonene in extracts with the total D-limonene content in the peel, amount D-limonene removed in percentage can be calculated (Table 4). As can be seen, n-hexane was the best solvent in term of extraction efficiency since it extracted much more D-limonene than other solvents.

Table 4. D-limonene analysis results

	Treated by hexane	Treated by diethyl ether	Treated by dichloromethane	Treated by ethyl acetate
Limonene extracted (%)	9.23	2.33	2.04	2.31

One of the purposes for this experimental series was to find out which solvent among those chosen was the most suitable in terms of their extraction efficiency as well as their effect on biogas production. Thus, biogas production of treated orange peel was carried out with different VS concentration, including 0.5%, 1%, 1.5% and 2% so that our assessment for different solvents will be more accurate to a greater extent. Anaerobic digestion of untreated orange peel with different VS concentrations were also studied since these experiments were expected to become a standard so that some comparison between biogas production of treated orange peels and untreated one can be made. The biogas production of blank itself was also examined.

There was significant difference in biogas production performance from different treated peel types at the early stage of digestion time. Moreover, our current purpose was to just look for the appropriate solvent rather than determining methane production potential of treated materials.

Therefore, the result for digestion process was decided to be picked up after 10 days of incubation. Some typical results are presented in Fig. 7. Notice that these results were mean values calculated from three values since each batch digestion was carried out in triplicates.

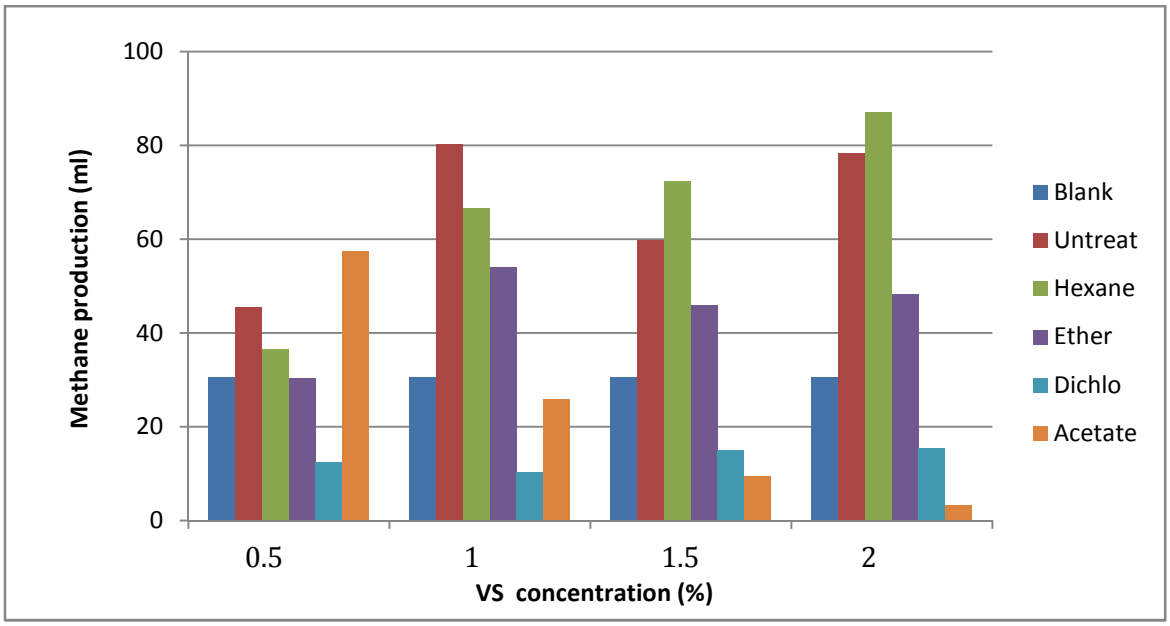

Figure 7. Methane production after 10 days of digestion of different substrates at different VS concentration (%)

From Fig. 7, there was significant difference between biogas production of untreated orange peel and that of treated orange peel, as well as difference between biogas production of orange peel treated by different solvents. Actually, orange peel waste treated by dichloromethane and ethyl acetate gave significantly lower methane amount in almost all the studied VS concentrations. Moreover, these samples even gave lower methane production compared to that of blank. This means that dichloromethane and ethyl acetate are very toxic to bacteria. It can be seen that the samples with 0.5% VS concentration of peel treated by ethyl acetate produced quite high methane amount. The reason that can be taken into consideration was decomposition of ethyl acetate in which methane can be produced. However, this phenomenon did not happen with the samples of other % VS concentrations prepared from the same substrate. Otherwise, these samples gave worse results of biogas production compared to the others. Therefore, it seems to be no need to put more interest on ethyl acetate as solvent for our extraction purpose. The higher produced methane amount was observed in the case of orange peel treated by diethyl ether. More interestingly, the higher produced methane amount was observed when untreated peel and peel treated by n-hexane were digested for all the studied VS concentrations. For the 0.5% and 1% VS, untreated peel gave significantly higher methane amount in comparison with peel treated by n-hexane; however, the opposite thing occurred when higher VS concentrations, 1.5% and 2%, were used. One reasonable explanation is that at the lower VS concentrations, D-limonene seems to cause less bad effect than extraction solvent does. However, as the VS concentration increases the accumulated D-limonene also increased and the effect of D-limonene is more obvious compared to effect of organic solvents. One more interested thing that can also be deduced is that some of D-limonene in orange peel was extracted by n-hexane as being confirmed by GC (FID) results.

Generally, treated orange peel seems to give unexpected results concerning biogas production. In fact, treated orange peel was not as good as untreated peel in this case. Actually, only 1.5% and 2% VS concentrations of peel treated by n-hexane can give slightly higher methane production

potential than for untreated peel. It can be explained by the fact that extraction organic solvent remained inside the peel caused toxic to bacteria. Moreover, one thing we know for sure is that D-limonene presented in the peel cannot be extracted completely. Actually, at the known condition the highest amount extracted D-limonene can be obtained only when the equilibrium of this compound in the peel and in the solvent is established. In addition, the fact that some extraction solvent still remains in the solid after completion of solid-liquid extraction cannot be completely avoided.

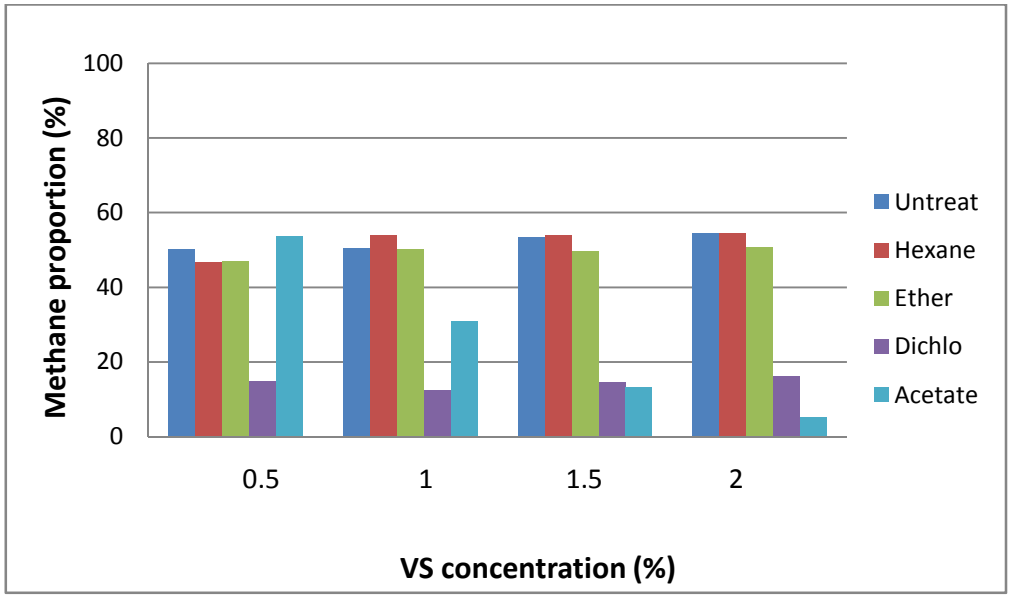

Figure 8. Proportion of methane measured in biogas (at the digestion time of 10 days)

It is shown in Fig. 8 that the samples prepared from the peel treated by dichloromethane and ethyl acetate gives very low proportion of methane in biogas. Otherwise, samples prepared from untreated and treated peel by n-hexane and diethyl ether gave higher proportion of methane in biogas. In the case of the samples with 0.5 %VS concentration of substrate prepared from peel treated by ethyl acetate, the proportion of methane was rather high.

