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Biological utilization of pyrolytic acetic acid for lipid-rich algal biomass production: substrate detoxification, inhibition mechanism, and fermentation optimization

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Biological utilization of pyrolytic acetic acid for lipid-rich algal biomass production: Substrate detoxification, inhibition mechanism, and fermentation optimization

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NOMENCLATURE

D	Dilution rate or inverse residence time
F	Flow rate
K_S	The "half-velocity constant"—the value of S when $\frac{\mu}{\mu_{max}} = 0.5$
P	Product concentration
S	The concentration of the limiting substrate for growth
S_0	Initial concentration of the limiting substrate for growth
X	Cell concentration (dry weight basis)
X_0	Initial cell concentration (dry weight basis)
V	The working volume of reactor vessel
$Y_{P/S}$	Yield coefficient (mass product formed/mass substrate consumed)
$Y_{X/S}$	Yield coefficient (mass cell produced/mass substrate consumed)
t	Cultivation time
α	Growth associated product formation constant
β	Non-growth associated product formation constant
μ	Specific growth rate of the microorganisms
μ_{max}	Maximum specific growth rate of the microorganisms

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ABSTRACT

Acetic acid derived from fast pyrolysis of lignocelulosic biomass is a promising substrate for microalgae *Chlamydomonas reinhardtii* fermentation for producing lipid-rich biomass. However, pyrolytic-acetate-containing substrate has an extremely complex composition and highly inhibitory to microorganism due to the various toxic contaminants. In this dissertation, this work is to improve the *C. reinhardtii* fermentation performance through various detoxification practice for enhancing the fermentability of the *C. reinhardtii.* The inhibitory mechanisms of toxic compounds to the algal culture were also studied. Finally, the algal fermentation on pyrolytic acetic acid was optimized though the development of various fermentation strategies.

When growing in raw bio-oil without any detoxification treatment, the algae can only tolerate up to 0.1 wt% of pyrolytic acetic acid stream. Alkaline treatment and oxidative treatment were applied to detoxification of pyrolytic acetic acid substrate. Addition of KOH, NaOH or $Ca(OH)_2$ can significantly reduce the toxicity and improved algal fermentability by 20, 40 and 55 times, respectively. Alkali species used, treatment pH and treatment temperature were found all influencing the effectiveness of the alkali treatment.

When oxidative treatment was further applied to NaOH-treated pyrolytic acetic acid substrate, the maximum cell density and biomass productivity was improved under each level inclusion of treated pyrolytic substrate although the cell still cannot tolerate the higher inclusion of the pyrolytic substrate. It was also found that directed evolution of algal strain increased the tolerance of algae strain; the maximum tolerance level of pyrolytic substrate by

the evolved strain increased to 5.5wt% as comparted to the maximum tolerant level of 4% by the wild type strain.

Studies also show that the contaminant compounds in crude acetic acid substrate such as furans, phenols, ketones, aldehydes, ethers, esters, alcohols were contributing the inhibition of pyrolytic acetic acid stream. Such an inhibitory was mainly due to the cell membrane damage. Reemoval of these compounds by by various detoxification methods developed above was mainly t through oxidation and precipitation The result also shows that the disruption of cell membrane integrity is correlated with algal growth inhibition caused by the pyrolytic acetic acid, and the enhanced growth performance of algal strain after directed evolution and surfactant protection may due to the enhanced ability to keep the cell membrane integrity.

Different fermentation strategies were used for heterotrophic microalgal fermentation using pyrolytic acetic acid as substrate. Perfusion culture with partially bleeding of algal cells from the reactor resulted in a 2.05 g/day/L of biomass productivity, 10 times higher than that in the batch culture process. In summary, this work demonstrated that pyrolytic acetic acid is a feasible substrate for producing lipid-rich biomass though the fermentation of microalga *C. reinhardtii*.

CHAPTER 1 GENERAL INTRODUCTION

1.1 Thermochemical-biological conversion of lignocellulosic biomass for fuels and chemicals

1.1.1 Hybrid processing

Worldwide increasing demand of petroleum is in the risk of volatile prices, depletion of high quality of oil resources, environmental concerns and energy security (Anwar et al., 2014; Balan., 2014; Bardhan et al., 2015). Arising effort from government agencies, industry and research institute has been pursued to promote the production of environmental-friendly sources as replacement for fossil fuels. As a potential feedstock for biofuels production, lignocellulosic biomass is abundantly available, non- edible, bio-renewable and carbon neutral. Thus, the conversion of lignocellulosic biomass into fuels and chemicals has gained more and more interest in the recent years (Sims et al., 2010).

Producing fuels and chemicals from lignocellulosic biomass is often achieved through biochemical pathways, which commonly consists of three steps: pretreatment of biomass to break down its recalcitrance, enzymatic hydrolysis of treated biomass into reducing sugars, and fermentation of sugars into desirable products (Saxena et al., 2009). This process, however, is limited by several technical and economic barriers such as the high costs for pretreatment and enzymes, lack of robust microbes capable of fermenting mixed sugars (hexose and pentose), and under-utilization of lignin compounds in the biomass (Kato et al., 2014; Naik et al., 2010; Sarkar et al., 2012).

Thermochemical processing, like combustion, gasification, liquefaction and fast pyrolysis, is another promising method to convert lignocellulosic biomass into fuels. With heat and/or pressure used to decompose biomass, and sometimes boosted by chemical catalyst, thermochemical processing can avoid almost all of the disadvantages of biochemical processing

listed above (Jarboe et al., 2011a); however, the economic viability is always a challenge due to the high capital cost and high energy input for thermochemical processing.

