Thesis for the Degree of Master of Engineering

Optimization of Biofuels Production from Alginate by Response Surface Methodology

by

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Department of Chemical Engineering

The Graduate School

Pukyong National University

February 2013

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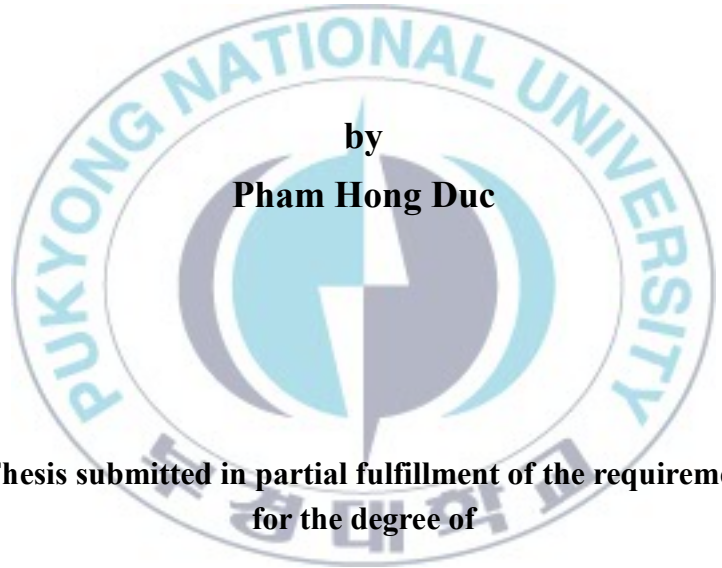

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Pham Hong Duc

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Optimization of biofuels production from alginate

by response surface methodology

A dissertation

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

Algin: Alginate

ANOVA: Analysis of Variance

BBD: Box-Behnken design

CCD: Central Composite Design

Coef: Coefficient

EtOH: Ethanol

FAO: Food and Agriculture Organization

FID: Flame Ionization Detector

FT: Fischer-Tropsch process

G: Guluronic acid

GC: Gas Chromatograph

Glu: Glucose

H-A: Acid alginate

HAc: Acetic acid

HBu: Butyric acid

HCa: Caproic acid (or Hexanoic acid)

HPLC: High Performance Liquid Chromatography

HPr: Propionic acid

HVa: Valeric acid

K-A: Potassium alginate

LC: Liquid Chromatography

M: Mannuronic acid

Na-A: Sodium alginate

Na-Algin: Sodium Alginate

P: P value

PG: Propylen glycol

PG-A: Propylen glycol

rpm: rotation per minutes

RSA: Response Surface Analysis

RSM: Response Surface Methodology

SE Coef: Standard Error of Coefficient

T: T value

TCD: Thermal Conductivity Detector

TVFAs: Total Volatile Fatty Acids

VFAs: Volatile Fatty Acids

WWTP: Waste Water Treatment Plant

Optimization of Fermentative Biofuels Production from Alginate by Response Surface Methodology

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ABSTRACT

This study was conducted to evaluate the optimum anaerobic fermentative conditions of alginate with the respect to the simultaneous effects of sodium alginate concentration and pH to maximize volatile fatty acids and ethanol productions. The optimization was based on central composite design (CCD) and performed by response surface methodology (RSM) on Minitab software (version 15.1.1.0., Minitab Incorporation, USA).

Sodium alginate using as sole carbon source was obtained from Wako company. Anaerobic sludge from local municipal wastewater treatment plant was acidic pretreated by HCl 2N for 24 hours at 35^{o}C in order to enhance volatile fatty acids (VFAs) and ethanol production. A series of anaerobic

fermentative processes was carried out in amber reactors at 35°C and 120 *rpm*.

Total volatile fatty acids (TVFAs) production in alkaline condition was more efficiency than that of acidic condition. According to response surface analysis (RSA), optimum conditions for maximum TVFAs yield were sodium alginate 9.0 g/L and pH 7.8. The maximum response value was estimated approximately 4.3 g/L. The VFAs produced from the system consisted of acetic, propionic, and butyric acids. Among them, acetic acid was the major component. In addition, H_2 and CO_2 was produced biogas in the system, whereas CH_4 (methanogenesis) was inhibited in order to improve biofuels production during acidogenesis. H_2 yield in acidic and alkaline condition was 1.66% and 4.03%, respectively.

반응표면분석을 이용하여 알지네이트(alginate)로부터 바이오연료(biofuels) 생산 최적화

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요 약

본 연구에서는 sodium alginate를 대상으로 농도와 pH의 영향을 동시에 평가하고, 혐기성 조건에서 alginate로부터 휘발성 유기산(VFAs) 및 에탄올 생산의 최적화 조건에 대해 연구하였다. 최적화 실험은 중심합성계획법(CCD)을 이용하여 디자인하였고, Minitab 프로그램의 표면반응법(RSM)을 이용하여 분석하였다.

탄소원으로 사용한 sodium alginate는 Wako 회사로부터 구입한 것을 사용하였다. 하수처리장에서 채취한 혐기성 소화 슬러지는 VFAs 및 에탄올 생산 미생물의 활성을 향상시키기 위해 35°C 24시간 동안 HCL 2N로 산 처리를 한 후 식종하였다. 혐기성 발효 실험은 35°C, 120 *rpm*인 조건에서 수행되었다.

실험결과, TVFAs는 산성 조건보다 알칼리 조건에서 더 많이 생성되었다. RSM 분석에 의하면, TVFAs 생산수율을 최대화하기 위한 최적 조건은 sodium alginate 농도 9.0 g/L와 pH 7.8으로 나타났다. 이 때 TVFAs 생산량은 약 4.3 g/L로 분석되었다.

생성된 VFAs 조성은 아세트산, 프로피온산, 뷰트릭산 순으로 나타났다. VFA 중에서는 아세트산이 가장 많이 생성되었다. 또한, 바이오연료 생산을 향상시키기 위해 메탄생산 반응을 저해시킨 후, 바이오가스 중 수소 및 이산화탄소가 많이 생성되었다. 산성 및 알칼리 조건에서 수소 수율은 각각 1.66%, 4.03%로 나타났다.

1. INTRODUCTION

1.1. General Backgrounds

The current energy system has been facing with fundamental dependency on fossil fuels energy production and its consequence such as numerous emissions of CO_2 and NO_x, dramatically rising fossil fuel prices, sharply depletion of a finite resource, and increase of climate change. Therefore, it has led scientists to find out new alternative energy resources. Among them, biofuels production from aquatic biomass such as algae has considered as promising future solution to energy issues.

Macroalgae are a potential biomass resource due to fast growth rates, growth ability in marine environment, and their exclusion from lignin compound that is tough to break out. Part of their composition, alginic acid is the most abundant, approximately 34.52 ± 1.00% (dry weight) [1], and simultaneous as the major component highly yielded biofuels. However, on account of chemical structure, its low solubility and degradation are the main drawbacks for biofuels production in anaerobic fermentation [2]. There is a lot of other bioenergy production (ethanol, volatile fatty acids, methane, hydrogen, and so on) research from pretreated brown algae, mixed substrate. However, past attempts have not been reported the maximum biofuel production from alginic acid. Thus, our work is to use sodium alginate chemical to find out the maximized bioconversion to biofuels.

1.2. Research Objectives

In this study, a series of anaerobic fermentative processes is conducted on two independent variables such as alginate (algin) concentration and pH. Alginate is regarded a novel organic substrate for bioconversion process to produce biofuels during the anaerobic condition. The use of alginate is mainly depended on its solubility. Alginate solubility has reported to be affected from the pH condition of the solution. Also, pH is a key variable to optimize the anaerobic microbial growth.

The aim of this examination is to evaluate the optimum fermentative conditions of alginate with the respect to the simultaneous effects of algin concentration and pH to maximize volatile fatty acids and ethanol production. The optimization is based on central composite design (CCD) and performed by response surface methodology (RSM).

2. LITERATURE SURVEY

2.1. Brown algae

Seaweeds can be classified into three broad groups based on pigmentation: brown, red, and green. Botanists refer to these as *Phaeophyceae*, *Rhodophyceae* and *Chlorophyceae*, respectively. Brown seaweeds are usually large, and range from giant kelp, often 20 m-long, through thick, leather-like seaweeds 2 to 4 m long, and to smaller species 30-60 cm long. Red seaweeds are usually smaller, generally ranging from a few centimetres to approximately a meter in length. However, red seaweeds are not always red, they are sometimes purple, even brownish red, but are still classified as *Rhodophyceae* because of other characteristics. Green seaweeds are also small, of similar size to the red seaweeds [3].

Brown algae are classified into 9 orders, 265 genera and, more than 1500 species. They absorb medium wavelength green light, which enables them to live even at 30 to 50 m depths, but the majority live in the intertidal belt and upper sublittoral zone. Brown algae prefer cooler water temperatures than red and green algae. Immersed in water, the seaweeds have no need for internal transport of nutrient or water. This saves energy, and many macroalgae have a very high productivity. Productivity of non-cultivated brown algae has been reported in the range 3.3 to 11.3 kg dry weight/m^2/year. While uses of seaweeds in the West have been based on natural beds, China has been cultivating *Laminaria japonica* since the early 1950s. Rope cultures of L. japonica have been reported to produce 13.1 kg dry weight/m^2 for a 7-month growing period. Sugarcane, the most productive of the cultivated land plants, has in the USA productivity from 6.1 to 9.5 kg fresh weight/m^2/year. Thus, brown algal have a high potential for biomass production and CO_2 fixation,

and may be an attractive alternative source for energy and chemicals [4].

In recent years, biofuels production from brown algae has been a remarkable topic of scientist due to their low percentage of lignin and hemicellulose as compared to other lignocellulose plants. In addition, algae can grow successfully in wastewater and recycled nutrients from agricultural sources. The versatility of algae biofuels is a good solution to the economic obstacles and the lifecycle challenges faced in renewable energy resources [5].

Table 1. Composition of algal biomass [6, 7]

Division	Macroalgae			Microalgae
	Green algae	Red algae	Brown algae	
Specices	Green laver, seastaghorn	Laver, agar-agar	Brown-seaweed, kelp	*Chlorella, Spirulina, Porphyridium*
Water (% of wet)	70 ~ 85	70 ~ 80	79 ~ 90	80 ~ 90
Minerals (ash, % of dry)	10 ~ 25	25 ~ 35	30 ~ 50	-
Carbohydrates (main components)	25 ~ 50 (Cellulose, Starch)	30 ~ 60 (Agar, Carrageenan)	30 ~ 50 (Alginate, Fucoidon)	4 ~ 57
Cellulose (%)	20 ~ 40	2 ~ 10	2 ~ 10%	-
Proteins (%)	10 ~ 15	7 ~ 15	7 ~ 15	26 ~ 63
Lipids (%)	1 ~ 2	1 ~ 5	2 ~ 5	2 ~ 40

2.2. Alginate [8-12]

2.2.1. Definition

Alginic acid, also called algin or alginate, is an anionic polysaccharide distributed widely in the cell walls of brown algae, where it, through binding water, forms a viscous gum. In extracted form it absorbs water quickly; it is capable of absorbing 200 to 300 times its own weight in water. Its colour ranges from white to yellowish-brown. It is sold in filamentous, granular or powdered forms.

Based on partial acid hydrolysis, alginate is separated into three fractions. Two of these contain almost homopolymeric molecules of guluronic acid (G) and mannuronic acid (M), respectively, whereas the rest consists of nearly equal proportions of both monomers. Moreover, the insoluble fractions are composed of molecules which have either predominantly M rich or mostly G rich residues, whereas the hydrolysable fractions are made up of a high proportion of alternating MG residues. A structure of the alginate is illustrated in Figure 1 where (a) indicates alginate monomers, (b) shows chain conformation, and (c) is block distribution.

2.2.2. Chemical structures

Alginates are salts of alginic acid (empirical chemical formula is $NaC_6H_7O_6$) and are an important water soluble polysaccharide containing a linear unbranched chain of β (1 → 4)-linked-d-mannuronic acid and α-(1 → 4)-linked-l-guluronic acid residues.