In summary, untreated orange peel seems to be better than treated peel in terms of biogas production. The reason may lie in the toxicity caused by the organic solvents and that the pretreatment procedure is not efficient enough. However, it seems that n-hexane was the best one among solvents that were chosen for pretreatment purpose and it will be the most interesting solvent that should be focused more in our case. However, diethyl ether is also another interesting alternative.

Table 5. Methane amount produced after 10 days of digestion

ml	Untreated	Treated by hexane	Treated by diethyl ether	Treated by dichloromethane	Treated by ethyl acetate
0.5% VS	45.6	36.6	30.5	12.5	57.6
1 % VS	80.3	66.5	54.0	10.3	25.9
1.5 % VS	59.7	72.5	45.9	15.0	9.6
2 % VS	78.5	87.1	48.3	15.6	3.2

From the Table 5, it can be seen that produced methane varies a lot depending on the kind of substrate used as well as VS concentration. The highest methane amount, 87.1 ml, obtained in the

case of 2 % VS concentration of orange peel treated by n-hexane and the lowest methane amount, 3.2 ml, obtained when 2% VS concentration of the peel treated by ethyl acetate was digested.

At the end of digestion time, pH values were measured and the results are shown in table below.

Table 6. pH value

pH value	Untreated	Treated by hexane	Treated by diethyl ether	Treated by dichloromethane	Treated by ethyl acetate
0.5% VS	7.8	7.8	7.7	7.4	7.7
1 % VS	7.6	7.7	7.9	6.8	5.1
1.5 % VS	7.9	7.8	7.8	6.3	5.1
2 % VS	7.8	7.7	7.7	6.3	5.0

pH values ranging from 6.8 to 7.2 is considered optimum for digestion. Lower pH value will cause failure for digestion [7]. It can be seen from the obtained pH values that the samples with 1, 1.5 and 2% VS of orange peel treated by ethyl acetate and dichloromethane had very low pH value. This is probably one of the reasons why those samples gave low methane production.

Since we have just seen the comparison of effect of different solvents and different VS concentrations by above visual charts, the logical way to analyze the experimental results will be needed. In our case, ANOVA for two-stage nested design was performed to confirm whether biogas production was really affected by different kinds of treated orange peel and/or different VS concentrations or not. From the data obtained, ANOVA was carried out assuming fixed factors. The results are summarized in Table 8. Minitab was used for ANOVA and constructing plots. As one can see from the results of ANOVA the F value is significant larger than the P value, the conclusion is that there were significant differences in biogas production among different types of substrates used, including untreated orange peel and the orange peel treated by different organic solvents. In addition, there was also significant difference in biogas production among different VS concentration.

Table 7. Methane production results for ANOVA calculation of nested design

ml		Untreated				Treated by n-hexane				Treated by diethyl ether				Treated by dichloromethane				Treated by ethyl acetate			
	% VS	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2	0.5	1	1.5	2
Obs	1st	41.6	80.2	49.1	82.2	33.2	65.9	70.4	84.3	30.7	44.4	47.7	47.2	12.8	10.3	16.5	16.5	54.3	27.1	9.2	4.9
	2nd	48.6	80.8	59.8	74.6	31.0	74.2	75.7	83.3	32.6	60.0	44.6	50.7	10.4	10.3	15.0	17.0	62.2	26.5	8.7	2.1
	3rd	46.4	79.8	70.3	78.6	42.6	59.5	71.4	93.6	28.0	57.7	45.6	46.9	14.4	10.5	13.7	13.2	56.2	24.0	10.9	2.7
	Average	45.6	80.3	59.7	78.5	35.6	66.5	72.5	87.1	30.5	54.0	45.9	48.3	12.5	10.3	15.0	15.6	57.6	25.9	9.6	3.2
	StDev	3.6	0.5	10.6	3.8	6.2	7.3	2.8	5.7	2.3	8.4	1.6	2.1	2.0	0.1	1.4	2.1	4.1	1.6	1.2	1.5

Table 8. Analysis of Variance for the data in Table 7

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Type of substrate	4	27393.6	6848.4	8.0	0.001
VS %	15	12762.9	850.9	45.1	0.000
Error	40	754.1	18.9		
Total	59	40910.7			

Table 9. Variance components

Source	Variance Comp.	% of Total	Standard Dev.
Type of substrate	499.8	62.8	22.4
VS %	277.3	34.8	16.7
Error	18.9	2.4	4.3
Total	796.0		28.2

The diagnostic checking was performed to confirm the conclusion of the ANOVA results to a greater extent. In order to make diagnostic and model checking, residual analysis was used as a tool. For a two-stage nested design, the residuals will be calculated as follows:

$$e_{ijk} = y_{ijk} - \hat{y}_{ijk} = y_{ijk} - \bar{y}_{ijk}$$

Where

y_{ijk}: Observed value

$\hat{y}_{ijk}$: Fitted value (Model)

$\bar{y}_{ijk}$: Estimated value of $\hat{y}_{ijk}$

Residuals were calculated and scatterplots were constructed (Fig. 9-11). As one can see from scatterplots above the spread of the residuals was significant different between % VS concentration as well as between the types of substrate, showing that there were significant differences in biogas production among different types of substrates and among different VS concentration as well.