A thermochemical biological hybrid processing for lignocellulosic biomass is recently proposed as an alternative to conquer the problem in current lignocellulosic biomass conversion process. It involves the use of thermochemical processing to break down lignocellulosic biomass into fermentable intermediates, and utilization of those intermediates as carbon sources in biochemical processing (fermentation) for producing fuels and chemicals. (Jarboe et al., 2011a). The first step of thermochemical processing can overcome the recalcitrance of lignocellulosic biomass and utilize lignin that is commonly treated as waste byproducts, while second stage of biological processing is featured with high product selectivity of the biological catalyst. Common studies about hybrid processing including fast-pyrolysis-bio-oil-fermentation and gasification-syngas-fermentation.

Fast pyrolysis is the thermal decomposition of biomass in the absence of oxygen. This process can convert biomass into an energy rich liquid (bio-oil), a flammable gas (syngas) and a carbon- and nutrient-rich solid (biochar) (Brown & Brown, 2013). The raw bio-oil is an extreme complex mix of chemical compounds. Some component substrates, such as levoglucosan and acetic acid, are promising to be used as fermentative substrates to produce various fuels and chemicals (Layton et al., 2011; Lian et al., 2013b; Lian et al., 2012b; Liang et al., 2013; Zhao et al., 2013c).

Gasification is a process that lignocellulosic biomass is converted to syngas under high temperature (>700 °C) and controlled amount of oxygen and/or steam. Syngas, the product of gasification, is a flammable gas mixture mainly composed of carbon monoxide (CO), hydrogen (H_2) and carbon dioxide (CO_2). Syngas has the potential to be converted into fuels and chemicals,

particularly bioethanol, via biocatalysts fermentation. Due to the low solubility of CO and H_2 in water, gas-to-liquid mass transfer limitation needs to be conquered to allow for successful syngas fermentation (Shen, 2013).

1.1.2. Lignocellulosic biomass composition

Biomass resources can be categorized into four groups: i) woody biomass such as forest residues; ii) agricultural residue; iii) organic waste, such as animal waste and sewage sludge, and iv) aquaculture, like algae and seaweeds. As one the most abundant renewable energy resources on earth, current estimated global biofuel production from lignocellulosic biomass can potential reach to 15–70 EJ final transport fuel energy with 30–140 EJ primary energy (Deng et al., 2015). The chemical composition of lignocellulosic biomass varies depending on the initial feedstock and is also largely affected by the conversion methods used. (Goyal et al., 2008).

Lignocellulose is composed of three major compounds, cellulose, hemicellulose and lignin. The structure of each compound is shown on Figure 1-1. The composition of lignocellulose derived from various types of biomass was listed on Table 1-1 (Iqbal et al., 2013). Cellulose is a linear polymer of β-1,4-D-glucopyranose units with the formula $(C_6H_{10}O_5)n$. The chain length varies from 300 to 1700 units for wood pulp, while from 800 to 10,000 units for bacterial cellulose. Reported average molecular weight of cellulose is around 100,000 (Goyal et al., 2008). Cellulose consists of crystalline and amorphous regions and is very rigid. As the largest constituent of plant cell, the rigidity of cellulose protects plant cell from interiors.

Hemicellulose is a branching polymer of various monosaccharides, including glucose, mannose, xylose, galactose, arabinose, and rhamnose. The chain length of hemicellulose varies from 500 to 3,000. Hemicellulose has an amorphous structure and not as rigid as cellulose. The

main role of hemicellulose is to strengthen the cell wall by interaction with cellulose and lignin (Scheller & Ulvskov, 2010).

Lignin is a complex, cross-linked polymer aromatic rings, corresponding approximately to the formula $(C_{31}H_{34}O_{11})n$. The monomers are p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which are methoxylated to various degrees. The molecular masses of lignin is in excess of 10,000 units. The rigidity of lignin imparts strength to plant cell and do not rot easily.

1.1.3. Fast pyrolysis

Among different types of lignocellulose processing, fast pyrolysis shows a promising process for biomass-to-biofuel conversion (Braimakis et al., 2014; Nanda et al., 2014). Fast pyrolysis is carried out under moderate temperature (~500 ℃) and high heating rate (tens to hundreds of ℃/s), biomass is decomposed in the absence of oxygen, while syngas (13~25 wt%), bio-oil (60~75 wt%) and biochar (12~25 wt%) are produced (Sarat Chandra et al.; Xiu & Shahbazi, 2012). Fast pyrolysis is featured of very high heating rates and heat transfer rate, carefully controlled reaction temperature to maximize liquid yield (60~75%) for most biomass, short hot vapor residence time (less than 2s), rapid removal of product char and rapid cooling of vapors. The main product of fats pyrolysis, bio-oil, can be used as feedstock to produce fossil fuel substitute through upgrading (Xiu & Shahbazi, 2012), valuable chemicals through extraction (Wang et al., 2014; Xiu & Shahbazi, 2012), and fuels and bulk chemicals through fermentation (Jarboe et al., 2011a). Use of bio-oil as road deicers was also reported. (Oehr & Barrass, 1992).