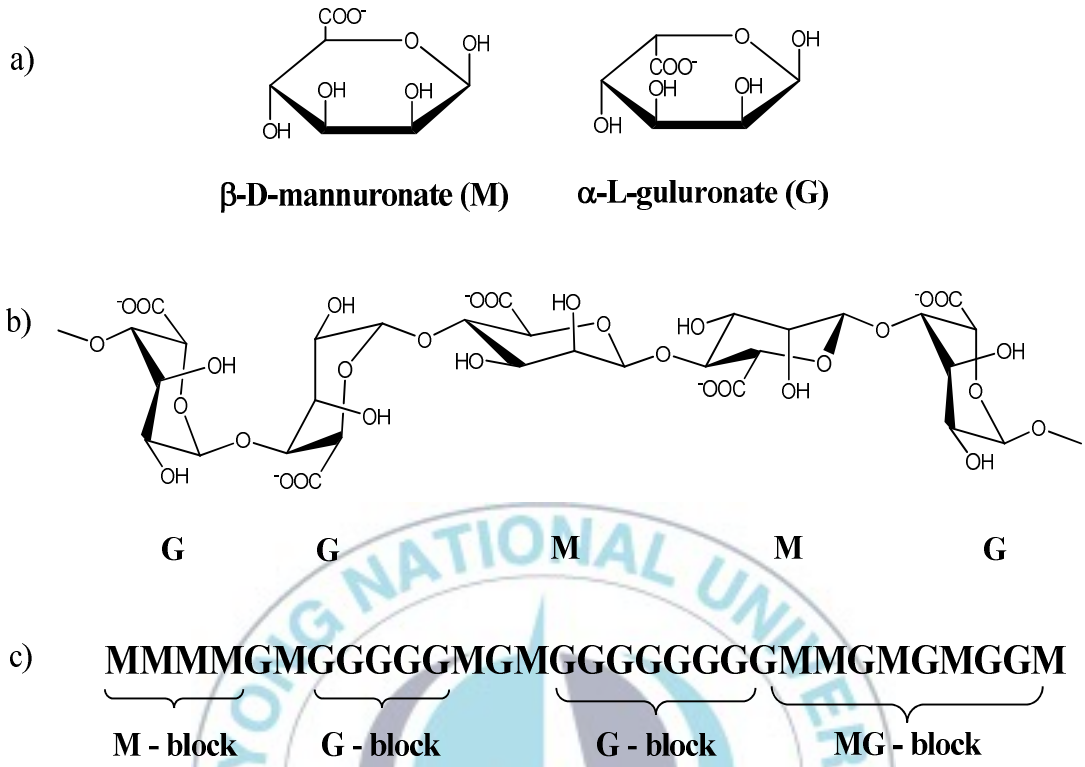


Figure 1. Structural characteristics of alginates: (a) alginate monomers, (b) chain conformation, and (c) block distribution.

2.2.3. Extraction of alginate from brown algae

The commercial alginate products have been dependent entirely on algal sources. Alginic acids exist in brown algae in the intracellular matrix as gels containing sodium, calcium, magnesium, strontium, and barium ions, such that the counterion component is determined by the ion-exchange equilibrium with seawater.

First of all is the extraction process where raw materials are removal of the counterions by proton exchange using mineral acid (i.e., hydrochloride). Following this, insoluble alginic acid is solubilized by neutralization in alkaline condition (sodium carbonate or sodium hydroxide) to form sodium

alginate. After that, strict separation operations such as sifting, flotation, centrifugation, and filtration are performed for the purpose of removing particulate matter. Sodium alginate is then precipitated directly by calcium chloride or a mineral acid. The product is dried and milled.

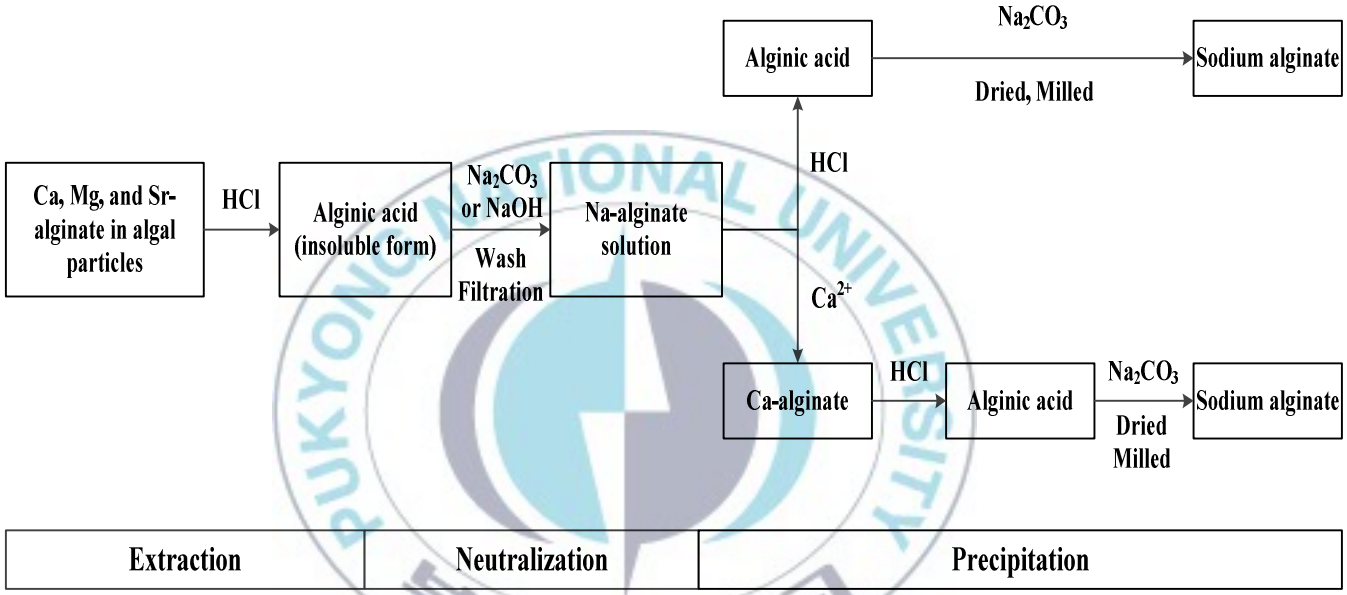


Figure 2. Scheme for the extraction of alginate from brown algae.

Table 2. Composition and sequence parameters of algal alginates

Source	F_G	F_M	F_{GG}	F_{MM}	$F_{GM,MG}$
Laminaria japonica	0.35	0.65	0.18	0.48	0.17
Laminaria digitata	0.41	0.59	0.25	0.43	0.16
Laminaria hyperborean, blade	0.55	0.45	0.38	0.28	0.17
Laminaria hyperborean, blade	0.68	0.32	0.56	0.20	0.12
Laminaria hyperborean, blade	0.75	0.25	0.66	0.16	0.09
Lessonia nigrescens	0.38	0.62	0.19	0.43	0.19
Ecklonia maxima	0.45	0.55	0.22	0.32	0.32
Macrocystis pyrifera	0.39	0.61	0.16	0.38	0.23
Durvillea Antarctica	0.29	0.71	0.15	0.57	0.14
Ascophyllum nodosum, fruiting body	0.10	0.90	0.04	0.84	0.06
Ascophyllum nodosum, old tissue	0.36	0.64	0.16	0.44	0.20

2.2.4. Advantages and disadvantages

As well as other compound, alginate has not only favour points, but also drawbacks. Based on them, we can apply them to proper fields in life. These issues are shown in the following Table 3.

Table 3. Advantages and disadvantages of alginate

Advantages	Disadvantages
_ Inexpensive _ Easy to use _ Hydrophilic -displace moisture, blood, fluids _ Stock trays	_ Tears easily _ Dimensionally unstable • immediate pour • single cast _ Lower detail reproduction _ Unacceptable for fixed pros _ High permanent deformation _ Difficult to disinfect

2.2.5. Applications

Alginate absorbs water quickly, which makes it useful as an additive in dehydrated products such as slimming aids, and in the manufacture of paper and textiles. It is also used for waterproofing and fireproofing fabrics, as a gelling agent, and for thickening drinks, ice cream and cosmetics.

Alginate is used in various pharmaceutical preparations such as Gaviscon, Bisodol, and Asilone, extensively as an impression-making material in dentistry, prosthetics, lifecasting and occasionally for creating positives for small-scale casting, and for thickening soups and jellies in the food industry.

Calcium alginate is used in different types of medical products, including burn dressings that promote healing and can be removed with less pain than conventional dressings. Also, due to alginate's biocompatibility and simple gelation with divalent cations such as Ca^{2+}, it is widely used for cell

immobilization and encapsulation. Alginic acid (alginato) is also used in culinary arts, most notably in the Sphereification techniques of Ferran Adrià.

Due to its ability to absorb water quickly, alginate can be changed through a lyophilization process to a new structure that has the ability to expand. It is used in the weight loss industry as an appetite suppressant.

2.3. Biofuels production

2.3.1. Dark fermentation

Dark Fermentation is the process of extracting energy from the oxidation of organic compounds, such as carbohydrates, using endogenous electron acceptor with the absence of light and oxygen.

Dark fermentation (or called Acidogenesis) is the second stage in the four stages of anaerobic digestion (Figure 3).

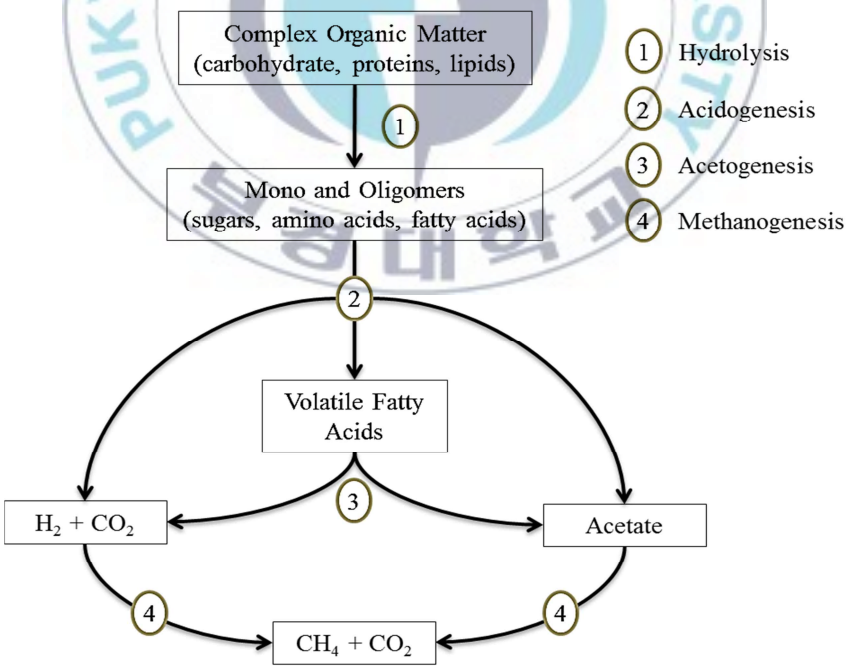


Figure 3. Schematic of the anaerobic digestion process.

Anaerobic digestion occurs in four steps:

1. Hydrolysis: Complex organic matter is decomposed into simple organic molecules using water to split the chemical bonds between the substances.
2. Fermentation or Acidogenesis: The chemical decomposition of hydrolyzed organic by enzymes, bacteria, yeasts, or molds in the absence of oxygen.
3. Acetogenesis: The fermentation products are converted into acetate, hydrogen, and carbon dioxide by what are known as acetogenic bacteria.
4. Methanogenesis: Is formed from acetate and hydrogen/carbon dioxide by methanogens (Archeare and bacteria).

By its side, the acidogenic activity was found in the early 20th century, but it was not until mid-1960s that the engineering of phase's separation was assumed in order to improve the stability and waste digesters treatment. In this phase, complex molecules (carbohydrates, lipids, and proteins) are depolymerized into soluble compounds by hydrolytic enzymes (cellulases, hemicellulases, amylases, lipases and proteases). The hydrolyzed compounds are fermented into volatile fatty acids (acetate, propionate, butyrate, and lactate), neutral compounds (ethanol, methanol), ammonia, hydrogen and carbon dioxide.

During the acidogenesis step, the hydrolysis products (amino acids, simple sugars, long chain fatty acids), which are relatively small soluble compounds, are diffused inside bacterial cells through the cell membrane and subsequently fermented or anaerobically oxidized. Acidogenesis is a very common reaction and is performed by a large group of hydrolytic and non-

hydrolytic microorganisms. About 1% of all known bacteria are (facultative) fermenters. The acidification products consist of a variety of small organic compounds, mainly VFAs, i.e. acetate, propionate, and butyrate, as well as H_2, CO_2, some lactic acids, ethanol and ammonia.

2.3.2. Generations of Biofuels

The division of fuels generation from biomass can be based on the competitiveness with food and the available supply of biomass in a limited land area (Table 4).