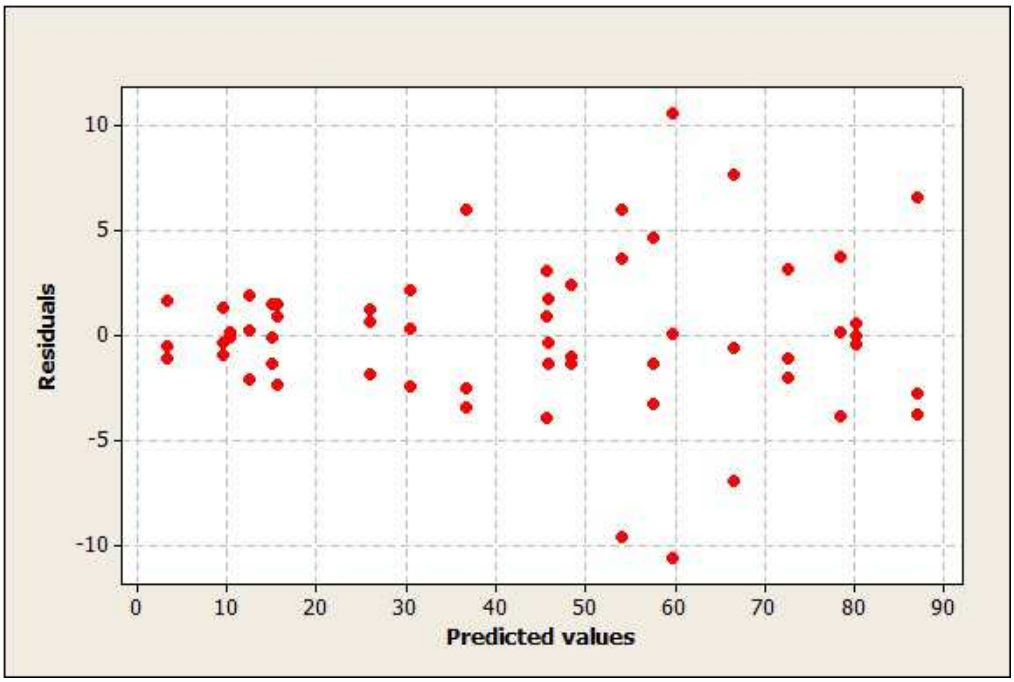

Figure 9. Scatterplot of residual versus predicted value

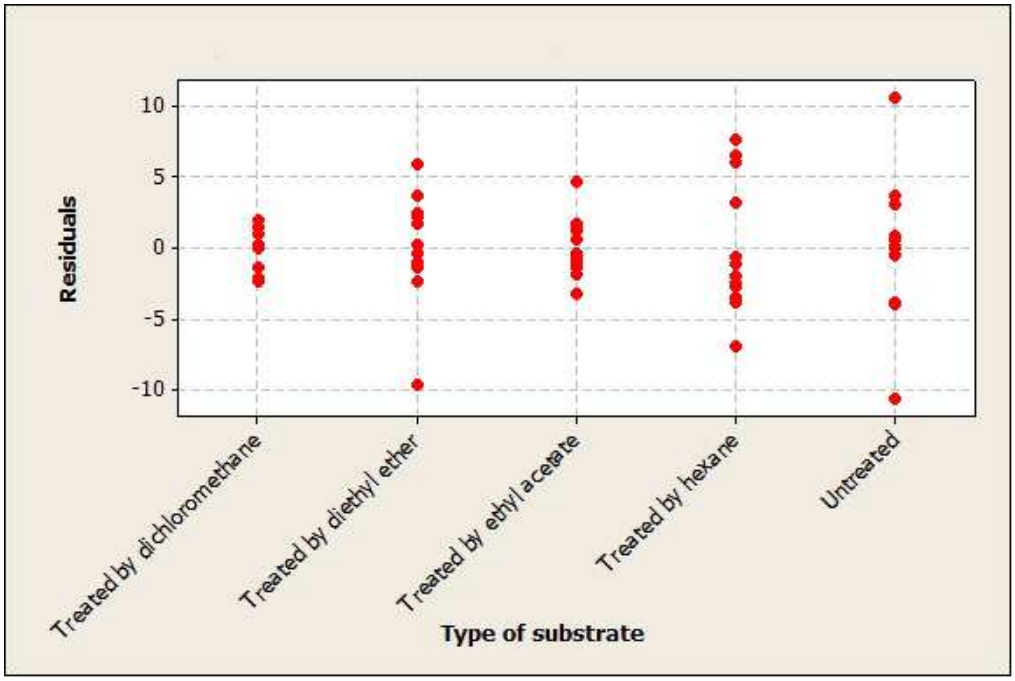

Figure 10. Scatterplot of residual versus different types of substrate

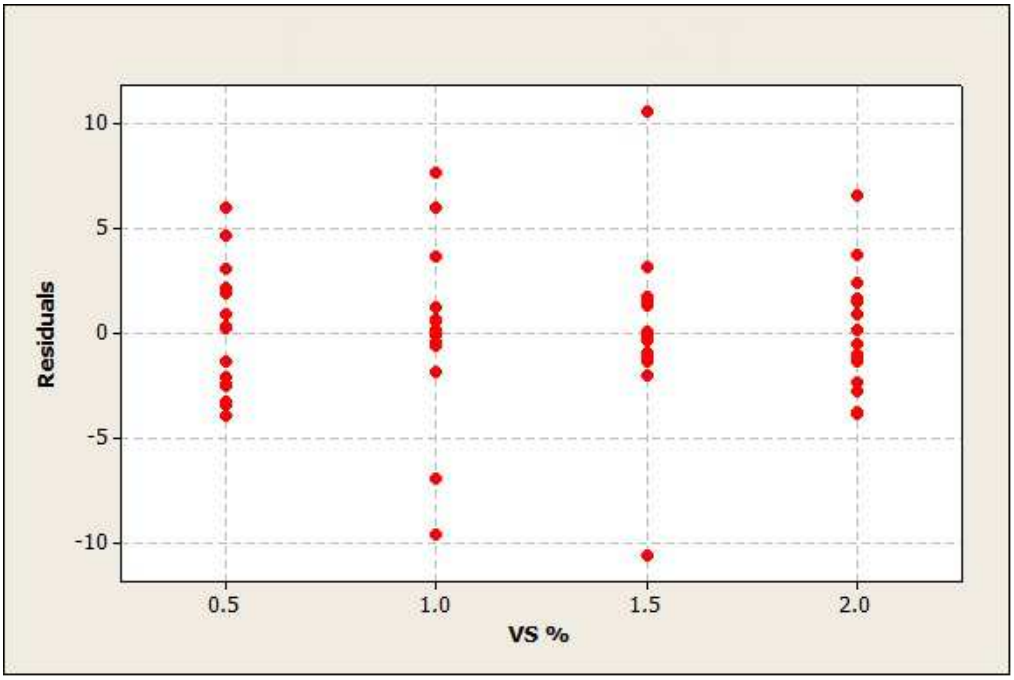

Figure 11. Scatterplot of residual versus different VS concentration (%)

4.5 Summary of Results

- A simple pretreatment procedure was designed and four solvents were investigated, including n-hexane, dichloromethane, diethyl ether and ethyl acetate. A two-stage nested design was applied to conduct experiments. Experimental results and ANOVA showed that there were significant differences in biogas production at four different VS concentration (0.5, 1, 1.5, 2 %) from peels treated with different solvents. Untreated peel often produced more methane than orange peel treated by chosen organic solvents following the suggested pretreatment method. The reason for unexpected biogas production can lie in the fact that there was solvent remaining in orange peel after pretreatment step and that remaining solvent caused the toxicity to biogas production.
- n-hexane was the most interesting solvent since it showed higher extraction efficiency toward D-limonene and orange peel treated by n-hexane gave higher methane production than the peel treated by other solvents. Behavior of n-hexane can be due to either its high extraction efficiency or its less toxicity to bacteria. Further experiments should be focused on pretreatment using n-hexane as solvent and the peel treated by n-hexane for biogas production. In addition, the pretreatment step should be improved and the conditions for pretreatment should be investigated so that biogas production from treated orange peel will increase in comparison with untreated one.

5. Improvement of Pretreatment Stage and Application of Single Two-Level Factorial Design

5.1 Introduction

This is the second part of the thesis where pretreatment procedure was improved and different pretreatment conditions were investigated so that the biogas production from treated peel would increase as much as possible compared to that of untreated one. In other words, the purpose was to construct models that consist of factors causing strong effect on pretreatment process resulting in effect on biogas production and then to optimize the biogas production from treated peel. In fact, several factors affecting pretreatment process that were considered as the most important ones were studied. Moreover, two-level factorial design was employed to conduct experiments and Design-Expert 8 software package was chosen to assist the experimental design. Experiments and results in detail will be presented below.

5.2 Materials and Instrumentations

- Chemicals including n-hexane, diethyl ether and sodium sulfate were used as received without further purification.
- Orange peel waste was collected from Brämhults Juice AB in Boras, Sweden.
- Active inoculum was collected from Sobacken, thermophilic biogas plant in Boras, Sweden. The collected inoculum was kept at the thermophilic condition (55°C) for 3 days before it was used for digestion process
- GC with TCD for biogas measurement was the same as the one specified previously in section 4.2.
- GC-MS analyses were performed using HP G1800C GCD Series II (Gas Chromatography Electron Ionization Detector) with the capillary column (HP 5, 30 m x 0.25 mm). The carrier gas was helium with the flow rate of 1ml/min. The detector gas was hydrogen with the flow rate of 1ml/min. The injection temperature was 200°C, the temperature of the detector was 280˚C. Initial temperature is 50°C, kept for 2 minutes and then increased 15°C /minute to 250°C and finally kept for 3 minutes.
- Design-Expert 8 package was employed as a tool for experiment design.