1.1.4. Bio-oil characterization and chemical compositions

Bio-oil derived from lignocellulosic biomass is commonly a sticky, dark brown liquid. The property of bio-oil is depended on the initial feedstock. Water content of the bio-oil is ranging from about 15 wt% to an upper limit of about 30–50 wt% . High water content would

reduce bio-oil viscosity and improve bio-oil stability. On the other hand, however, the high water content leads to the high oxygen content and low heating value of bio-oil. The viscosity of the bio-oil can vary from as low as 25 $m^2 s^{-1}$ to as high as 1000 $m^2 s^{-1}$ (40 °C). The oxygen content of bio-oil is around 35~40 wt%. The higher heating value of bio-oil is about 17 MJ/kg, which is about 37% that of crude oil (Czernik & Bridgwater, 2004; Venderbosch & Prins, 2010). Bio-oil has 42% of the energy content of fuel oil on a weight basis; however, due to the high density of bio-oil (~1200 kg t^{-1}), on a volumetric basis, bio-oil contains 61% of that in fuel oil

In general, bio-oil has a very complex chemical composition, including anhydrosugars, carboxylic acids, pyrans, furans and phenolics and as well as other trace amount of compounds. Major compounds in bio-oil can be quantified via GC-FID equipped with MS. The organic chemical composition of bio-oil derived from lignocellulosic biomass is listed at Table 1-2 (Goyal et al., 2008).

The formation mechanisms of major compounds in bio-oil gains lots of interests. Previous study shows that during fast pyrolysis, cellulose undergoes two phases of decomposition reactions. During the first phase, depolymerization and dehydration reactions occur with the corresponding C5-C6 products being produced, mainly anhydrosugars, pyrans and furans (Mettler et al., 2012). These products are not stable under pyrolytic conditions and are further decomposed in the following cracking phase. The chemicals produced during the second decomposition phase are mainly aliphatic oxygenated C2-C4 organic compounds as well as some other light species/gases (Shen et all., 2012). As to hemicellulose decomposition, the predominant product is acetic acid. Fast pyrolysis of lignin leads to the yield of aromatic compounds with guaiacyl-units or phenolic-units. Some small molecules, such as methanol, acetic acid, and acetone are produced during decomposition of lignin (Mohan et al., 2006).

Pyrolytic bio-oil is composed of a complex mixture of organic compounds that provide both the potential and challenge for its utilization. Bio-oil has potential as a fuel substitute without additional processing, but can also be upgraded to higher value fuel such as biodiesel (Xiu & Shahbazi, 2012). Additionally higher value chemicals can be extracted from bio-oil (Wang et al., 2014; Xiu & Shahbazi, 2012). Previous study shows that bio-oil contains significant amount of anhydrosugars and carboxylic acids, which are suitable for fermentation. Acetic acid derived from bio-oil can also be used as road deicers. The phenolic oligomers contained in bio-oil can be upgraded into hydrocarbon, which can be further refined into drop-in fuels by using existing petroleum refining technology and infrastructure (Mortensen et al., 2011).

1.1.5 Bio-oil utilization:

Due to its unstable physical properties and complex chemical composition, the utilization of pyrolytic bio-oil often faces challenges (Lehto et al., 2014). Unlike conventional fuels, bio-oil is featured by high oxygen content, high solids content, high viscosity and high chemical instability, which may adversely affect bio-oil fuel quality. Some physical or chemical techniques can be used to upgrade pyrolytic bio-oil prior to convert into transport fuel. The hybrid processing proposed hereby apply pyrolytic bio-oil in biological utilization for bioenergy or value-added chemical production.

Physical and chemical upgrading to transport fuel: Physical filtration can be applied to reduce the ash content to less than 0.01 wt%. Solvent addition is helpful to homogenise bio-oil and reduce the viscosity of bio-oil. Bio-oil emulsified with diesel oil with the aid of surfactants can be applied as transport fuel or a fuel used in no-need-for-modification engines. Catalytic upgrading of bio-oil to conventional transport fuel, such as biodiesel, requires full deoxygenation

and conventional refining. The principle of catalytic upgrading of bio-oil is reject oxygen reject oxygen as H_2O via hydrotreating or rejects oxygen as CO_2 via zeolite cracking.

Biochemical utilization: Biochemical upgrading of bio-oil can be realized via fermentation with bacteria, yeast or microalgae to produce bioethanol, carboxylic acids or polymers. Various pyrolysis derived compounds, such as anhydrosugars and carboxylic acids, have been explored as potential fermentation substrates, which made hybrid processing of lignocellulosic biomass possible. Improve the fermentability of bio-oil can be achieved by two different ways: detoxification of bio-oil and adapt biocatalyst to tolerate to the toxicity of pyrolytic substrate. Some physical, chemical and combinations methods have been applied to bio-oil detoxification. In addition, metabolic engineering were used to improve the biocatalysts robustness. Previous study about hybrid processing was reviewed and listed in Table 1-3. In the following section (Section 1.2), the various aspects of biological utilization of pyrolytic substrates are discussed.

1.2 Bio-oil fermentation process

1.2.1 Fermentable substrates from pyrolytic bio-oil

The fermentability of anhydrosugars, 1,6-anhydro-β-D-glucose or levoglucosan, have been widely investigated. The concentration levoglucosan in pyrolytic bio-oil can reach ~30 wt% on a moisture-free basis (Liang, 2012), which shows a great potential for pyrolytic levoglucosan to be used as a carbon resource in fermentation.

Acetic acid is a suitable fermentation substrate for some biocatalysts. (He et al., 2009; Nakamura et al., 2011; Chen and Johns, 1996), although it is undesirable from energy production of view in the fast pyrolysis process due to its low energy density and extensive corrosion possibility. Utilization of acetic acid has been demonstrated in yeast (Lian et al., 2012a) or

microalgae fermentation (Liang et al., 2013; Zhao et al., 2013a; Zhao et al., 2015) for producing lipid-rich biomass.