From the perspective of categorization, sugar, starch, vegetable oil, or animal fats are known as main biomass source in the first generation biofuels. The primary feedstocks for its product are often seeds or grains (i.e. corn, rapeseeds) that can be produced bioethanol and biodiesel. A more feasible solution is to produce biofuels from non-food crops, called second-generation biofuels. These include waste biomass, the stalks of corn, grass, and wood and special energy or biomass crops.

However, due to huge amount of biofuels that would be adequate to replace fossil fuels role, highly productive plants have to be used as biomass resources. Therefore, the third generation biofuels are approached to solve it. It has low input; high yield feed stocks such as algae and genetically modified plants. Algae produce 30 times more energy per acre than land crops such as soybeans.

Table 4. Classification of biofuels generations [6]

Generation	1st	2nd	3rd
Target	Partial use of biomass	Whole use of biomass	Effective solar energy use
Biomass	Sugar-based ethanol, plant oil-based biodiesel (~50% CO_2 ↓)	Non-edible biomass (wastes, lignocellulose) –based biofuel (80~90% CO_2 ↓)	Improved plants or algae-based biofuel
Process	Bioethanol - Fermentation Biodiesel - Transesterification	Bioprocess - Pretreatment, Saccharification, Fermentation, Algal biotechnology Chemical process - Gasification, Catalysis, FT (BTL)	Bioprocess - Pretreatment, Saccharification, Fermentation, Algal biotechnology Chemical process - Gasification, Catalysis, FT (BTL)
Product	Bioalcohol - Ethanol Biodiesel - FAME	Gasoline - Cellulosic Ethanol - Cellulosic Butanol - Long-chain alcohols - Hydrocarbons Diesel - FT Diesel - Hydrocarbon	Gasoline - Cellulosic Ethanol - Cellulosic Butanol - Long-chain alcohols - Hydrocarbons Diesel - FT Diesel - Algal oil

2.3.3. Volatile fatty acids

Volatile fatty acids (VFAs) are fatty acids with a carbon chain of six carbons or fewer such as acetic acid, propionic acid, butyric acid, valeric acid, hexanoic acid, and their isomers.

Acetic, propionic, and butyric acids are intermediates of the acidogenic and acetogenic stages of anaerobic digestion. Compared with biomethane production, a higher productivity can be expected under conditions conducive to VFAs production since this process occurs within two to three days as compared to 15 to 20 days for methane production. The VFAs can be converted to a mixed alcohol fuel (e.g., isopropanol, 2-butanol, and 3-pentanol) by hydrogenation with a catalyst [13].

Anaerobic digestion process converts protein, lipids, and carbohydrates to mixed volatile fatty acids. Thus, if VFAs can be converted to fuels and chemicals such as ethanol and Butanol via economical processes, mixed VFAs fermentation could supply a new platform with versatile applications for the production of biofuels and biochemical as shown in Figure 4 [6].

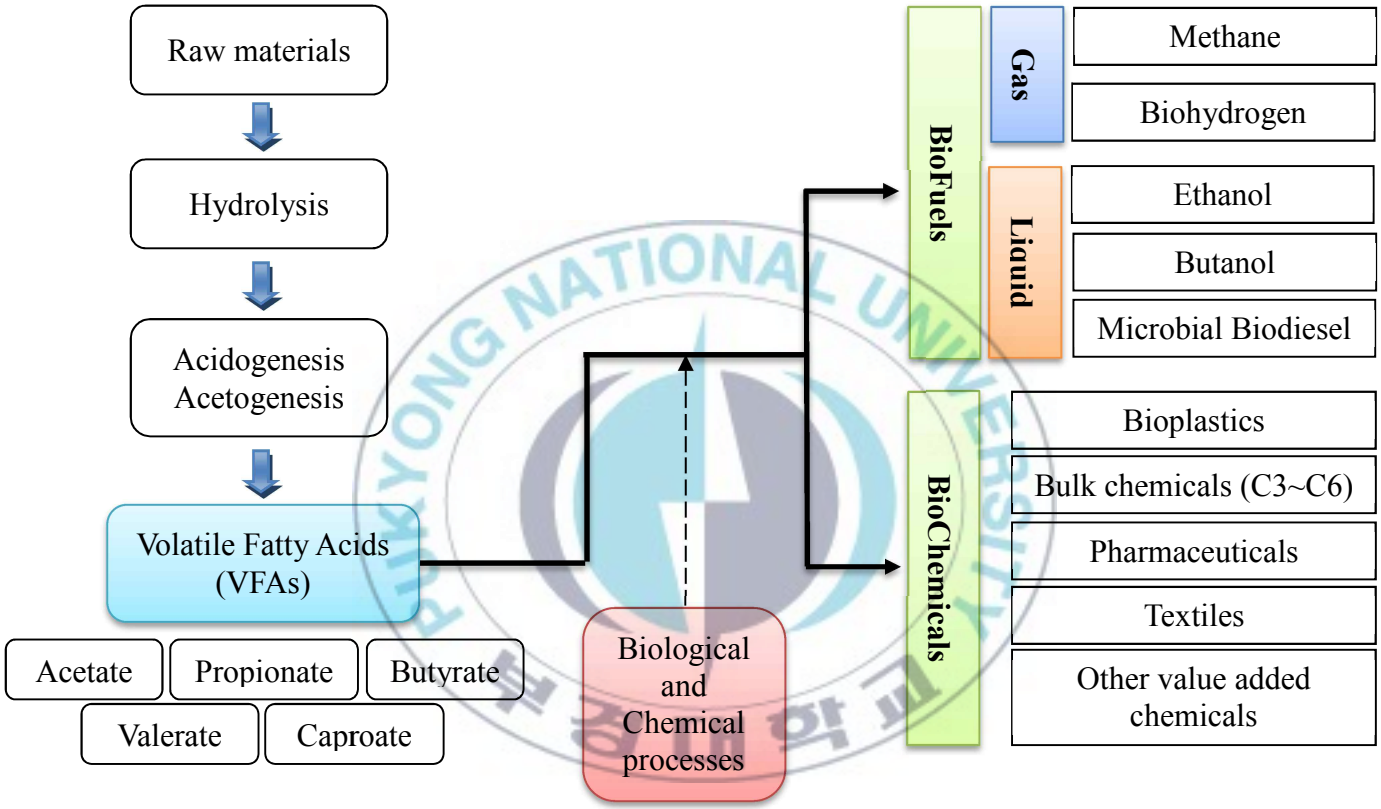


Figure 4. Volatile fatty acids platform [6, 14].

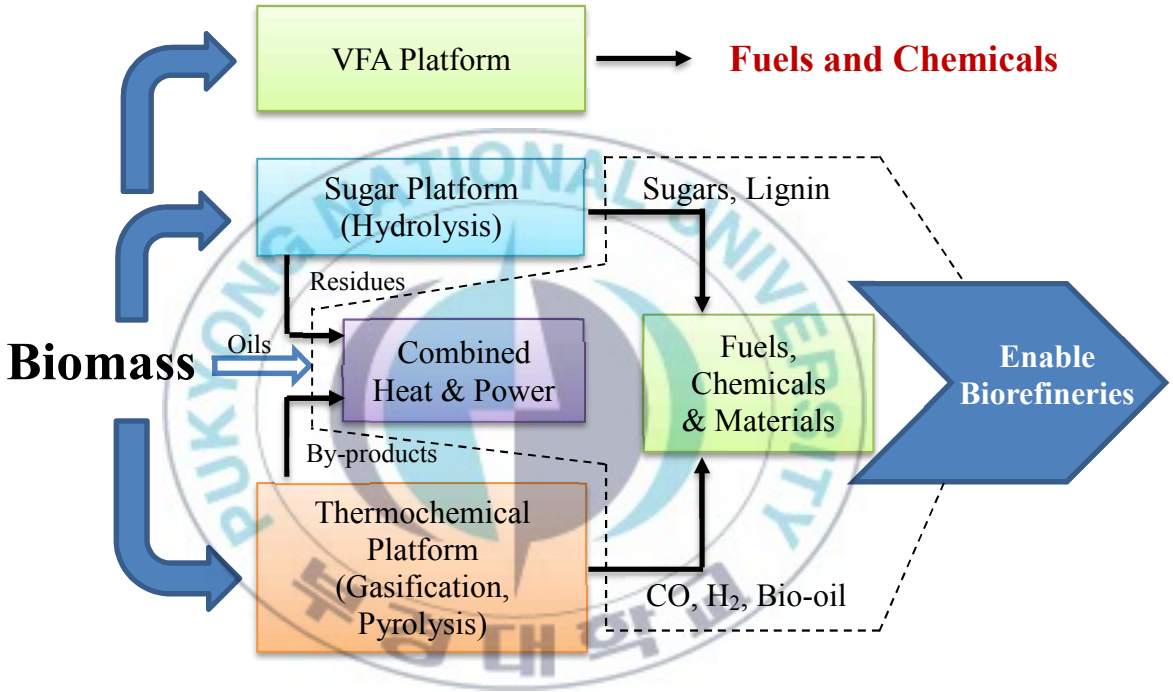


Figure 5. Two current actively researched platforms and VFA platform suggested.

2.3.4. Biogas production

Hydrogen (H_2) is the lightest gas known, with no colour, taste or smell, in the Universe. It consists of more than 75% of the earth's atmosphere. The current hydrogen production methods can be classified into three main categories as shown in the Figure 4.

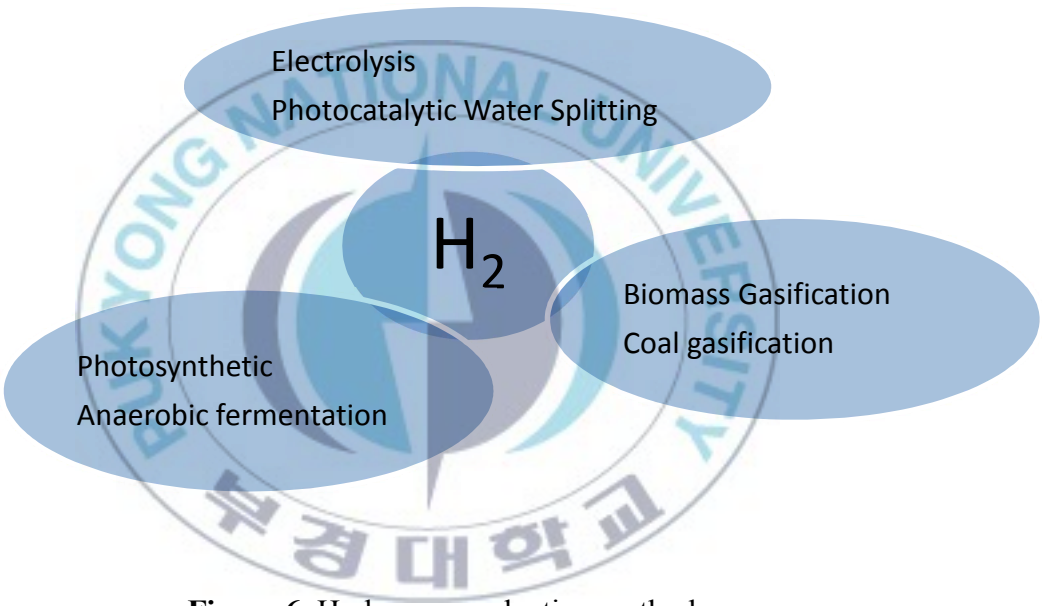


Figure 6. Hydrogen production methods.

Fossil fuel reforming: In general all these processes involve the need for high temperatures and/or pressures (as in the case of coal gasification) as well as the production of other gases, mainly carbon dioxide. Partial oxidation of natural gas as well as coal gasification methods generate less CO_2 than conventional process.

Biomass gasification: This process is similar to conventional fossil fuel

reforming. However, its added benefit is to use waste sludge material as the process influent. Because of the costs as well as the impracticality associated with the transportation of the biomass to a centralized location, however, it is unlikely that this method will ever be employed in anything but small-scale applications. More importantly, expensive equipment as well as other economically competitive opinions for biomass use makes the gasification process. The use of biomass as feedstock for a biologically based on hydrogen production process is more attractive both in the potential for large application and economical viability.

Electrolysis: Electrolysis of water involves passing an electric current through water. Following that, it is split into oxygen and hydrogen gas. After fossil fuel reforming, electrolysis is the most utilized method of hydrogen production. The advance point of this process is that it does not produce any green house gases, however its overall “environmental-friendliness” depends on the source used for electricity generation. Hydrogen produced via this manner has a potential of being completely emission free if electricity is generated using a renewable source such as wind or solar. However, electrolysis cannot compete with reforming processes in large volume production.