5.3 Experiments

As indicated previously n-hexane was chosen as pretreatment solvent for further study. Four factors, including orange peel types (homogenized peel and chopped peel), pretreatment temperature, pretreatment time, hexane/peel ratio (volume/weight) were investigated. Single complete factorial design was chosen for constructing the experimental setups. Biogas yield (produced methane) was chosen as the response. The investigated two levels for variables were as follows:

Table 10. Two levels of four variables

Variable	Lowest value (−)	Highest value (+)
Temperature (A)	20°C	40°C
Time (B)	10 minutes	300 minutes (5 hours)
Hexane/peel ratio (C)	2	12
Peel type (D)	Homogenized	Chopped

Since complete factorial design was carried out for four factors at two levels, 2^4 experiments, 16 experiments of pretreatment were conducted, resulting in 16 kinds of pretreated peel. In addition, 6 experiments at the center points were also carried out so that pure error can be estimated. Notice that experiments were made in random order. The factorial design matrix is presented below:

Table 11. Factorial design matrix

Experiment No.	Sample name	Factors			
		A	B	C	D
1	1H	-	-	-	Homogenized
2	2H	+	-	-	Homogenized
3	3H	-	+	-	Homogenized
4	4H	+	+	-	Homogenized
5	5H	-	-	+	Homogenized
6	6H	+	-	+	Homogenized
7	7H	-	+	+	Homogenized
8	8H	+	+	+	Homogenized
9	1C	-	-	-	Chopped
10	2C	+	-	-	Chopped
11	3C	-	+	-	Chopped
12	4C	+	+	-	Chopped
13	5C	-	-	+	Chopped
14	6C	+	-	+	Chopped
15	7C	-	+	+	Chopped
16	8C	+	+	+	Chopped
17	1HC	0	0	0	Homogenized
18	2HC	0	0	0	Homogenized
19	3HC	0	0	0	Homogenized
20	1CC	0	0	0	Chopped
21	2CC	0	0	0	Chopped
22	3CC	0	0	0	Chopped

Homogenized peel and chopped peel were treated to remove D-limonene following the diagram below. In this new pretreatment procedure, the washing step was added. The purpose of washing step was to remove remaining n-hexane. After pretreatment step, the peel was washed three times with water before it was digested for the production of biogas. The extracts obtained were further purified and then analyzed by GC-MS to determine D-limonene content. TS and VS measurements were carried out for pretreated peels following the method presented previously in section 4.3.2. The treated peel and untreated one were then digested under thermophilic condition and the gas production was monitored with the same procedure specified in section 4.3.3. The only diffidence should be noticed is that the total volume of the mixture in each bottle was 30 ml, instead of 25 ml. VS concentration of 2% was chosen. The digestion time was 33 days. pH measurement was also carried out at the end of digestion time.

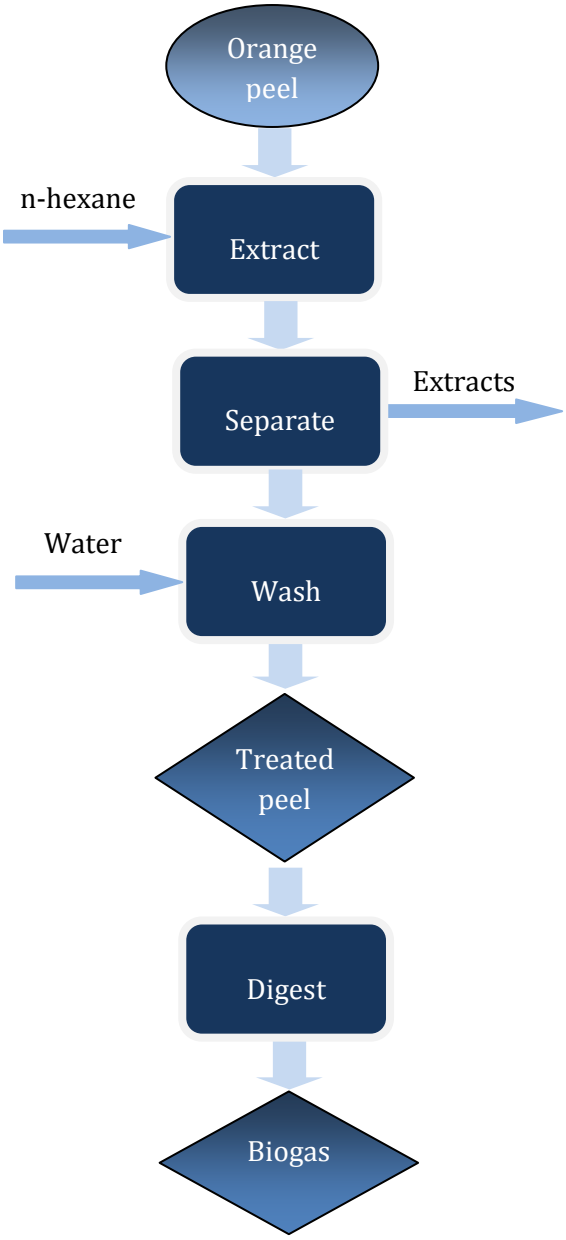

Figure 12. Block diagram of pretreatment and anaerobic digestion process

5.4 Results and Discussion

Three samples from each type of pretreated peel were prepared to be determined TS and VS content. The average values are shown in the Table 12. As one can see from the results, there were significant differences in TS and VS values between treated homogenized peel and chopped peel. From these results, the total VS content of treated peel was calculated and compared with the total VS content of untreated peel. Likewise, values of loss of VS (%) were estimated.

Table 12. TS and VS results for treated peel

Samples	TS (%)	VS (%)	Loss of VS (%)
Homogenized peel	6.4	6.0	51.0
Chopped peel	11.6	11.0	15.0

All peels treated at different conditions were digested to produce biogas. The digestion for each peel types was made in triplicates and average values were taken as results. Accumulated biogas production of treated peels and untreated ones as function of time is shown in Fig. 13-14. The digestion process was decided to stop after 33 days of digestion when the amount of produced methane stabilized.