Glycolaldehyde present in pyrolysis bio-oil is another potential fermentative substrate. The concentration of glycolaldehyde was within the rage of 5-13 wt% in wood-derived pyrolysis oil has been reported (Zhang et al., 2011). Vitasari (2012) developed a lab-scale conceptual process to isolate glycolaldehyde from pyrolysis bio-oil, and this renewable glycolaldehyde can be utilized as fermentation feedstock for ethylene glycol production (Vitasari et al., 2012)

Acetol composes 8 wt% of softwood-derived pyrolysis bio-oil. It is well known that l,2-propanediol can be produced via bioreduction of acetol under the mediation of baker's yeast. The lab-scale was developed 70 years ago, in which a large amount of sucrose (100 g/L) is needed as energy source and the yield was 5-6g/L (Levene & Walti). Kometanik et al. (1993) developed a large-scale system for l,2-propanediol production from acetol in a bubble-column reactor (10 L), in which 300 mM of ethanol is needed as energy source and the yield increased to 220 g/L (Kometani et al., 1993). In addition, acetol was found to be reduced to R(–)–1,2–propanediol via *Clostridium thermosaccharolyticum* (Cameron & Cooney, 1986).

1.2.2. Inhibition of crude pyrolytic substrates

The fermentation of pyrolytic bio-oil via alga *C. reinhardtii* is hindered by the toxicity of pyrolytic bio-oil. For example, previous study showed that pyrolytic acetic acid over 0.01 wt% inhibited the microalgae growth (Liang *et al.*, 2013). Thermal conversion process. to the complex composition of bio-oil and imitation of analytical methods, only a few toxic compounds, such as methanol, furfural, 5-HMF, phenolic compounds and carboxylic acids has been identified, with a large potential toxic compounds still being unknown. A rigorous Identification of compounds in bio-oil, and investigation of these compounds on microorganism morphology

and genotype property change are needed to better understand the inhibitory mechanisms and conquer pyrolytic substrate inhibition on cell growth.

Previous research on the mechanism of inhibition on microorganism culture might be helpful to understand the inhibitory effect of bio-oil on algal fermentation. Inhibitors from lignocellulosic dehydrates, lignin derived sugars, bio-oil and syngas hinder the biochemical processing of lignocellulosic biomass and hence impede biofuel and biochemical production. Research has been done to investigate the mechanism behind the inhibition on bacteria, fungi and microalgae.

As the product of fast pyrolysis of lignocellulosic biomass, acetic acid at certain level has proved to inhibition to microbial fermentation. Acetic acid-rich pyrolytic bio-oil contains ~80 mg/L acetic acid, 10 times as high as the concentration for pure acetic acid to completely inhibit the growth of *C. reinhardtii* (Zhao *et al.*, 2013). Culture of *E. coli* can be inhibited by acetic acid at a concentration even as low as 50 mM. Inhibition of other organisms has also been reported. For example, yeast used in wine fermentation such as *Saccharomyces cerevisiae* can be inhibited by high levels of acetic acid secreted by other competitor yeasts (Peter Piper, 2001).

The inhibition of acetate on yeast growth was explained by intracellular accumulation of H^+ and CH_3COO^-. Acetic acid presents in yeast fermentation broth with pH lower than 4.75 (pKa of acetic acid) as its uncharged form, CH_3COOH, which is freely permeable to cell membrane. The difference of CH_3COOH concentration outside and inside cells drive the CH_3COOH entering cell membrane via diffusion until same concentration of CH_3COOH is achieved on both sides of cell membrane. Under the relatively high pH inside the cell membrane, H^+ and CH_3COO^- were produced via CH_3COOH dissociation and accumulated to very high levels for its relatively low permeability to cell membrane. The accumulation of H^+ and CH_3COO^- would lead

to intracellularly acidification, free radical increase, and high turgor pressure, and thus cell growth inhibition. Yeast species protect cells from the inhibition of acetic acid by consuming acetate, changing cell envelope to limit CH_3COOH diffusional entry or catalytically accelerating CH_3COOH extruding.

Lipophilic weak acids inhibit cell growth more likely through a different mechanism from acetic acid inhibition. When the broth pH is 4.5, sorbate (pKa=4.76) even at a low concentration, can disorder cell membrane structure and completely inhibit cell growth. In addition, sorbate may affect respiratory chain function and glycolysis pathway, and bring oxidative stress and severe energy depletion respectively to yeast cells. Associated with oxygen, lipophilic weak acid can affect membrane transport processes and energy coupling. Intracellularly acidification was not found in sorbate-treated yeast cells. The difference of acetic acid and lipophilic acid inhibition mechanism is more apparent when at neutral pH. When pH of the broth is 7, both acetic acid and sorbate are completely dissociated. The acetic acid shows slight inhibition and provides a potential fermentation substrate. While sorbate still shows notable threat on yeast cell growth. In addition, it is reported that palmitoleic acid may increase cell osmotic pressure, leading to plasmolysis and destroy of cell wall and cell membrane.

Acetate inhibition on *E. coli* culture has been previously studied. For batch culture (pH<4.3), significant inhibition on *E. coli* growth was found when acetic acid concentration is above 20 mM. As to continuous culture (pH 6), *E. coli* cell growth can still be inhibited but fourfold concentration of acetate is needed compared to low pH culture. It was reported that the inhibition may come from acetate anion accumulation, which was mainly driven by the difference of extracellular pH (~6) and intracellular pH (6.8~7.6), or ΔpH. High acetate tolerance

E. coli species was expected to have the ability both to decrease intracellular pH and then decrease ΔpH, and metabolic system to tolerant low intracellular pH.