2.4. Mathematical model building

2.4.1. Response surface methodology

Experimental design can be regarded as a process by which certain factors are selected and deliberately varied in a controlled manner to obtain their effects on a response of interest, often followed by the analysis of the experimental results. According to the number of the factors to be investigated at a time, the experimental design can be classified into two categories: one-factor-at-a-time design (or single-factor design) and factorial design (or multiple-factor design) [15].

Due to extremely complicated and influenced by many factors such as substrate concentration, temperature, pH, and so on of a biological experiment, thus an appropriate experimental design is essential and vital to study simultaneously the effects of various factors on the process in order to make it better understood and even optimized to improve its performance [16]. To perform this issue, response surface methodology, one kind of factorial design, is considered to proper solution.

Response surface methodology is a collection of mathematical and statistical techniques that is useful for modelling and analysis in applications where a response of interest is influenced by several variables and the objective is to optimize this response [17].

In most RSM issues, the form of the relationship between the response and the independent variables is unknown. Thus, the first step in RSM is to find a suitable approximation for the true relationship between Y and the independent variables. Usually, a low-order polynomial in some region of the independent variables is employed. If the response is well modeled by a linear function of the independent variables, the approximating function is the first-order model:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_k x_k + \varepsilon \qquad \text{Eq. 1}$$

where, Y is the response (yield), β_0 is the constant coefficient, β_i is the i^{th} linear coefficient, ε is the experimental error, and x_1, x_2, …, x_k, is independent parameters.

If there is curvature in the system, a polynomial of higher degree must be used, such as the second-order model:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \ldots + \sum_{i<j} \sum \beta_{ij} x_i x_j + \varepsilon \qquad \text{Eq. 2}$$

where, Y is the response, β_0 is the constant coefficient, β_i is the i^{th} linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interactive coefficient, ε is the experimental error, and x_i or x_j is independent parameters.

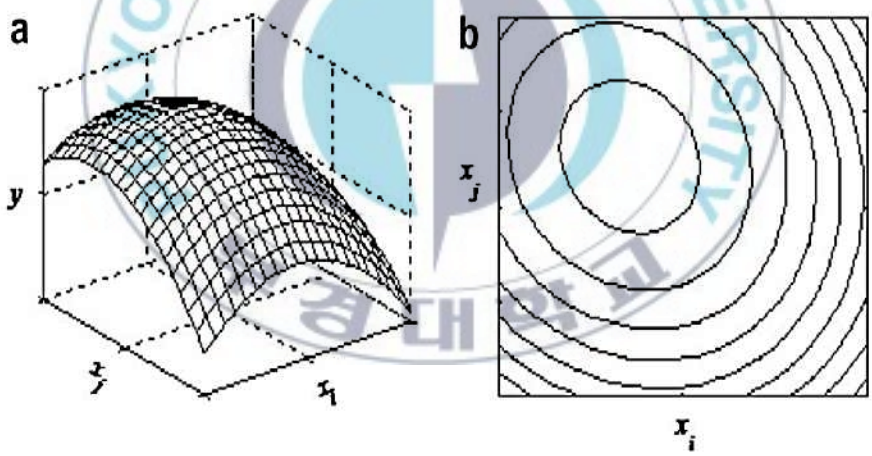


Figure 7. Surface plot (a) and contour plot (b) for a response.

As shown in Figure 7, the estimated second-order polynomial model can be displaced as a surface plot and a contour plot, by varying only two factor levels, while keeping other factor levels constant. The surface plot and contour plot will visually show the response over a region of the interesting

factor levels. In addition, they will indicate how sensitive the response is to the change of each factor levels and to what degree the factors interplay as they affect the response. Furthermore, based on the analysis of variance (ANOVA) of the estimated model, terms which have significant effects on the response can be determined. In addition, with the aid of the regression model, the optimal response can be estimated by calculating the derivatives of the model.

In RSM, central composite design (CCD) and Box-Behnken design (BBD) are widely used experimental designs to estimate a second-order polynomial approximation to a response in that region. CCD is a five-level fractional factorial design developed by Box and Wilson. The design usually consists of a 2n full factorial design, 2 x n axial designs and m central designs. The axial design is identical to the central design except for one factor, which will take on levels either above the high level or below the low levels of the 2n full factorial design. Whereas, BBD is a three-level factional factorial design developed by Box and Behnken. The design can be thought of as a combination of a two-level factorial design with an incomplete block design. In each block, a certain number of factors are put through all combinations for the factorial design, while other factors are kept at the central levels. BBD provides an economical alternative to the CCD, for it has less factor levels than the CCD and does not contain extreme high and extreme low levels [15, 18-20].

An experimenter is supposed to find the levels of variables x_k that optimize the predicted response. This point will be the set of variables x_k for which the partial derivatives equal to 0. This point, say $x_{k,s}$, is called the stationary point.

$$\frac{\partial \hat{y}}{\partial x_1} = \frac{\partial \hat{y}}{\partial x_2} = \cdots = \frac{\partial \hat{y}}{\partial x_k} = 0 \qquad \text{Eq. 3}$$

The stationary point could represent a point of maximum response, a point of minimum response, or a saddle point.

Contour plots (Figure 7-b) play a very important role in the study of the response surface. By generating contour plots using computer software for response surface analysis, the experimenter can usually characterize the shape of the surface and locate the optimum with reasonable precision [21].

2.4.2. Experimental design

Factorial designs and modified factorial designs are widely used in experiments involving several factors where it is necessary to investigate the joint effects of the factors on a response variable. 2^k factorial design, 2^k factorial design containing interaction, and the simplex design are usually used when the first order regression model is proper to describe the empirical response. In the case that an empirical response is adequate to be fit with the second order regression model, the composite design is applied to experimental designs. Part of them, the central composite design involving F factorial points, 2k axial points, and n_0 center runs is without a doubt the most popular class of second-order designs and a tool regressing the response with minimum of work and expense. It was introduced by Box and Wilson in 1951.

The full-factorial central composite design consists of a complete 2k factorial design, where k is the number of independent variables, n_0 center points ($n_0 \geq 1$) and two axial points on the axis of each design variable at a distance of a, which is determined depending on the number of factors in the factorial part of the design, from the design center. Hence, the total number of design points is described the equation (4) and Figure 8 below. A full-

factorial central composite design is usually used to acquire data to fit an empirical second-order polynomial model.

$$N = 2^k + 2k + n_o \qquad \text{Eq. 4}$$

The axial points contribute in a large way to estimation of quadratic terms. Without the axial points, only the sum of the quadratic terms, $\sum_{i=1}^{k} \beta_{ii}$, can be estimated. In the central composite design the center runs provide an internal estimate of error and contribute toward the estimation of quadratic terms [22].

a) b)

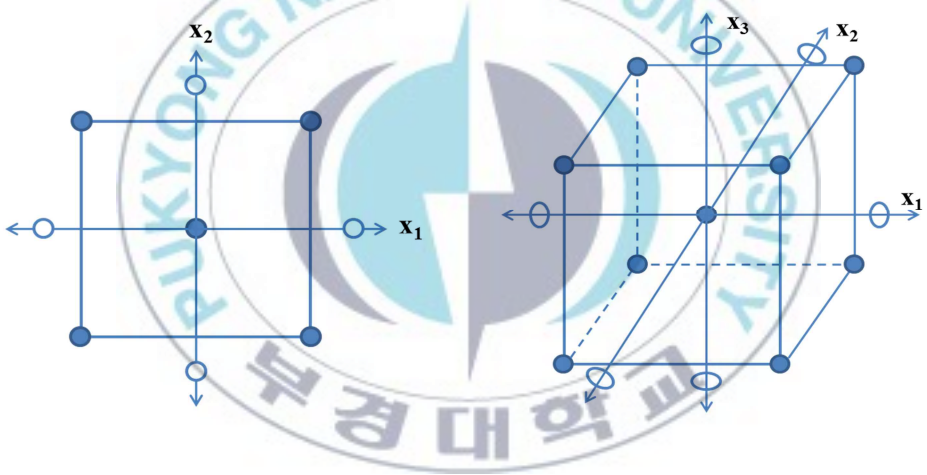


Figure 8. (a) Two-factor Central Composite Design model and (b) Three-factor Central Composite Design model.

3. MATERIALS AND METHODS

3.1. Inoculum system

The anaerobic sludge was taken from an anaerobic digester in Su-young wastewater treatment plant (WWTP), Busan, Korea. It was pretreated by 2 N HCl (37%, Samchun, Korea) at 35°C for 24 hours in order to enhance activity of VFAs-producing bacteria and inhibit methanogenesis phase. Following this, it was seeded and operated with a fermenter in order to dominate the microorganisms for the dark fermentation at a constant status. An artificial medium made with glucose as sole carbon source was used for the VFAs fermentation. The inoculum operation was conducted as same as Lee et al. [23]. Before the experiments, the glucose-acclimatized sludge was centrifuged at 1000 rpm for 3 min and supernatant part was removed. The rest part was washed out by distilled water to remove substrate and other nutrients. After a three-time repeat performance, the microorganism was applied to the experiment.

3.2. Experimental design

In this study, the alginate concentration and value of pH were regarded as two independent variables in RSM. According to Haug and colleagues, the rate of alginate degradation is strongly dependent on acidic (pH less than 5.0) or alkaline (pH more than 9.0) condition [24]. The pH are also important in the solubility of alginate in water [2].

To analyze the effect of alginate concentration and initial pH as well as any interaction between the two independent variables on VFAs and ethanol production, CCD of RSM was employed with Minitab (version 15.1.1.0., Minitab Incorporation, USA) software.

The first region of exploration was decided as 3.0 to 9.0 g/L for alginate

concentration and 2.5 to 6.5 for initial pH. Alginate concentration at central point (6.0 g/L) was chosen based on the optimum alginate concentration pretreated by enzyme, whereas pH range was picked out based on optimum pH of acidogenesis – producing bacteria and acidic environment for alginate degradation. The design boundary and a thirteen trial design were shown in Figure 9 and Table 5, respectively.

Based on statistical analysis of the first result, the result showed that pH was the most significant factor and there was slightly effect of alginate concentration on biofuel production. As a consequence, the experiment design was transferred to alginate concentration of 3.0 g/L and a new series of pH from 5.0 to 8.0 because initial pH over than 8.0 is so alkaline condition, has harmful effect to acidogenic activity [25].

After performing statistical analysis of two experiments, it was clearly shown that pH also was the most significant factor, whereas alginate concentration did not. Thus, the method of steepest ascent involves sequential moves in the direction of maximum increase in response. The new design of exploration for alginate concentration was [6.0 g/L, 12.0 g/L] and for initial pH it was [6.0, 8.0]. Based on the experimental data in this research, the design boundary and an eleven trial-design were shown in Figure 10 and Table 5, respectively.

In order to correlate the relationship between variables and response, a full quadratic polynomial equation was used for model fitting. The general equation of the predictive polynomial quadratic form is [15]:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \ldots + \sum \sum \beta_{ij} x_i x_j + \varepsilon \qquad \text{Eq. 5}$$

where y is the response, β_0 is a constant, β_i is a linear coefficient, β_{ii} is a quadratic coefficient, and β_{ij} is an interactive coefficient.

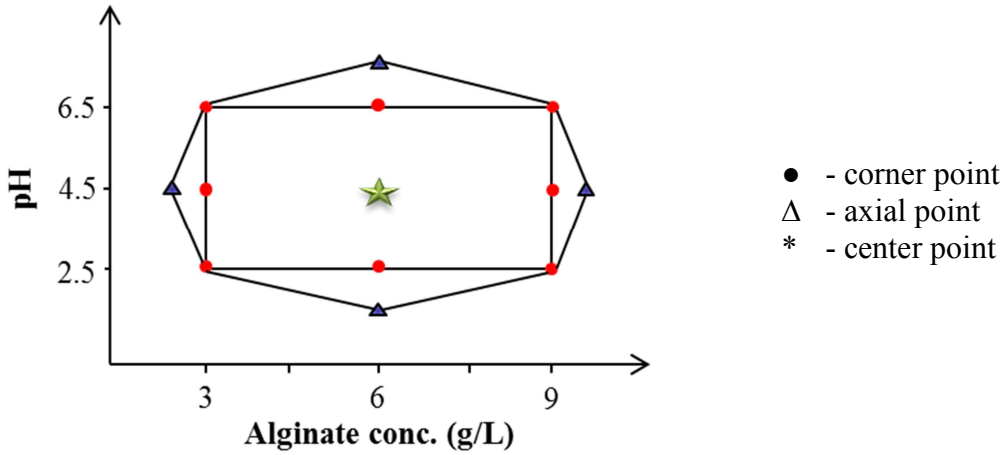


Figure 9. Design boundary of experiment I.