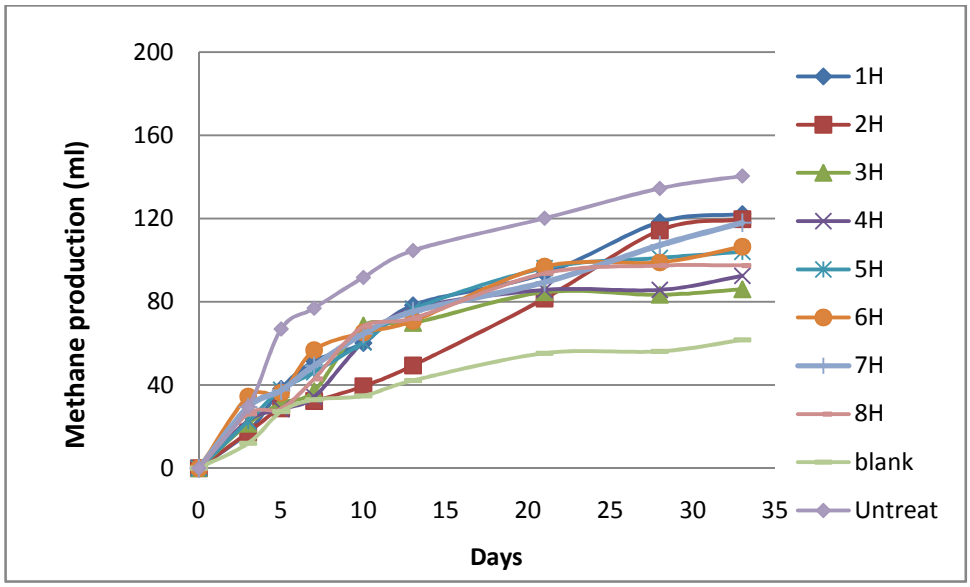

Figure 13. Accumulated methane production of treated homogenized peel and blank

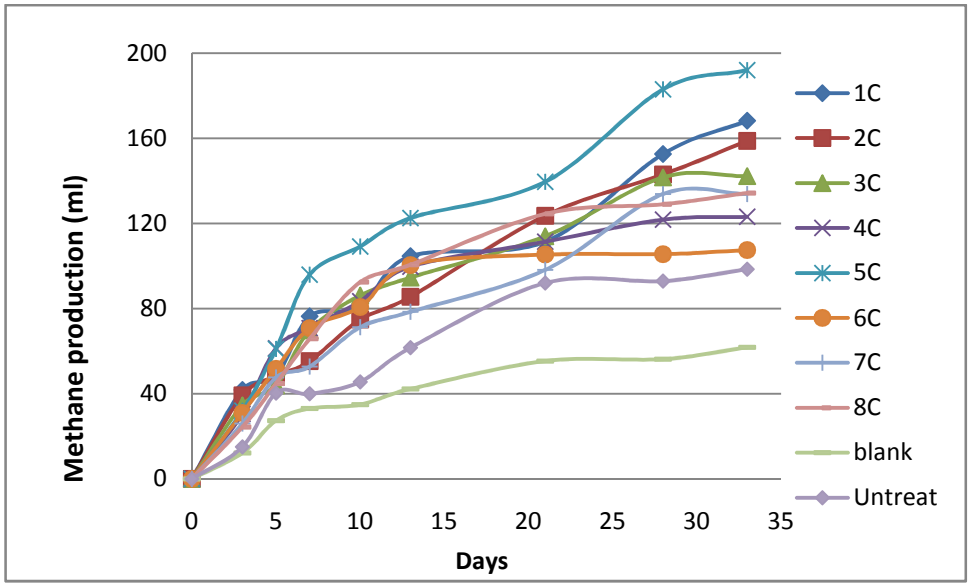

Figure 14. Accumulated methane production of treated chopped peel and blank

The final results of methane production after 33 days were measured and standard deviations were calculated to construct the bar charts above (Fig. 15 and Fig. 16 with names of the samples specified in Table 11) so that the comparison of methane production of different substrates will be performed for all experiments. As one can see, for the homogenized peel, the untreated peel gave higher methane results than that of treated ones. Otherwise, chopped peels that were treated gave higher methane production than untreated ones did. Moreover, the treated chopped peel gave significantly higher methane production compared to that of treated homogenized peel. As one can notice from loss of VS (%) values presented previously, there was a significant decrease of total VS content of treated peels compared to that of untreated ones. The decrease of VS was even worst for homogenized treated peel. The reason that should be considered for these phenomena was either the mass transfer of some interested compounds from the peel into the surrounding solvent (n-hexane) during the pretreatment time or dissolution of some compounds e.g. sucrose, fructose, glucose and pectin in water during washing step. When the peel was homogenized, the size of the peel particles was smaller, resulting in greater interfacial area between the solid and liquid. Therefore, it became easy for substances staying in the peel being to leach to surrounding liquid. In other words, in the case of homogenized peel the loss of compounds that are interesting for biogas production was more significant, resulting in decrease in biogas production compared to that of untreated peel. It can be noticed from D-limonene extraction results that homogenized peels often was extracted more D-limonene compared to chopped peel at the same condition. However, the treated homogenized peel gave lower methane production. This means that the loss of VS in the case of homogenized peel resulted in more significant effect than effect of extracted D-limonene amount.

In the case of chopped peel, biogas production of treated ones was improved compared to that of untreated one. There were some reasons that should be considered for this improvement e.g. the partly removal of D-limonene, the swelling of the peel due to contact with solvent and water resulting in more easily digestion for bacteria. There was also loss of VS caused by the pretreatment stage; however, these losses were significantly lower than that of homogenized peel.

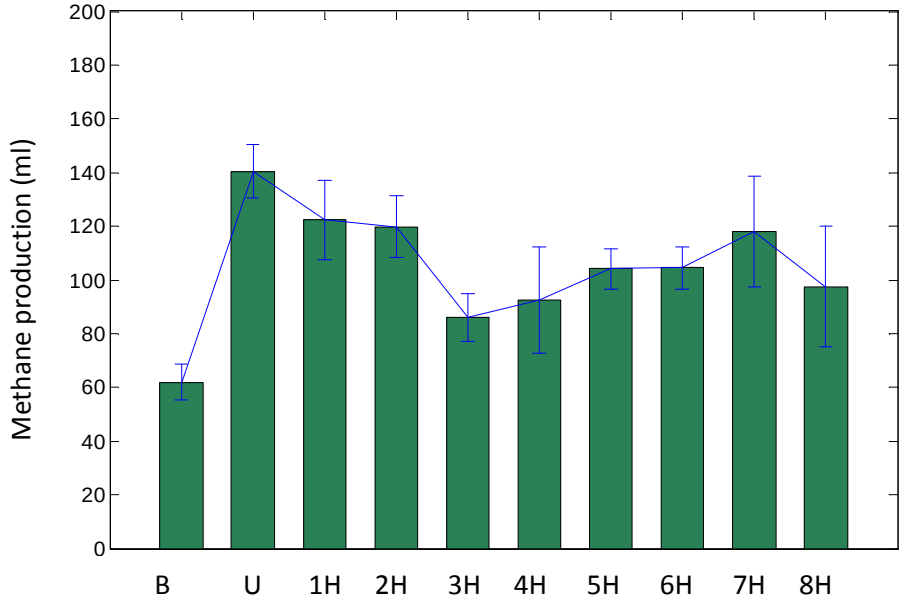

Figure 15. Methane production of homogenized peel and blank after 33 days of digestion (B: blank, U: untreated peel)

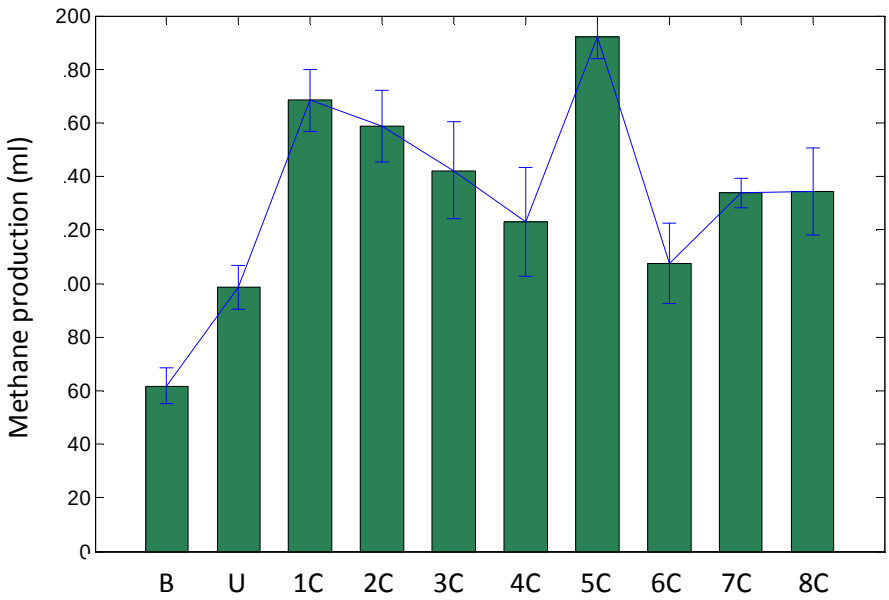

Figure 16. Methane production of chopped peel and blank after 33 days of digestion (B: blank, U: untreated peel)

It can be noticed from D-limonene analysis results that the treated peels which had higher D-limonene removal did not give as high biogas production (Table 13). Notice also that amount of D-limonene removal varied depending on pretreatment conditions, ranging from 7.04% to 82.01%. The loss of substances inside the peel has to be emphasized in our case. One more important thing that should be considered is that there may be differences in component that was lost, also resulting in difference in biogas production. Likewise, the effect of solvent at different treatment conditions on the peel may be another factor that should be highly concerned.

To sum up, there were some factors causing effects on treated peel during pretreatment step, resulting in effect on biogas production. The effects of these factors together with effect of D-limonene have to be focused more carefully. However, no more experiments focused on these features have been executed in this thesis work. The proportions of methane in biogas were from 45 to 68.5% and 62.3 to 78.4 % for treated homogenized peel and treated chopped peel, respectively. pH measurement was conducted at the end of digestion time and results were reasonable since the pH values ranged between 7.26 and 7.77.