In addition to acetic acid, other compounds in bio-oil also exhibit strong inhibition to microorganisms. Phenolic compounds were reported to have adverse effect on bacteria, fungi and microalgal cell membrane integrity. The degree of inhibition depend on the category, lipophilicity and degree of ionization and the specific chemical structure of phenolic compounds. Reported lowest effective concentration for phenolic acid, phenyl aldehyde and polyphenolics to significantly decrease the viability of cell are 250 mg/L, 50 mg/L and 0.25 mg/L. These phenolic compounds may to inhibit the biosynthesis and simultaneously bind with target structural component of membrane, i.e. ergosterol, to disrupt cell membrane integrity. Also the decrease of cell membrane integrity resulted from phenolic compounds may be associated with increased intracellular pH, ion leakage, proton influx, oxidative stress and membrane depolarization. With a concentration of 20g/L in SF5, phenolic compounds may significantly decrease the cell viability and inhibit cell growth.

In summary, short chain carboxylic acids (formic acid, acetic acid and propanoic acid) can lead to intracellular acidification, free radical increase, and high turgor pressure; lipophilic weak acids (phenolic acids) may affect respiratory chain function glycolysis pathway, increase cell osmotic pressure, and bring oxidative stress to cells; and phenolic compounds have also been reported to damage the cell membrane structure by inhibiting the biosysthesis of structural components and/or binding the structural components, which causes an increase in leakage through the membrane. Most of the reported damage described above are related to the cell viability, which can be evaluated by microscopic or cytometric examination of cells stained with dyes or other chemicals.

1.2.3. Detoxification of bio-oil through various treatments

Previous study showed that inhibitory compounds removal by absorbent treatment (Prosen et al., 1993), solvent extraction (Lian et al., 2010), acid treatment- (Prosen et al., 1993) alkali treatment (Chi et al., 2013), or combination of some methods, followed by levoglucosan hydrolyzation to glucose (Lian et al., 2010; Prosen et al., 1993), can significantly improve the fungi, *E. coli* and yeasts fermentation of pyrolytic levoglucosan for bioethanol or lipids production. Treated pyrolytic anhydrosugars has been reported for bioethanol production through yeast (Lian et al., 2013; Luque et al., 2014; Sukhbaatar et al., 2014) and *E. coli* (Chang et al., 2015; Chi et al., 2013; Jarboe et al., 2011a; Rover et al., 2014; Wang et al., 2012), and for producing lipids through yeast fermentation (Lian et al., 2013).

Aqueous extract: Solvent extraction is utilized to separate a mixture of compounds with solubility difference in immiscible solvents. In bio-oil detoxification, solvent extraction was used to remove fermentation inhibitors and separate fermentation substrates such as lignin (Prosen et al., 1993), phenolics (Lian et al., 2010), carboxylic acids (Chan & Duff, 2010), and recovery of glycolaldehyde (Vitasari et al., 2012).

Distillation: Distillation can separate component substances with different boiling points from a liquid mixture by selective evaporation and condensation. To simplify the bio-oil composition and facilitate further bio-oil refinery, a fractionation system has been developed in Iowa State University to separate raw bio-oil into different stage fractions (SFs) with distinct chemical and physical properties (Pollard et al., 2012). For example, the stage fraction 1 (SF1) contains the majority of levoglucosan and phenolic oligomers; while stage fraction 5 (SF5) contains the majority of water and acetic acid (Pollard et al., 2012). The whole system is shown

on Figure 1-2. The composition of different stage fractions are listed on Table 1-4 (Pollard et al., 2012).

Due to the complex nature, stage fractionations of pyrolytic bio-oil are still toxic and not ready to be applied in biological processing. Thus, distillation was commonly used in combination with other treatment methods, such as activated carbon adsorbent, solvent extraction or overliming (Chan & Duff, 2010; Chi et al., 2013; Liang et al., 2013; Vitasari et al., 2012).

Adsorption: Activated carbon has strong absorption capability due to its high degree of microporosity. It is widely applied for metal extraction, water purification, medicine, wastewater purification, filters, filter masks, and hydrocarbon adsorption. Previous study has shown that activated carbon is an efficient absorbent, and can absorb many compounds such as formic acid, acetic acid, phenols, furfural and HMF (Lee et al., 2011; Dąbrowski et al., 2005). Thus it is promising to mitigate the toxicity of pyrolytic bio-oil via activated carbon absorption of part of the inhibitory compounds. Activated carbon has been applied to detoxify pyrolytic substrates (Lian et al., 2010; Lian et al., 2012b; Liang et al., 2013) along with some other treatment methods. Activated biochar, the solid product of fast pyrolysis, instead of activated carbon, can be used as the adsorbent to detoxify pyrolytic substrates, which decrease the atmospheric carbon levels.

Acid hydrolysis: Acid hydrolysis can be used to convert levoglucosan, the most common pyrolytic sugar, into glucose (Bennett et al., 2009; Wang et al., 2012). Bennett et al. (Bennett et al., 2009) quantified the relationship between glucose yield and the effect of time, temperature and sulphuric acid concentration. Wang et al. (2012) established an economic analysis for the fermentation of pyrolytic sugar, detoxified through both water extraction and acid hydrolysis,

and the cost of ethanol in a large scale plant is approximately $14 per gallon, which makes the whole processing not feasible.