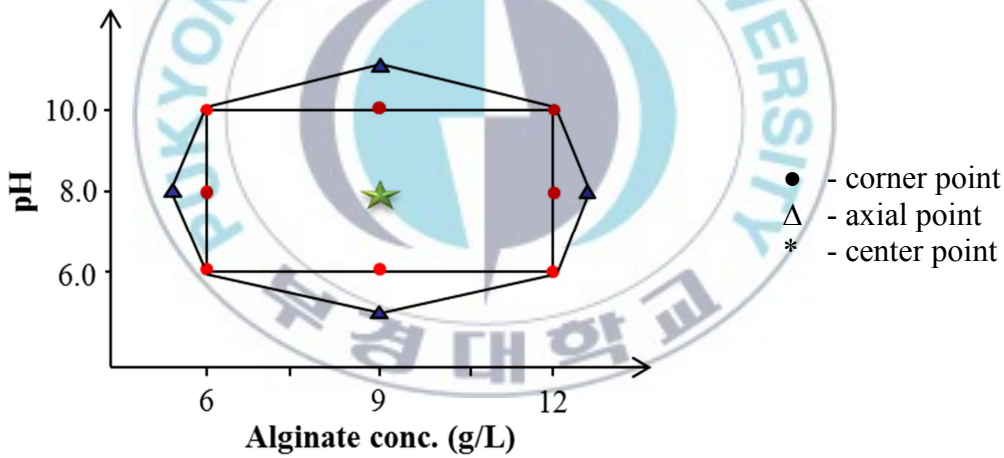


Figure 10. Design boundary of experiment III.

Table 5. Central composite design of overall experiments

Experiment	Trials	Coded Variables		Alginate conc. (g/L)	pH
		x_1	x_2		
I	01 – ●	-1.0	-1.0	3.0	2.5
	02 – ●	1.0	-1.0	9.0	2.5
	03 – ●	-1.0	1.0	3.0	6.5
	04 – ●	1.0	1.0	9.0	6.5
	05 – Δ	-1.4	0.0	1.8	4.5
	06 – Δ	1.4	0.0	10.2	4.5
	07 – Δ	0.0	-1.4	6.0	1.7
	08 – Δ	0.0	1.4	6.0	7.3
	09 – *	0.0	0.0	6.0	4.5
	10 – *	0.0	0.0	6.0	4.5
	11 – *	0.0	0.0	6.0	4.5
	12 – *	0.0	0.0	6.0	4.5
	13 – *	0.0	0.0	6.0	4.5
II	01			3.0	5.0
	02			3.0	6.0
	03			3.0	7.0
	04			3.0	8.0
III	01 – ●	-1.0	-1.0	6.0	6.0
	02 – ●	1.0	-1.0	12.0	6.0
	03 – ●	-1.0	1.0	6.0	10.0
	04 – ●	1.0	1.0	12.0	10.0
	05 – Δ	-1.4	0.0	4.8	8.0
	06 – Δ	1.4	0.0	13.2	8.0
	07 – Δ	0.0	-1.4	9.0	5.2
	08 – Δ	0.0	1.4	9.0	10.8
	09 – *	0.0	0.0	9.0	8.0
	10 – *	0.0	0.0	9.0	8.0
	11 – *	0.0	0.0	9.0	8.0

3.3. Experimental procedure

Sodium alginate (80 to 120 mPa) was obtained from Wako Pure Chemical Industries and stored in fridge at 4 °C. Prior to using, because the minimum temperature for alginate degradation was reported at 130 °C [26], the substrate was mixed in distilled water and sterilized at 121 °C for 15 min in autoclave in order to enhance alginate solubility and remove contaminated microorganisms under the physical nondegradable conditions. Following this, it was stirred at room temperature to make solution be homogeneous and then this was used for the experiment.

A series 500-ml amber reactors as shown in Figure 11 with a working volume of 400 ml was seeded with the inoculum sludge (i.e. in this research, equivalent to 10% of working volume) and filled with a alginate solution, nutrient, and tap water. The nutrient component was 2 g/L NH_4HCO_3, 1 g/L KH_2PO_4, 0.01 g/L $MgSO_4$.$7H_2O$, 0.001 g/L NaCl, 0.001 g/L $Na_2MoO_4.2H_2O$, 0.001 g/L $CaCl_2.2H_2O$, 0.0015 g/L $MnSO_4.7H_2O$, and 0.00388 g/L $FeCl_2$.$4H_2O$ [27]. The pH was adjusted as required using 5 N HCl and 5 N NaOH. Nitrogen gas was purged for 5 min to provide an anaerobic condition. All batch reactors were incubated at 35 ± 1 °C and operated in an orbital shaker with a rotation speed for 120 *rpm* to provide better contact between substrate and microorganism.

Figure 11. Schematic of (a) a batch reactor and (b) biofuels production system.

3.4. Analytical methods

The carbon concentration was measured by total organic carbon (TOC) analysis. In addition, the analysis of volatile suspended solid (VSS) and volatile solid (VS) was performed like standard method of the Examination of Water and Waste Water [28].

Gas samples were collected in 1 L of Tedlar® bags. Its component was H_2, CH_4, and CO_2. H_2 gas was analyzed by GC-HP5890 with thermal conductivity detector and a packed column Hayesep Q (SS, 1.8 m x 1/8 ", 80/60 mesh). Carrier gas was Nitrogen gas at flow rate of 25 ml/min. The analytical condition of injector, oven, and detector were 90 ℃, 30 ℃, and 120 ℃, respectively. While, CH_4 and CO_2 were measured by GC-HP5890 with flame ionization detector and Ni catalyst and a packed column Porapak Q (SS, 2 m, 1/8 ", 80/100 mesh). The temperature of injector, oven, detector, and catalyst was 180 ℃, 35 ℃, 280 ℃, and 350 ℃, respectively. Carrier gas was Helium gas at flow rate of 35 ml/min. In addition, total gas volume in gas bag was calculated by water displacement methods at room temperature.

Furthermore, organic acids analysis was performed using a Shimadzu 17A gas chromatograph with capillary column (Agilent Technologies, Inc., model HP-FFAP, 50 m x 0.32 mm x 0.50 μm). The GC was operated with a flame ionization detector (FID) at 250 °C and injector at 200 ^{o}C. The oven temperature increased from 80 °C to 200 °C at 10 °C/min and was held for an additional 4 min at 200 °C. Carrier gas was Nitrogen gas at flow rate of 30 ml/min.

In addition, ethanol was measured using a Shimadzu LC 20A model HPLC with column Aminex HPX-87H. The analyses were performed at 75 °C under isocratic condition with 5 mM H_2SO_4 as mobile phase.

Liquid and gas samples were taken daily. The liquid sample was centrifuged at 3000 *rpm* for 10 min for acid and ethanol analysis. All analytical methods was summarized in brief and shown in the Table 6.

Table 6. Analytical parameters and methods

Items	Parameters	Sampling points	Analytic method
Monitor	pH	Everyday	pH meter
	Temperature	Everyday	Thermometer
Substrate	Soluble organic carbon (SOC)	Start – Middle – End	TOC analyzer (TOC-VCPH, Shimadzu, Japan)
Organics	Volatile suspended solid (VSS)	Start – Middle – End	Standard methods
	Volatile solid (VS)	Start – Middle – End	Standard methods
Products	H_2, CH_4, CO_2	Everyday	GC – TCD GC – FID methanizer (HP 5890, Agilent , USA)
	Volatile Fatty Acids (VFAs)	Everyday	GC – FID (Shimadzu 17A, Japan)
	Ethanol	Everyday	HPLC (Shimadzu LC 20A, Japan)

4. RESULTS AND DISCUSSION

4.1. Estimating maximum condition of volatile fatty acids and ethanol production (Exp. I and Exp. II)

From an overall perspective of both cases, organic acids were produced from pH 5.0 to pH 6.5. When initial pH was alkaline, the pH went up a first few days and there was no VFAs production during this period. This phenomenon was caused by remaining amount of N_2 gas flushing after making anaerobic condition. Following that, decrease of pH and VFAs production was took place simultaneously.

At the experiment I, there was a little TVFAs production at center points (6.0 g alginate/L and pH 4.5), while maximal TVFAs produced at alginate concentration 9.0 g/L and pH 6.5 was 4.8 g/L. In addition, according to contour and surface plots as shown Figure 12, the optimum point did not appear in the design boundary. Its trend was to be higher Algin concentration and pH.

Table 7. Estimated Regression Coefficients for TVFAs concentration

Term	Coef	SE Coef	*T* – value	*P* – value
Constant	0.152	0.289	0.525	0.616
Algin (g/L)	0.332	0.229	1.450	0.190
pH	1.261	0.229	5.511	0.001
Algin (g/L) * Algin (g/L)	0.287	0.245	1.168	0.281
pH * pH	1.015	0.245	4.137	0.004
Algin (g/L) * pH	0.887	0.324	2.741	0.029
R^2 = 89.15%				

Furthermore, based on estimated regression coefficients as shown in Table 6, pH term was more significant at the 0.5% α-level, whereas Algin concentration was not significant at the 5% α-level for the model.

According to experiment I, pH value was redesigned from 5.0 to 8.0 at fixed algin concentration (3 g/L) in order to find out the estimated optimum trend. To combine with 13-trial of first set and response surface analysis (RSA) (Figure 13), the trend showed the estimated point around pH 8.0 with high alginate concentration.

4.2. Estimating optimum condition of volatile fatty acids and ethanol production (Exp. III)

Therefore, design boundary of experiment III was made in alkaline environment. According to the results of experiment I, as a result in Figure 14, maximum TVFAs production was took place at center points (Algin concentration 9.0 g/L and pH 8.0). Based on response surface analysis, the estimated regression coefficients were described in Table 8.

Table 8. Estimated Regression Coefficients for TVFAs concentration

Term	Coef	SE Coef	*T* – value	*P* – value
Constant	3.404	0.417	8.170	0.001
Algin (g/L)	0.023	0.295	0.077	0.943
pH	-0.403	0.295	-1.368	0.243
Algin (g/L) * Algin (g/L)	-0.670	0.313	-2.143	0.099
pH * pH	-1.777	0.313	-5.688	0.005
Algin (g/L) * pH	0.230	0.466	0.493	0.648
R^2 = 90.23%				

According to Table 8, full quadratic model was used to describe the response surface of the TVFAs production and was

$$Y = 3.404 + 0.023X_1 – 0.403X_2 – 0.670X_1^2 – 1.777\ X_2^2 + 0.230X_1X_2 \quad \text{Eq. 6}$$

where Y = experimental value of TVFAs production (g/L)

X_i = independent variable i (i = 1 for sodium alginate concentration (g/L) and 2 for pH)

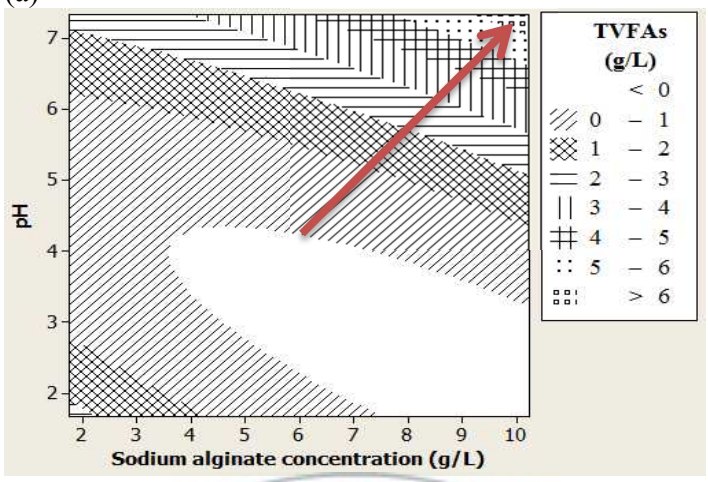
This RSA model estimated a maximal TVFAs production at Na-Algin concentration 9.0 g/L and pH 7.8.

The maximal TVFAs of this study was approximately 4.3 g/L after performing a validation trial. Comparing to other researches, it was approximately 5 times less than Phung [3] and Pham [29] because these used brown algae with another kinds of pretreatment as carbon source. After pretreatment, polysaccharides in carbohydrate compounds were converted to monomers that were well-situated structure for microorganisms' uptake and growth. While this study used sodium alginate that is known as a polysaccharides and without pretreatment performance to do experiment. Moreover, VFAs of this research were composed of acetic, propionic, and butyric acid. Mainly among them, acetic acid was the main component. Comparing to other studies, the result did not include iso-butyric, valeric acid, iso-valeric, and caproic acid with the exception of above organic acids [3, 29-32].