Table 13. Methane production and extracted D-limonene results

Experiment No.	Sample name	Factors				Methane production (ml)	Limonene extracted (%)
		A	B	C	D		
1	1H	-	-	-	Homogenized	122.3	36.12
2	2H	+	-	-	Homogenized	119.8	45.97
3	3H	-	+	-	Homogenized	86.0	66.56
4	4H	+	+	-	Homogenized	92.6	82.01
5	5H	-	-	+	Homogenized	104.1	9.78
6	6H	+	-	+	Homogenized	106.5	13.01
7	7H	-	+	+	Homogenized	118.1	31.54
8	8H	+	+	+	Homogenized	97.5	46.20
9	1C	-	-	-	Chopped	168.3	12.01
10	2C	+	-	-	Chopped	158.8	17.86
11	3C	-	+	-	Chopped	142.2	42.58
12	4C	+	+	-	Chopped	123.1	61.25
13	5C	-	-	+	Chopped	192.0	7.04
14	6C	+	-	+	Chopped	107.5	9.25
15	7C	-	+	+	Chopped	133.8	38.85
16	8C	+	+	+	Chopped	134.3	53.52

Table 14. Specific accumulated methane production (after reducing blank) of untreated peel and peel treated at different conditions (m^3/kg VS)

m^3/kg VS			Homogenized peel					
Untreated	**1H**	**2H**	**3H**	**4H**	**5H**	**6H**	**7H**	**8H**
0.131	0.101	0.097	0.040	0.051	0.071	0.074	0.094	0.060
m3/kg VS			**Chopped peel**					
Untreated	**1C**	**2C**	**3C**	**4C**	**5C**	**6C**	**7C**	**8C**
0.061	0.177	0.162	0.134	0.102	0.217	0.076	0.120	0.121

Measure of biogas production was also performed in terms of specific accumulated methane production after reducing blank and results can be seen in Table 14. The highest methane potential obtained in the case of sample 5C, chopped peel treated at the condition of 20°C, 10 minute and hexane/peel ratio 12. The obtained result was 0.217 m^3 methane/kg VS which is more than three times higher than that of untreated chopped peel. It also can be recognized that the chopped peel pretreated at the condition of 20°C, 10 minute and hexane/peel ratio 2 gave second highest specific accumulated methane production of 0.177 m^3 methane/kg VS. This should be considered when choosing the pretreated condition for further study since n-hexane is rather expensive.

Table 15. Methane production (ml) from different samples after 33 days of digestion (after reducing blank)

Experiment No.	Sample name	Factors				Methane production (ml)
		A	B	C	D	
1	1H	-	-	-	Homogenized	60.5
2	2H	+	-	-	Homogenized	58.0
3	3H	-	+	-	Homogenized	24.2
4	4H	+	+	-	Homogenized	30.8
5	5H	-	-	+	Homogenized	42.3
6	6H	+	-	+	Homogenized	44.7
7	7H	-	+	+	Homogenized	56.3
8	8H	+	+	+	Homogenized	35.7
9	1C	-	-	-	Chopped	106.5
10	2C	+	-	-	Chopped	97.1
11	3C	-	+	-	Chopped	80.5
12	4C	+	+	-	Chopped	61.3
13	5C	-	-	+	Chopped	130.2
14	6C	+	-	+	Chopped	45.7
15	7C	-	+	+	Chopped	72.0
16	8C	+	+	+	Chopped	72.5
17	1HC	0	0	0	Homogenized	33.6
18	2HC	0	0	0	Homogenized	40.2
19	3HC	0	0	0	Homogenized	32.3
20	1CC	0	0	0	Chopped	83.5
21	2CC	0	0	0	Chopped	77.7
22	3CC	0	0	0	Chopped	89.3

Produced methane was determined after reducing blank and these results that were presented in Table 15 were used as data for evaluating experimental results. Normal probability plot method and ANOVA were performed and results are shown in Table 16 and Table 17. From the results obtained, factor effect estimates and sums of squares as well as percent contribution of model terms for 2^4 factorial design were calculated.

Table 16. Effect Estimate and Sum of Squares

Model term	Effect Estimate	Sum of Squares	Percent contribution (%)
A	-15.84	*1004.62*	8.06
B	-18.96	*1437.02*	11.53
C	-2.44	*23.73*	0.19
D	39.14	*6130.75*	49.18
AB	7.68	*235.38*	1.89
AC	-9.72	*378.47*	3.04
AD	-12.3	*605.68*	4.86
BC	12.34	*610.09*	4.89
BD	-4.34	*75.65*	0.61
CD	-3.8	*57.95*	0.46
ABC	7.84	*245.86*	1.97
ABD	11.16	*498.21*	4.00
ACD	-4.14	*68.86*	0.55
BCD	4.78	*91.41*	0.73
ABCD	15.84	*1003.19*	8.05

In order to examine factor effect, normal probability plot was constructed. All of the effects that lie along a line are negligible and the large effects are far from the line. In our case, only the factor of peel type gave significant effect. This can also be seen from the percent contribution of effects results that the model term D, peel type, contributed nearly 50%. In addition, one can see from the biogas production results, chopped peel gave higher methane production compared to that of homogenized peel.

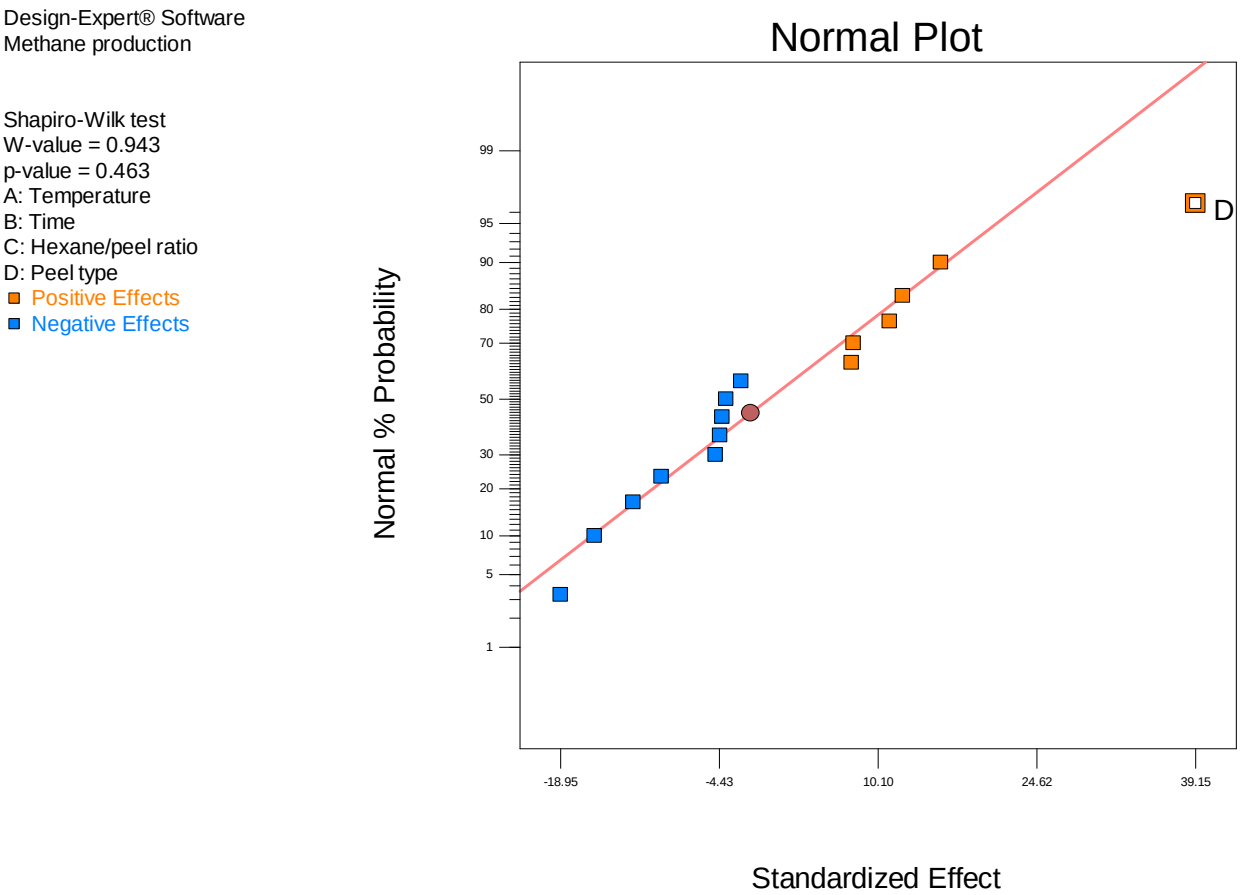

Figure 17. Normal probability plot of effects for the model with four factors (peel types, temperature, time, hexane/peel ratio)