Overliming: Overliming is established to precipitate inhibitors via adding $Ca(OH)_2$. It is widely used for detoxification of cellulose hydrolysates (Huang et al., 2009; Okuda et al., 2008; Wang et al., 2012), hemicellulose hydrolysates (Amartey & Jeffries, 1996; Martinez et al., 2000; Mohagheghi et al., 2006), lignocellulosic hydrolysates (Millati et al., 2002; Palmqvist & Hahn-Hägerdal, 2000a; Ranatunga et al., 2000) and pyrolytic bio-oil (Chi et al., 2013; Islam et al., 2015; Zhao et al., 2015) for following microbial fermentation. The significant improvement of substrates fermentability is commonly attributed to the neutralization of carboxylic acids and removal of furans, phenolics and other inhibitors as well.

Oxidative treatment: Remarkable unsaturated bonds, like double bands (-C=C-), aldehyde group (-CHO), ketone group (-C=O) and hydroxyl group (R-OH, Ar-OH) can be found from structures of identified compounds, which implies the possibility of bio-oil detoxification via oxidation. For example, phenolic compounds are present in fast pyrolysis bio-oil from decomposition of lignocellulosic biomass. They were implicated as causing compounds of solution taste and odor (Chrostowski et al., 1983) as well as the inhibition on microorganism fermentation (Liang et al., 2013; Palmqvist & Hahn-Hägerdal, 2000b; Zhao et al., 2013b). Phenolic compounds were as well known for being vulnerable under oxidative reagents. For example, ozone is reported to efficiently oxidize aqueous phenolics through hydroxylation, degradation and oxidative coupling pathways (Chrostowski et al., 1983). The toxicity of SF5 may come from aldehydes, ketones and phenolic compounds, and oxidative methods are promising for bio-oil detoxification.

Membrane technology: Membrane technology, driven by hydrostatic pressure, chemical potential gradient or thermal gradient, has been widely applied for biorefining and bioenergy production, particularly in common bioprocessing of biomass to ethanol (He et al., 2012). He et al. (2012) summarized the application of membrane process in inhibitor removal for detoxification of biomass hydrolysate, and the effectively removed inhibitors include acetic acid, formic acid, levulinic acid, furfural and hydroxymethylfurfural. With the advantage of high detoxification efficiency, possible inhibitor recovery, simple operation and ease of scale-up, membrane technology is promising to treat pyrolytic bio-oil.

Air stripping: Air stripping is used to convert volatile liquids, characterized by low aqueous solubility and high vapor pressure, into gasses. Wang et al. (2012) (Wang et al., 2012) established a stripping tower system to remove volatile compounds, such as acetic and formic acids, from aqueous fraction of pyrolytic bio-oil prior to pyrolytic sugar fermentation. However, due to water evaporation, air stripping cannot decrease the concentration of volatile inhibitors low enough for successful fermentation.

Combination of different methods: Bio-oil detoxification can also be realized commonly by a combination of several detoxification methods. Prosen et al. (1993) (Prosen et al., 1993) treated woody pyrolytic bio-oil through aqueous extraction, activated carbon adsorption and acid hydrolysis prior to fungi or yeast fermentation, which resulted to significant improvement on cell growth and biomass yields. Recently, Lian et al. (2010) (Lian et al., 2010) employed 1) solvent extraction (separate pyrolytic anhydrosugars from phenolics), 2) acid hydrolysis (convert anhydrosugars to glucose), 3) alkali treatment (neutralize pH), and 4) activated carbon adsorption (remove inhibitors), to detoxify pyrolytic sugar for following yeast fermentation and ethanol and lipids production.

1.2.4. Strain improvement to enhance microorganism tolerance to toxicity of pyrolytic substrates

In order to increase the fermentability of bio-oil, biocatalysts with high tolerance to the inhibitors are needed. The strong biocatalysts can be obtained through metabolic engineering technology. Due to the various types of toxic compounds contained in bio-oil, the mechanism of inhibition can hardly be investigated via rational metabolic engineering, whereas applying inhibitor-sensitive biocatalyst to metabolic evolution for high tolerance strain seems promising. In addition, the inhibition mechanism and metabolic change can be investigated via reverse engineering technology on inhibitor-resistant biocatalyst and thus the metabolic change can be implemented to other biocatalysts for required fermentation and product. In fact, metabolic evolution for strain with enhance tolerance to bio-oil based substrates has been reported for bioethanol (Chan & Duff, 2010), lipids (Liang et al., 2013) and carboxylic acid production (NAKAGAWA et al., 1984; Zhuang et al., 2001).

Metabolic evolution, or directed evolution, is widely used for enhancing biocatalysts' tolerance to inhibitory compounds (Miller et al., 2010; Miller et al., 2009a; Miller et al., 2009b). Directed evolution can be applied to synthetic biology at different levels such as protein, pathway, network, and whole cell (Cobb et al., 2012; Cobb et al., 2013). The principle of directed evolution is similar to that for Darwinian evolution process but on a much smaller scale and a much shorter time. In a typical directed evolution process, one or some types of inhibitor-sensitive biocatalysts would be cultured under selective pressure, when genomic DNA acquired randomized mutations and expressed in the biocatalysts. Via appropriate screening or selection methods, such as sub-culture under gradually increased growth pressure, mutations with particular properties to tolerant or confer the growth pressures would accumulate and become dominant in the culture. Single variant would be isolated and applied in production. The

genotype change of robust strain would be analyzed to investigate the benefit from directed evolution (Cobb et al., 2012; Cobb et al., 2013; Liang, 2012).