Table 9. CCD and response result for TVFAs production

Experiment	Trials	Coded Variables		Alginate conc. (g/L)	pH	TVFAs (g/L)
		x_1	x_2			
I	01	-1.00	-1.00	3.00	2.50	0.18 ± 0.03
	02	1.00	-1.00	9.00	2.50	0.47 ± 0.02
	03	-1.00	1.00	3.00	6.50	1.48 ± 0.03
	04	1.00	1.00	9.00	6.50	4.78 ± 0.15
	05	-1.41	0.00	1.76	4.50	0.46 ± 0.03
	06	1.41	0.00	10.24	4.50	0.17 ± 0.01
	07	0.00	-1.41	6.00	1.67	0.00 ± 0.00
	08	0.00	1.41	6.00	7.33	3.55 ± 0.83
	09	0.00	0.00	6.00	4.50	0.35 ± 0.21
	10	0.00	0.00	6.00	4.50	0.16 ± 0.16
	11	0.00	0.00	6.00	4.50	0.25 ± 0.12
	12	0.00	0.00	6.00	4.50	0.00 ± 0.00
	13	0.00	0.00	6.00	4.50	0.00 ± 0.00
II	01			3.00	5.00	0.34 ± 0.02
	02			3.00	6.00	1.10 ± 0.07
	03			3.00	7.00	1.22 ± 0.01
	04			3.00	8.00	1.36 ± 0.04
III	01	-1.00	-1.00	6.00	6.00	2.27 ± 0.02
	02	1.00	-1.00	12.00	6.00	3.12 ± 0.18
	03	-1.00	1.00	6.00	10.00	2.15 ± 0.25
	04	1.00	1.00	12.00	10.00	0.00 ± 0.00
	05	-1.41	0.00	4.76	8.00	2.74 ± 0.01
	06	1.41	0.00	13.24	8.00	2.60 ± 0.02
	07	0.00	-1.41	9.00	5.17	0.00 ± 0.00
	08	0.00	1.41	9.00	10.83	0.00 ± 0.00
	09	0.00	0.00	9.00	8.00	3.40 ± 0.23
	10	0.00	0.00	9.00	8.00	3.43 ± 0.20
	11	0.00	0.00	9.00	8.00	3.13 ± 0.05

(a)



(b)

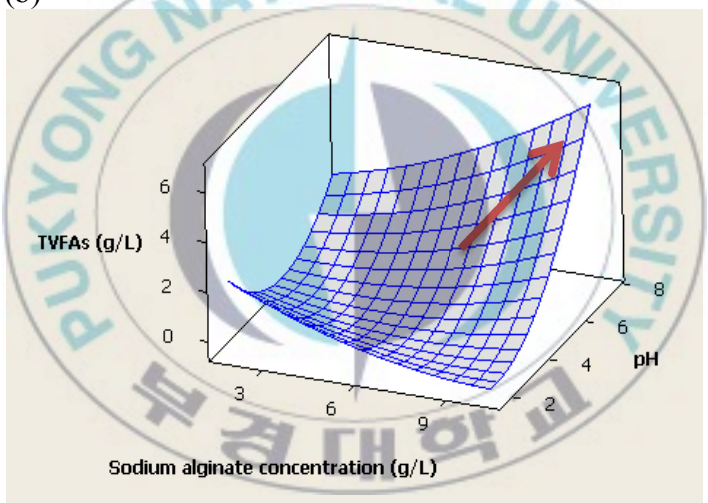
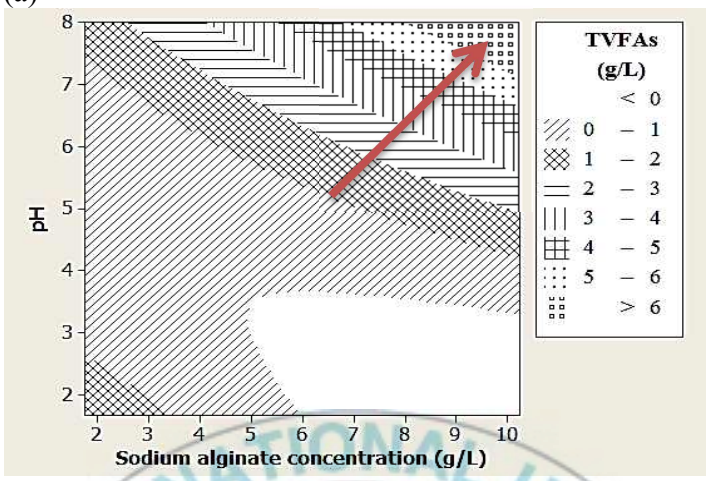


Figure 12. (a) Two- and (b) three-dimensional plots of the quadratic model for the TVFAs production (g/L) with an estimated maximum condition with respect to algin concentration and pH within the design boundary.

(a)



(b)

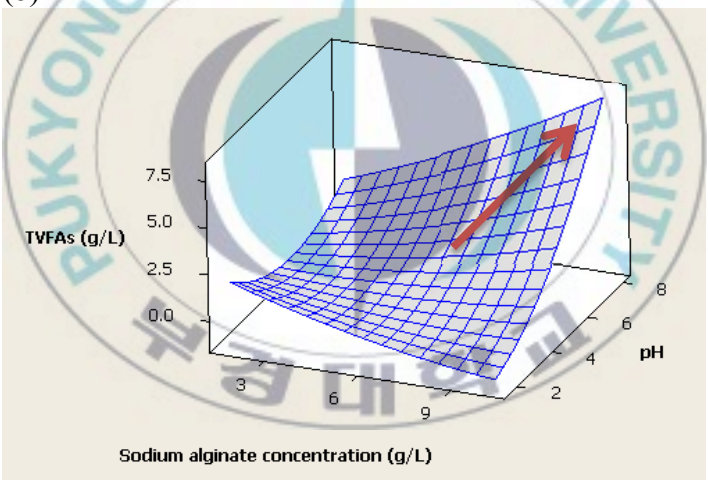
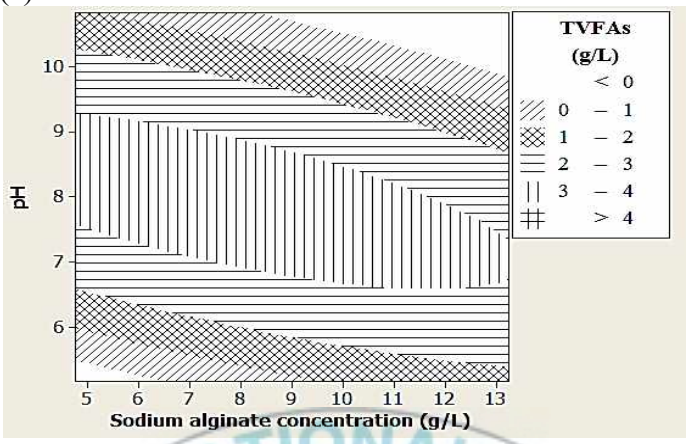


Figure 13. (a) Two- and (b) three-dimensional plots of the quadratic model for the TVFAs production (g/L) with an estimated maximum condition with respect to algin concentration and pH within the design boundary.

(a)



(b)

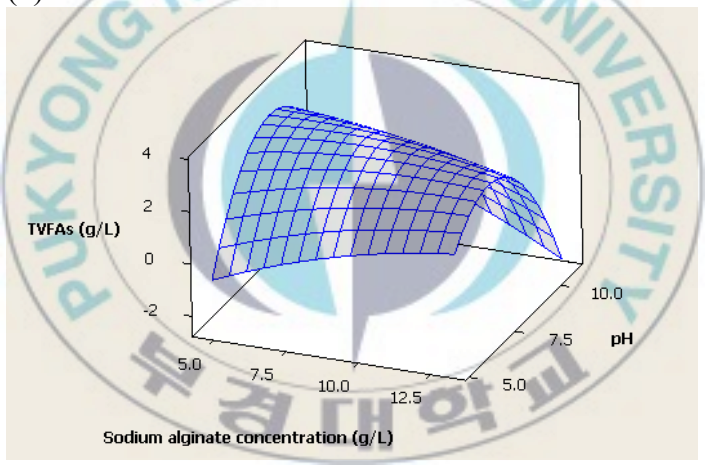


Figure 14. (a) Two- and (b) three-dimensional plots of the quadratic model for the TVFAs production (g/L) with an estimated maximum condition with respect to algin concentration and pH within the design boundary.

4.3. Biofuels production from different type of alginates

The optimum condition was applied in another experiment to find out the difference alginate forms and glucose for VFAs and ethanol production. In addition to sodium alginate of Wako company (Japan), this experiment used sodium, potassium, propylene glycol alginate, and alginic acids of FMC BioPolymer company (USA). Their product names are PROTANAL® LF200FTS, PROTANAL® KF200FTS, PROTANAL® ESTER SD-LB, and PROTACID® F120NM, respectively.

According to Figure 15 and 16, the TVFAs production was in the order of Glucose (3.0) > Na- (Wako) = Na- (1.9) > K- (1.7) > H- (1.5) > PG- (1.0). In general, TVFAs production from glucose fermentation was higher than alginate fermentation. Besides, compared with alginate fermentation, there were only C_2 to C_4 organic acids produced in glucose case. It means that different VFAs production pathway may be exist in the alginate fermentation.

Among alginate forms, sodium alginate showed the most efficiency biofuel production. Acetic acid was the major metabolite in all case with the exception of PG-alginate. There are a lot of applications based on acetic acid such as vinyl acetate monomers (i.e. paints, adhesives), its ester (i.e. solvents for inks, paints, and coatings), organic solvents (i.e. organic synthesis), or micro fuel cells. In addition, propionic acid was only produced in PG-alginate and glucose fermentation. This may be caused by their organic structure without minerals (i.e. sodium, potassium). Propionic acid has anti-fungal and bacterial effects to prevent human from physiology issues (i.e. obesity, inflammation) [33]. However, it is a drawback in biofuel production due to its inhibition on microbial activity.

Based on Figure 16-b, Ethanol was produced in all case with the exception of PG- and H- alginate. Part of them, K-alginate showed the maximum ethanol production, approximate 850 mg/L.

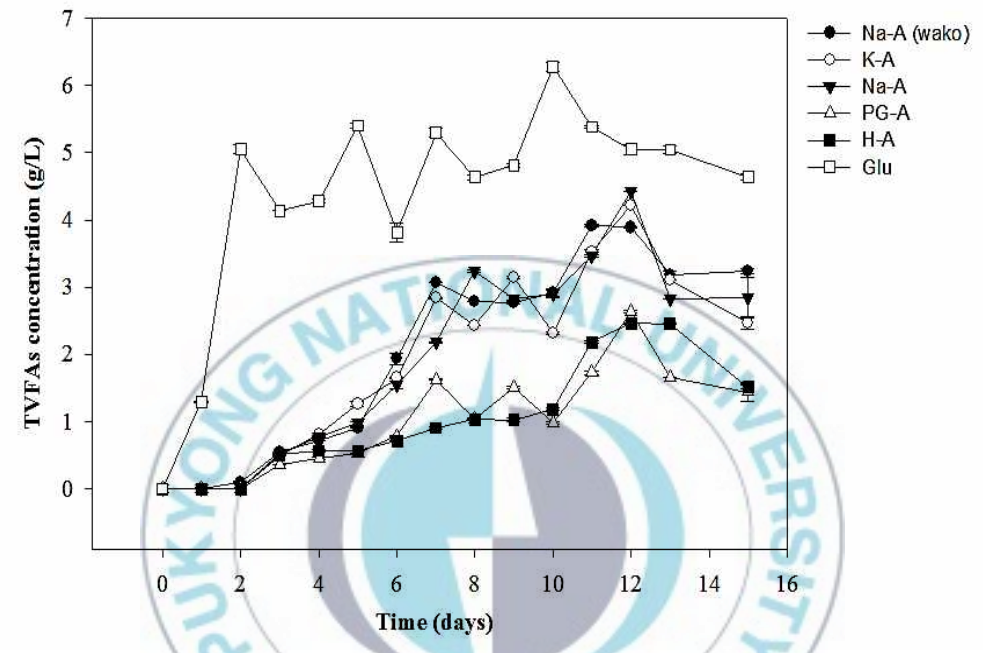
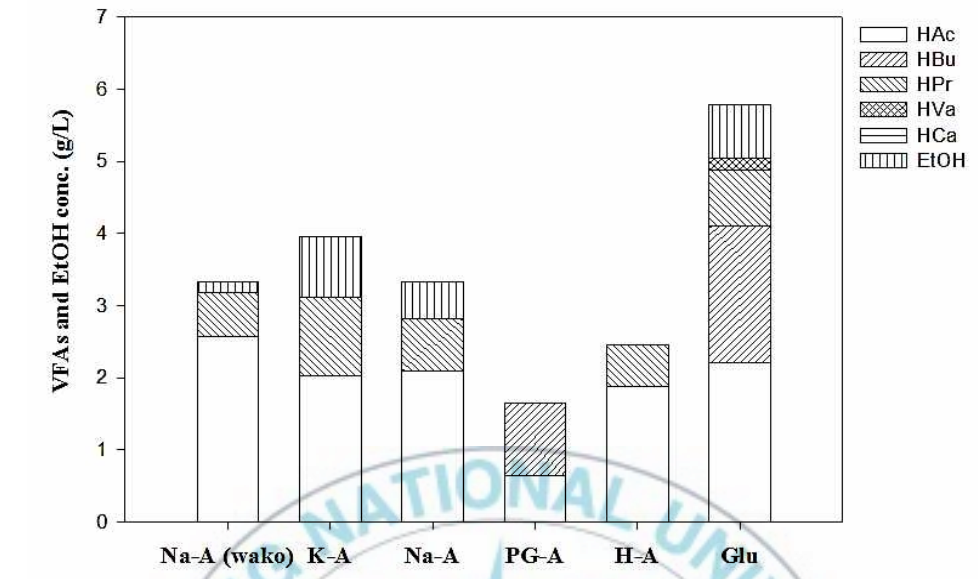


Figure 15. TVFAs concentration in the optimal application experiment.