With addition of center points, pure error can be estimated. From the results of ANOVA, the conclusion drawn from normal probability plot was confirmed. Notice that the model constructed was not meaningful since Lack of Fit was significant. The purpose of constructing this model was just to confirm the results from the normal probability plot. Consequently, only the factor of peel type was really significant. Our initial purpose was to construct a statistical model and optimize the biogas production. However, the fact that only peel type gave highly significant effect confirmed that there was not possible to construct any significant models. Otherwise, the simple way for choosing the best pretreatment conditions was to select one of the best among those from 16 experiment series in the factorial design results.

Table 17. Analysis of variance for factorial design

Source of Variance	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Model					
	1	9515.9	9515.9	28.8	<0.0001
D-peel type	1	9515.9	9515.9	28.8	<0.0001
Residual	20	6603.8	330.19		
Lack of Fit	16	6500.9	406.3	15.8	0.0081
Pure Error	4	102.8	25.7		
Total	21	16119.0			

The plot of methane production and the plot of residual versus the factor D-peel type were also constructed. It can be seen that the spreads of methane production and residual are different for two peel types. This confirmed that there was a significant difference in biogas production from two kinds of peel.

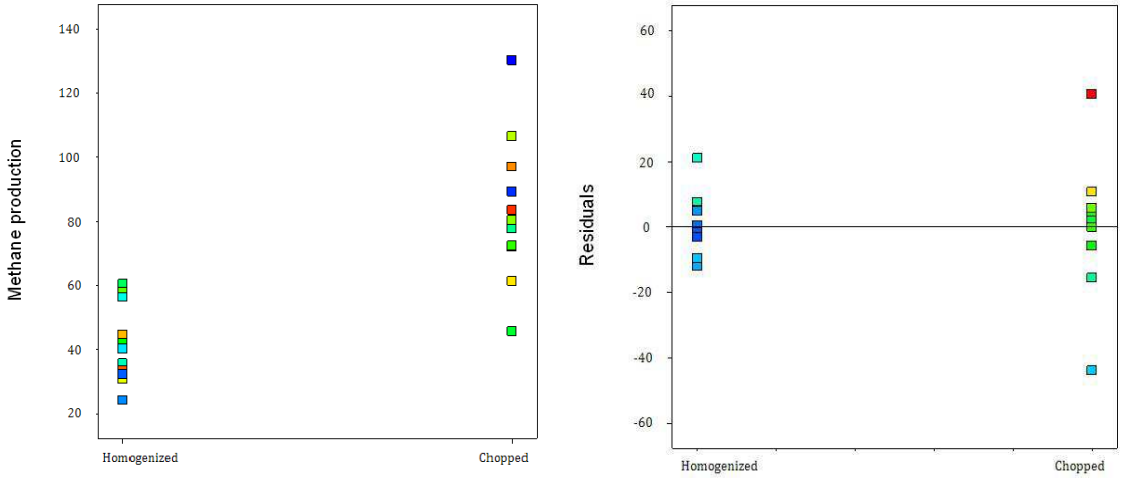

Figure 18. Scatterplots of methane production (left) and residual (right) versus peel type

5.5 Summary of Results

- Complete factorial design with four factors including orange peel types (chopped peel and homogenized peel), pretreatment temperature, pretreatment time, hexane/peel ratio was executed and evaluated. The results showed that only factor of peel type gave significant effect. The chopped peel gave higher methane production compared to that of homogenized peel. The purpose of constructing statistical model and then optimize biogas production from treated peel basing on the proposed model could not be achieved.
- The highest methane production was obtained in the case of chopped peel treated at 20°C, 10 minute and hexane/peel ratio of 12. The obtained methane production was 0.217 m^3 methane/kg VS which was more than three times higher than that of untreated chopped peel.
- The D-limonene analysis results showed that the treated peel that had higher D-limonene removal did not gave as high methane production. Other factors in pretreatment step rather than D-limonene removal affected treated material i.e. the loss of VS, the difference in component loss, the swelling of peel, etc., resulting in effect on biogas results.

6. Additional Testing Inhibitory Effect of n-hexane and D-limonene

In order to determine the inhibitory effect of n-hexane and D-limonene, some extra tests were conducted. Batch biogas production was carried out in 120 ml-glass bottles as reactors under thermophilic condition (55°C). The total sample-volume was 52 ml, including 50 ml of inoculum, 1 ml of test substrate and 1 ml of test substance (water, n-hexane or D-limonene). Test substrate was prepared from nutrient broth, yeast extract, D-glucose and water. The result showed that D-limonene and n-hexane inhibited bacterial activity since those samples with addition of n-hexane or D-limonene gave lower methane production compared to that of samples without these chemicals. n-hexane seemed to be more toxic to bacteria compared to D-limonene at addition of the same volumes of these substance since the samples with addition of n-hexane gave lower methane production than even the blank.

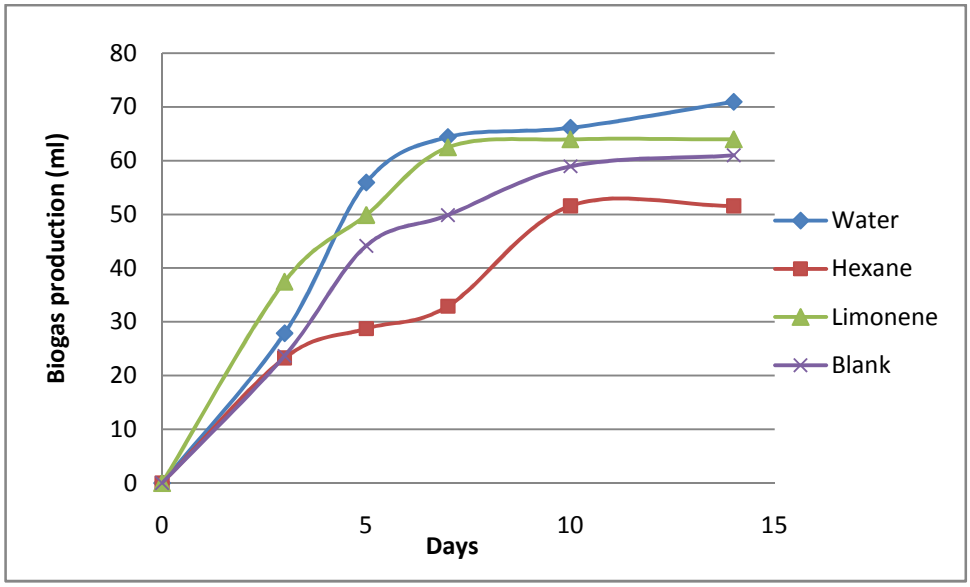

Figure 19. Accumulated methane production of tested samples

7. Conclusions

From this work, some important conclusions can be drawn, as follows:

- Four different solvents including n-hexane, dichloromethane, diethyl ether and ethyl acetate were investigated for pretreatment purpose. A two-stage nested design was applied to conduct experiments in the step of screening selection of solvent. Experimental results and ANOVA showed that there were significant differences in biogas production at four different VS concentration (0.5, 1, 1.5, 2 %) from peels treated with different solvents. n-hexane was the most interesting solvent since it showed higher extraction efficiency toward D-limonene and orange peel treated by n-hexane gave higher methane production than the peel treated by other solvents.
- Four important factors which cause effect on pretreatment step were studied, including orange peel types (chopped peel and homogenized peel), pretreatment temperature, pretreatment time, hexane/peel ratio so that the pretreatment step can be improved. Two-level factorial design was employed to conduct experiments in this experimental series. The results showed that only factor of peel type gave significant effect. The chopped peel gave higher methane production compared to that of homogenized peel. The purpose of constructing statistical model and then optimize biogas production from treated peel basing on the proposed model could not be achieved.
- Biogas production from orange peel was improved by solvent pretreatment. A simple pretreatment procedure following solid-liquid extraction technique using n-hexane as solvent was designed and analyzed. The highest methane production obtained in the case of chopped peel treated at 20°C, 10 minute and hexane/peel ratio of 12. The obtained methane production was 0.217 m^3 methane/kg VS, more than three times higher than that of untreated chopped peel.
- The D-limonene analysis results showed that the treated peel that had higher D-limonene removal did not gave as high methane production. The initial aim of the research was to improve biogas production from orange peel by employing solid-liquid extraction technique to remove D-limonene since this substance is toxic to bacteria. However, the research results showed that there were other factors i.e. the loss of VS, the difference in component loss, the

swelling of peel, etc. during pretreatment step together with D-limonene removal causing effect on treated peel, resulting in total effect on biogas production.

From this research, there are some limitations that should be considered, as follows:

- Due to limitation of the resources, only some experiments were repeated. More experiments should be carried in replicates to confirm if obtained research results are reliable.
- The research results showed that there were other factors i.e. the loss of VS, the difference in component loss, the swelling of peel, etc. during pretreatment step together with D-limonene removal causing effect on treated peel, resulting in total effect on biogas production. Therefore, the effect of organic solvent together with treatment conditions e.g. temperature, time, amount of solvent and the influence of washing step on treated peel should be highly considered. However, these features have not been solved in this thesis work and should be focused more carefully if the research is going to be continued.

8. Future Works

For the future, the following areas need to be examined:

- Investigating the real effect of D-limonene on the available orange peel to some greater extent and determining the threshold inhibitive D-limonene amount, the limiting load of D-limonene to anaerobic digestion if the available orange peel is used as a feedstock for biogas production.
- Analyzing the composition of the peel after pretreatment so that the effect of pretreatment step on the peel and the biogas production performance of treated orange peel can be clearly explained.
- Investigating the effect of n-hexane on orange peel, improving pretreatment procedure so that remained n-hexane amount in the treated peel will be reduced as much as possible while the interested compounds in the peel will not be lost that much.
- Studying biogas production of treated peel with different % VS concentration so that the best VS concentration can be chosen.
- Trying different pretreatment solvents with the new pretreatment procedure, the one with addition of washing step.
- Examining of solvent recycle to decrease cost and environmental concern.

Appendix

A. Data for nested design analysis

U: *Untreated* ***TH:*** *Treated by hexane* ***TDE:*** *Treated by diethyl ether*

TDM: *Treated by dichloromethane* ***TEA:*** *Treated by ethyl acetate*

Type of substrate	VS %	Biogas production	Predicted values	Residuals
U	0.5	41.62	45.55	-3.94
U	0.5	48.63	45.55	3.08
U	0.5	46.41	45.55	0.86
U	1	80.20	80.26	-0.06
U	1	80.80	80.26	0.54
U	1	79.78	80.26	-0.47
U	1.5	49.09	59.71	-10.62
U	1.5	59.76	59.71	0.05
U	1.5	70.27	59.71	10.57
U	2	82.18	78.46	3.72
U	2	74.59	78.46	-3.88
U	2	78.62	78.46	0.16
TH	0.5	34.10	36.64	-2.54
TH	0.5	33.20	36.64	-3.44
TH	0.5	42.62	36.64	5.98
TH	1	65.90	66.53	-0.64
TH	1	74.17	66.53	7.63
TH	1	59.54	66.53	-7.00
TH	1.5	70.45	72.50	-2.05
TH	1.5	75.69	72.50	3.19
TH	1.5	71.36	72.50	-1.14
TH	2	84.33	87.07	-2.74
TH	2	83.27	87.07	-3.80
TH	2	93.61	87.07	6.54
TDE	0.5	30.74	30.46	0.28
TDE	0.5	32.60	30.46	2.14
TDE	0.5	28.04	30.46	-2.42
TDE	1	44.42	54.03	-9.61
TDE	1	59.98	54.03	5.95
TDE	1	57.68	54.03	3.65
TDE	1.5	47.65	45.93	1.72
TDE	1.5	44.57	45.93	-1.36
TDE	1.5	45.57	45.93	-0.36
TDE	2	47.23	48.28	-1.05
TDE	2	50.68	48.28	2.40

TDE	2	46.93	48.28	-1.35
TDM	0.5	12.78	12.53	0.25
TDM	0.5	10.39	12.53	-2.15
TDM	0.5	14.43	12.53	1.90
TDM	1	10.29	10.34	-0.06
TDM	1	10.27	10.34	-0.07
TDM	1	10.47	10.34	0.13
TDM	1.5	16.50	15.04	1.46
TDM	1.5	14.95	15.04	-0.08
TDM	1.5	13.66	15.04	-1.38
TDM	2	16.48	15.56	0.92
TDM	2	17.03	15.56	1.47
TDM	2	13.16	15.56	-2.39
TEA	0.5	54.31	57.56	-3.25
TEA	0.5	62.19	57.56	4.64
TEA	0.5	56.17	57.56	-1.39
TEA	1	27.06	25.85	1.21
TEA	1	26.49	25.85	0.64
TEA	1	24.01	25.85	-1.84
TEA	1.5	9.18	9.58	-0.40
TEA	1.5	8.67	9.58	-0.91
TEA	1.5	10.89	9.58	1.31
TEA	2	4.90	3.24	1.66
TEA	2	2.12	3.24	-1.11
TEA	2	2.70	3.24	-0.54

B. Data for factorial design (with blank) (The numbering of samples was specified in Table 11)

Day	Blank	Untreated	1H	2H	3H	4H	5H	6H	7H	8H
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	12.11	29.63	17.51	17.28	21.44	22.79	22.59	34.53	29.42	26.15
5	27.30	67.03	38.22	28.84	31.33	28.54	37.78	36.33	37.39	28.71
7	33.01	76.99	51.54	32.30	37.08	34.32	46.16	56.83	49.29	42.98
10	34.86	91.74	60.19	39.50	68.60	60.85	60.56	65.32	64.73	68.32
13	42.18	104.64	78.40	49.43	69.87	76.67	76.44	70.88	75.35	72.12
21	55.33	120.21	93.56	81.57	84.61	85.80	96.26	96.95	89.45	93.68
28	56.26	134.58	118.39	114.36	83.43	85.80	101.05	99.04	107.29	97.37
33	61.79	140.47	122.27	119.80	86.03	92.57	104.09	106.46	118.10	97.50

Day	Blank	Untreated	1C	2C	3C	4C	5C	6C	7C	8C
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	12.11	15.05	41.75	39.18	34.51	30.53	32.02	31.00	25.99	24.37
5	27.30	40.35	47.93	48.23	43.84	61.34	61.05	51.66	47.70	44.77
7	33.01	39.93	76.37	55.26	69.82	70.85	95.96	70.81	52.71	65.78
10	34.86	45.51	80.66	74.85	86.15	83.47	109.24	80.79	71.24	92.43
13	42.18	61.68	104.58	85.49	94.55	99.73	122.60	100.46	78.44	100.57
21	55.33	91.98	111.50	123.62	114.04	111.44	139.49	105.37	98.18	124.53
28	56.26	92.91	152.65	142.95	141.69	121.79	183.04	105.55	133.77	128.97
33	61.79	98.44	168.27	158.84	142.25	123.12	192.02	107.48	133.77	134.25

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