1.3 Fermentation of microalgae for lipids production

1.3.1. The heterotrophic alga Clamydomonas reinhardtii

The alga *Clamydomonas reinhardtii* is widely used as a model strain for various cell culture system and genetic modification study (Rochaix, 1995). Wild type of *C. reinhardtii* is oval-shaped green unicellular algae, and featured that cells can be cultured phototropically (light with CO_2 as the carbon source), heterotrophically (dark with acetate as the carbon source), or mixotrophically (light with both CO_2 and acetate as carbon sources) (Rochaix, 1995). In addition, genetic tools and techniques for *C. reinhardtii* have been studied in deep and reviewed comprehensively (Mussgnug, 2015). In particular, *C. reinhardtii* cells can be used as model organism in metabolic modification for enhanced biolipid production because of its well-defined genetics (Work et al., 2010). Thus *C. reinhardtii* is a suitable platform to utilize pyrolytic acetic acid for biolipid production.

Sager and Granick (Sager & Granick, 1953) investigated the heterotrophic growth of *C. reinhardtii* with 15 mM of acetate under a series of pH. Chen et al. (Chen & Johns, 1994) reported significant substrate inhibition of *C. reinhardtii* by > 0.4 g/L acetate in heterotrophic culture, and conducted a single-stage continuous culture (1 L) with steady state cell density of 0.4 g/L and maximum cell productivity of 0.02 g/L/hr. Also, previous study showed that genetic modification can improve the lipid contents of *C. reinhardtii* from 20 wt% (Li-Beisson et al., 2015) to 25-30% (Liang et al., 2013) of dry biomass. Liang et al (Liang *et al.*, 2013) investigated the fermentability of pyrolytic acetate via *C. reinhardtii* for biolipids production, used activated

carbon to remove fermentation inhibitors in pyrolytic bio-oil and established directed evolution to obtain strains with enhanced tolerance to pyrolytic substrate.

1.3.2. Common fermentation strategies

Batch culture: Batch culture is where strain seeds and substrates, including carbon source, energy source and nutrients are added to a reactor only once before harvesting. Strength of batch culture includes low energy cost, less labor needed and less water used, low overall cost, quite simple system, easy to handle, low contamination risk. The weaknesses of batch culture is mainly low productivity. The differential equations for batch culture are listed below.

If the only product is microbial cell (Blanch, 1996), the cell growth and the substrate consumption can be written as

$$\frac{dX}{dt} = \mu X = \frac{\mu_{max} S X}{K_S + S}$$

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{\mu_{max} S X}{K_S + S}$$

If the culture forms a product partially growth and non-growth associated with growth rate, then the substrate consumption and production formation can be written as

$$\frac{dP}{dt} = \alpha \frac{\mu_{max} S X}{K_S + S} + \beta X$$

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \frac{\mu_{max} S X}{K_S + S} - \frac{1}{Y_{P/S}} (\alpha \frac{\mu_{max} S X}{K_S + S} + \beta X)$$

Fed-batch culture: Fed-batch culture is process in which one or more nutrients are supplied to the bioreactor during cultivation to compensate the depletion of the nutrient(s). The major advantage of fed-batch culture is to control the limiting substrate concentration at optimal

level and to achieve higher productivity than batch culture. However, the cell yield and product yield can be decreased if feeding flow rate is high.

If the only product is microbial cell, the cell growth and the substrate consumption can be wirten as (Blanch, 1996)

$$\frac{dX}{dt} = \mu X - (\frac{F}{V})X = \frac{\mu_{max} S X}{K_S + S} - (\frac{F}{V})X$$

$$\frac{dS}{dt} = \left(\frac{F}{V}\right)(S_0 - S) - \frac{1}{Y_{X/S}}\mu X = \left(\frac{F}{V}\right)(S_0 - S) - \frac{1}{Y_{X/S}}\frac{\mu_{max} S X}{K_S + S}$$

If the culture forms a product partially growth and non-growth associated with growth rate, then

$$\frac{dP}{dt} = \alpha\frac{\mu_{max} S X}{K_S + S} + \beta X - (\frac{F}{V})P$$

$$\frac{dS}{dt} = \left(\frac{F}{V}\right)(S_0 - S) - \frac{1}{Y_{X/S}}\mu X - \frac{1}{Y_{P/S}}\frac{dP}{dt}$$

$$= \left(\frac{F}{V}\right)(S_0 - S) - \frac{1}{Y_{X/S}}\frac{\mu_{max} S X}{K_S + S} - \frac{1}{Y_{P/S}}[\alpha\frac{\mu_{max} S X}{K_S + S} + \beta X - \left(\frac{F}{V}\right)P]$$

Continuous culture: In a continuous culture, fresh media are fed to the bioreactor while the broth is removed, the flow rates of influent and effluent are usually maintained constant to keep the reactor working volume unchanged. Continuous culture shows a way to obtain high cell/product productivity; however, the cell density is commonly low due to constant elution of the cells and/or products.

if the only product is microbial cell, the biomass and the substrate change can be written as (Blanch, 1996)

$$\frac{dXV}{dt} = F_{in}X_0 - F_{out}X + \mu XV$$

$$\frac{dSV}{dt} = F_{in}S_0 - F_{out}S - \frac{1}{Y_{X/S}}\mu XV$$

When the volumetric feed rates, into and from the vessel, $F_{in}\ and\ F_{out}$, are maintained constant and dilution rate is $D = F/V$ and $X_0 = 0$, the equations can be simplified as

$$\frac{dX}{dt} = \mu X - DX$$

$$\frac{dS}{dt} = D(S_0 - S) - \frac{1}{Y_{X/S}}\mu X$$

where F/V, or dilution rate $D = F/V$, is between 0 and μ_{max}.