(a)



(b)

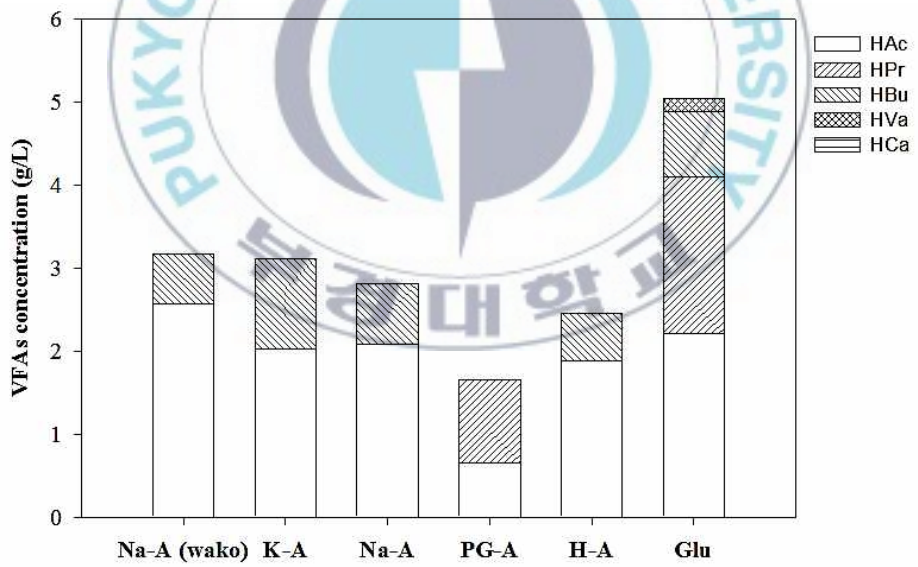


Figure 16. (a) VFAs and Ethanol production and (b) VFAs profile at 13th day in the optimal application experiment.

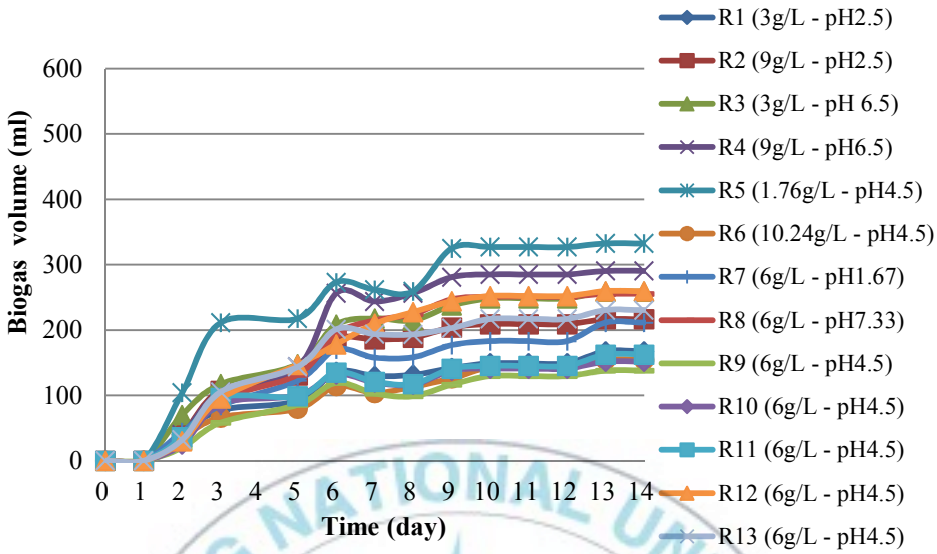
4.4. Biogas production and Hydrogen yield

In this system, biogas consisted of H_2 and CO_2. CH_4 was blocked by inhibitor 100 mM $CHCl_3$ [34] during experiment. Among them, CO_2 was the main gas, whereas H_2 was the minor thing.

As depicted in Figure 17, an amount of biogas production in alkaline condition (504 ml) was obviously higher than acidic condition (332 ml). CO_2 accumulation in alkaline environment was higher than 80 times that in acidic environment (Figure 18). H_2 production in acidic environment was around 4 ml, whereas its in alkaline environment reached 10 ml (Figure 19). In addition, H_2 yield in acidic and alkaline condition was 1.7 % and 4.0 %, respectively.

To summarize, alkaline condition was favour of biogas production using sodium alginate as a sole carbon substrate.

(a)



(b)

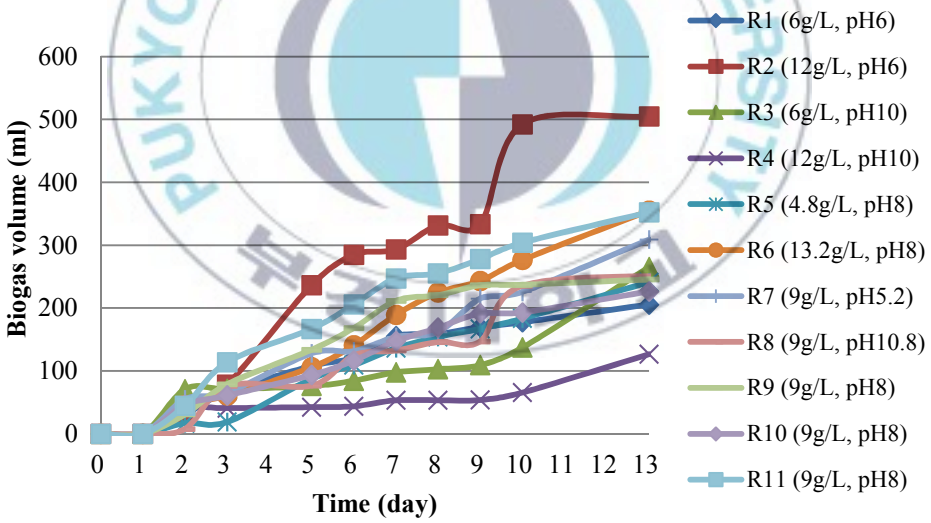


Figure 17. Total Biogas accumulation production in acidic (a) and alkaline (b) condition.

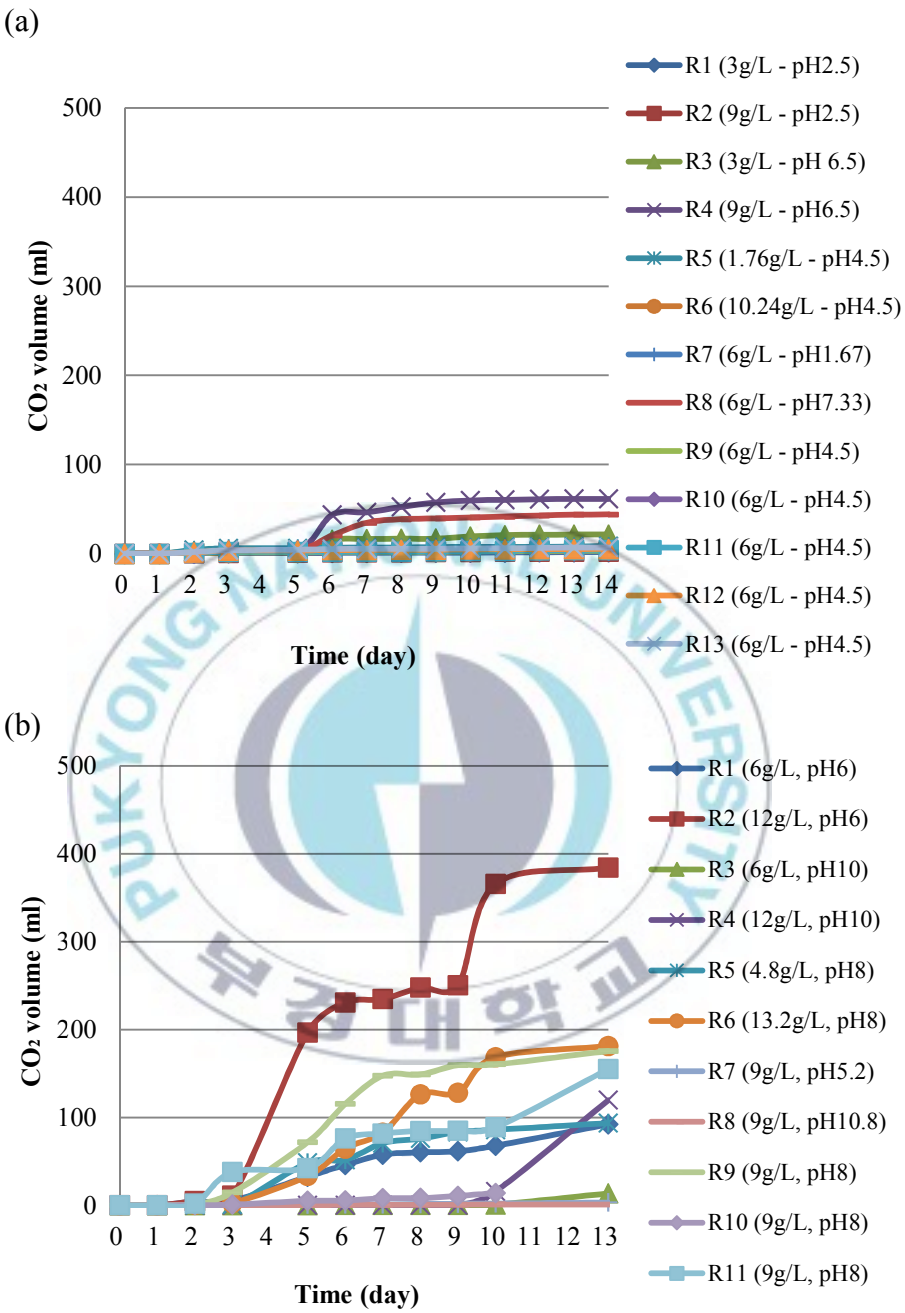
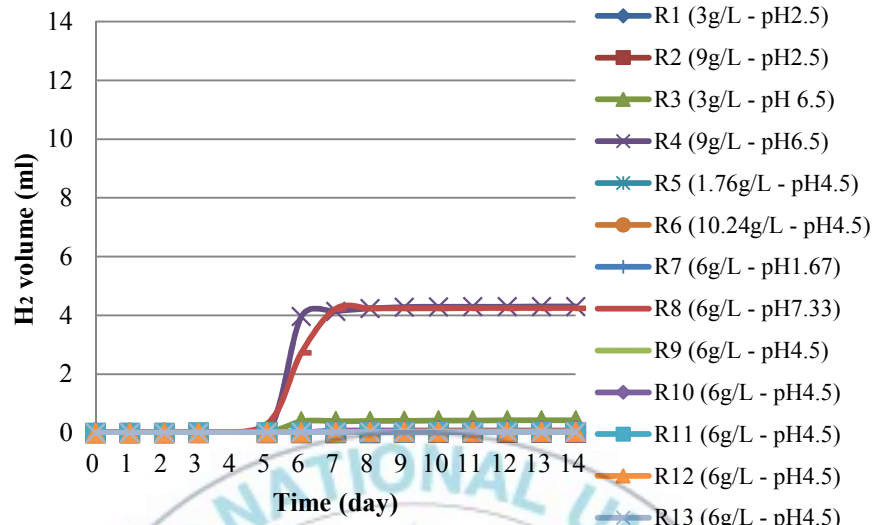


Figure 18. Carbon dioxide accumulation production in acidic (a) and alkaline (b) condition.

(a)



(b)

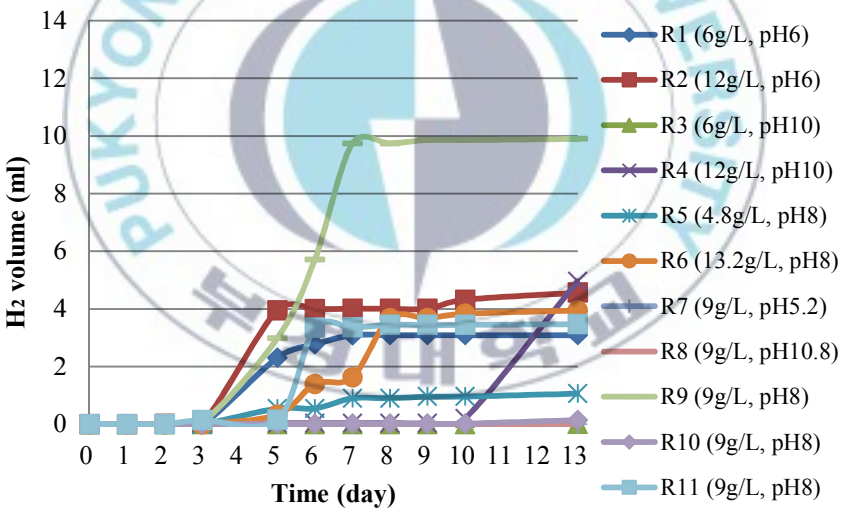


Figure 19. Hydrogen accumulation production in acidic (a) and alkaline (b) condition.

5. CONCLUSIONS

In this study, response surface methodology was applied to evaluate the optimum anaerobic fermentative conditions to produce biofuels from alginate. Based on the results, the following conclusions were pointed out:

- Response surface analysis was successfully applied for optimum conditions of biofuel (VFAs and bioethanol) production from alginate.
- The estimated optimum condition for maximum total VFAs production was 9.0 g/L of sodium alginate and pH 7.8 from the RSM.
- pH represented more significant effect of 99%-level on VFAs production than alginate concentration.
- Maximal total VFAs was approximately 4.3 g/L during the alginate fermentation.
- Acetic acid was the major component for alginate fermentation.
- Individual VFAs profiles represented that only acetic acid and butyric acid were produced from alginates except PG-alginate.
- Biogas from alginate were mainly composed of hydrogen (H_2) and carbon dioxide (CO_2). H_2 yield was 0.04 (mol/mol.SOC.day) and H_2 production rate was 5.62 (ml/g.SOC.day).

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APPENDIX

VOLATILE FATTY ACIDS ANALYSIS

Carboxylic acids were analyzed by at least 3 mL of liquid being drawn from the reactor and placed in a 15 mL conical bottomed centrifuge tube. If not used immediately, the samples could be stored at 2°C. For analysis, frozen samples required thawing and vortexing before beginning the procedure.

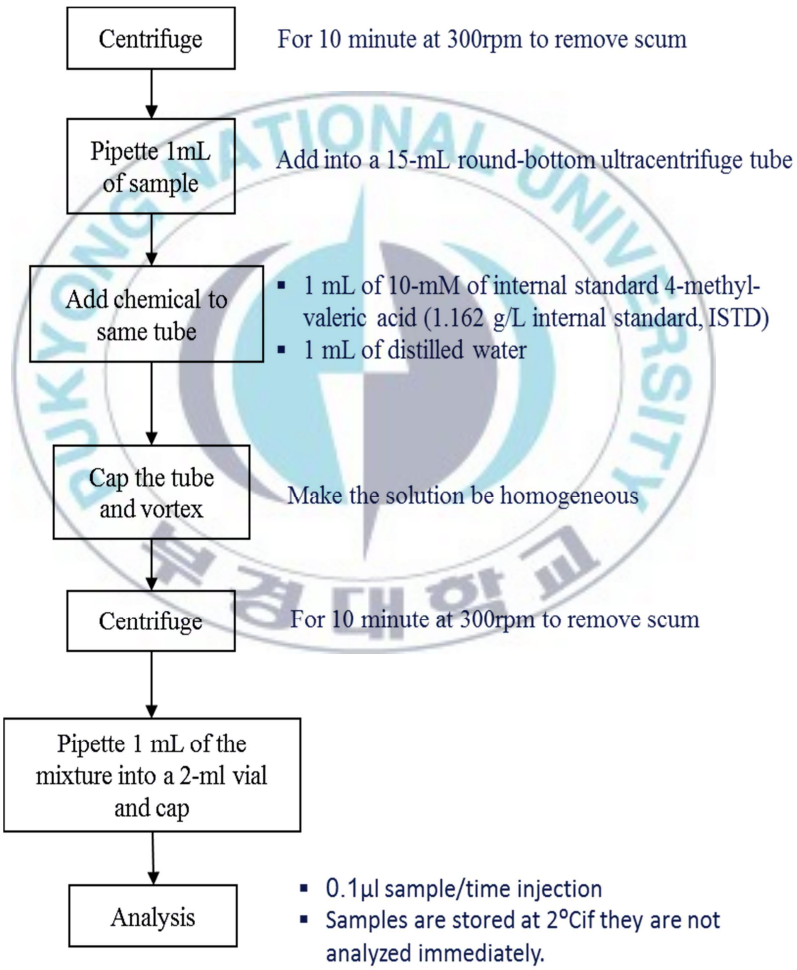

Figure A-1. GC liquid sample preparation.

GC OPERATION

1. Before starting the GC, check the gas supply cylinders (compressed hydrogen, zero-grade helium, and compressed zero-grade air) to ensure at least 100 psig pressure in each. If there is not enough gas, switch cylinders and place an order for new ones.
2. Establish gas flow by setting the regulators to 40 psig for hydrogen, 60 psig for helium, and 50 psig for air.
3. Check the solvent and waste bottles on the injection tower. Fill the solvent bottles with methanol, and be sure the waste bottles are empty.
4. Make sure the column head pressure gauge on the GC indicates the proper pressure (15 psig). Low head pressure usually indicates a worn-out septum. Replace the septum before starting the GC.
5. Use standard mix acids solution for calibration after every 50 samples.
6. Check the setting conditions in the method:
    a. Oven temperature = 80 ºC
    b. Ramp = 10 ºC/min
    c. Inlet temperature = 200 ºC
    d. Detector temperature = 250 ºC
    e. H_2 flow = 40 mL/min
    f. He flow = 88 mL/min
    g. Air flow = 400 mL/min
7. Start the GC on the computer by selecting the method with the abovementioned setting conditions. Set and load the sequence of samples to run. Once the conditions are reached and the green start signal is on the screen, start the run sequence.
8. Periodically check to ensure that the equipment is working properly. Be sure to indicate the number of samples and any maintenance performed (changes of septum, gas cylinders, liner, etc.) in the GC logbook.

9. When finish running the sequence, turn the GC on standby and close the air and hydrogen cylinder valves.

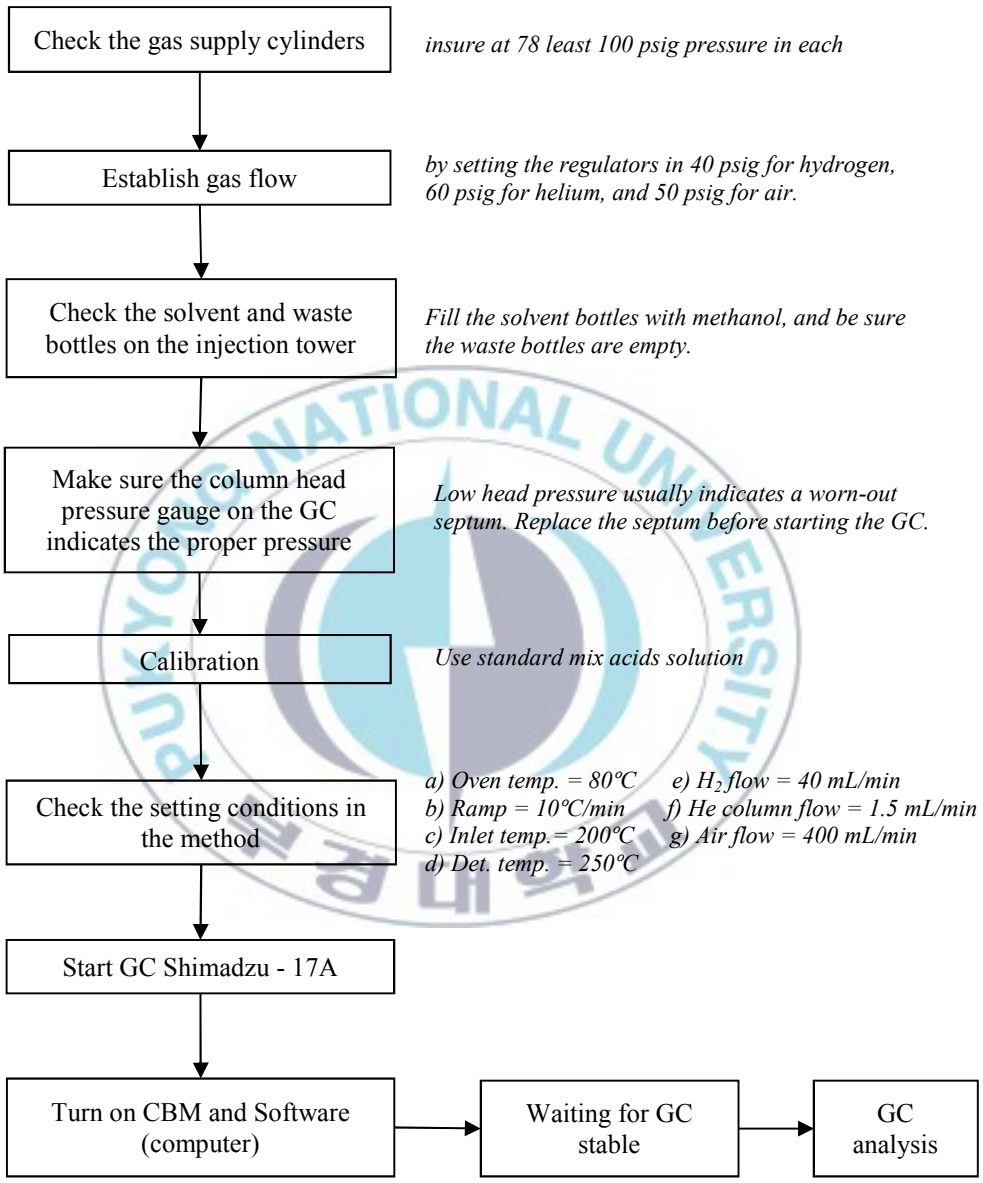

Figure A-2. GC operation.

GAS CHROMATOGRAPHY ANALYSIS OF VFAs

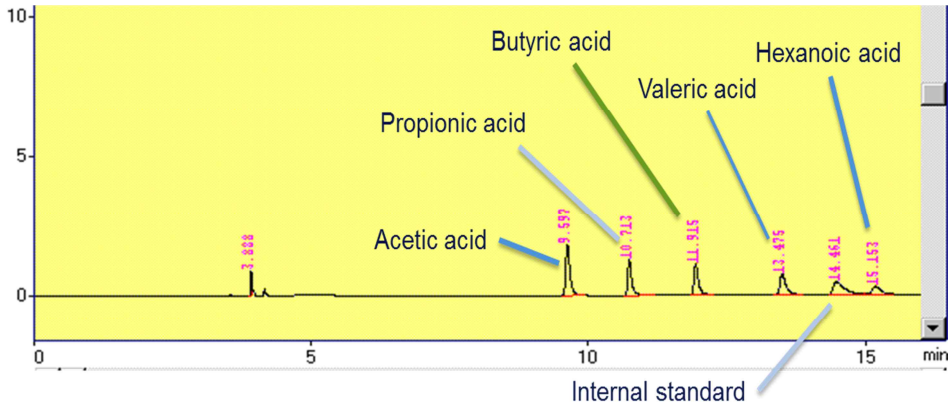

Figure A-3. Gas chromatography analysis of each organic acid.

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Pham Hong Duc