In the culture where the product formation is partially growth and non-growth associated, the product formation can be written as

$$\frac{dP}{dt} = \alpha\frac{\mu_{max}SX}{K_S + S} + \beta X - DP$$

$$\frac{dS}{dt} = D(S_0 - S) - \frac{1}{Y_{X/S}}\mu X - -\frac{1}{Y_{P/S}}(\alpha\frac{\mu_{max}SX}{K_S + S} + \beta X - DP)$$

Perfusion culture: Perfusion culture refers to isolation of cells from spent medium and retaining cells in the bioreactor, with continuously feeding fresh medium to and withdrawing spend medium from the reactor. It can be operated at batch or continuous mode to improve cell density and productivity. Different techniques can be used for isolation of cells, such as i) capillary fibers and membranes to bind cells, ii) filtration systems to separate cells and media, and iii) techniques to concentrate cells via centrifuge or precipitation. The strength of batch culture with cell perfusion includes very high cell density and alleviation of metabolites inhibition (Wen & Chen, 2001). The weakness of this culture method includes no cell productivity; the separation system can be very expensive (for example, hollow fiber membrane); media with high cell density can easily clog the separation system

As to cell growth kinetics, if the microbial cells does not produce any product, is the cell growth and substrate consumption can be written as

$$\frac{dXV}{dt} = F_{in}X_0 + \mu XV$$

$$\frac{dSV}{dt} = F_{in}S_0 - F_{spent}S - \frac{1}{Y_{X/S}}\mu XV$$

When the volumetric feed rates, F_{in} and F_{spent}, into and from the vessel are maintained constant and equal F, and $X_0 = 0$, the equations can be simplified as

$$\frac{dX}{dt} = \mu X$$

$$\frac{dS}{dt} = (\frac{F}{V})(S_0 - S) - \frac{1}{Y_{X/S}}\mu X$$

If the culture forms a product partially growth and non-growth associated with growth rate, then

$$\frac{dP}{dt} = \alpha\frac{\mu_{max}SX}{K_S + S} + \beta X - (\frac{F}{V})P$$

$$\frac{dS}{dt} = (\frac{F}{V})(S_0 - S) - \frac{1}{Y_{X/S}}\mu X - -\frac{1}{Y_{P/S}}[\alpha\frac{\mu_{max}SX}{K_S + S} + \beta X - \left(\frac{F}{V}\right)P]$$

Perfusion strategy with cell bleeding: Wen and Chen (2001) have evaluated the microalgae heterotrophic growth in a combined operation of perfusion and particle cell bleeding in order to achieve both high cell density and productivity. The strength of this system includes i) optimal productivity can be obtained via adjusting parameters, ii) both high cell density and high cell/product productivity can be obtained, which means the highest maximum possible conversion rates per unit reactor volume; iii) high tolerance to toxic media when high cells density is achieved; and iv) alleviating metabolites inhibition. However, this system has some

weakness includes: i) complex system; ii) high overall cost than other systems due to high energy, labor, and water requirement; iii) expensive perfusion system such as hollow fiber membrane; and iv) concern about the efficiency and clogging of cell perfusion system.

As to differential equations, if the only product are microbial cells

$$\frac{dXV}{dt} = F_{in}X_0 - F_{bleeding}X + \mu XV$$

$$\frac{dSV}{dt} = F_{in}S_0 - F_{spent}S - F_{bleeding}S - \frac{1}{Y_{X/S}}\mu XV$$

When the volumetric feed rates, $F_{in}, F_{spent}, F_{bleeding}$, into and from the vessel are maintained constant and keep $F_{in} = F_{spent} + F_{bleeding}$, and $X_0 = 0$, the equations can be simplified as

$$\frac{dX}{dt} = \mu X - \frac{F_{bleeding}}{V}X$$

$$\frac{dS}{dt} = (\frac{F_{in}}{V})(S_0 - S) - \frac{1}{Y_{X/S}}\mu X$$

If the culture forms a product partially associated with growth and non-growth associated, then

$$\frac{dP}{dt} = \alpha\frac{\mu_{max}SX}{K_S + S} + \beta X - (\frac{F_{in}}{V})P$$

$$\frac{dS}{dt} = (\frac{F_{in}}{V})(S_0 - S) - \frac{1}{Y_{X/S}}\mu X - -\frac{1}{Y_{P/S}}[\alpha\frac{\mu_{max}SX}{K_S + S} + \beta X - \left(\frac{F_{in}}{V}\right)P]$$

Table 1-1 The composition of lignocellulose derived from various types of biomass

Lignocellulosic material	**Lignin (%)**	**Hemicellulose (%)**	**Cellulose (%)**	**Reference**
Sugar cane bagasse	20	25	42	Kim & Day (2011)
Sweet sorghum	21	27	45	Kim & Day (2011)
Hardwood	18–25	24–40	40–55	Malherbe & Cloete (2002)
Softwood	25–35	25–35	45-50	Malherbe & Cloete (2002)
Corn cobs	15	35	45	Prassad *et al.* (2007)
Corn stover	19	26	38	Zhu *et al.* (2005)
Rice straw	18	24	32.1	Prassad *et al.* (2007)
Nut shells	30–40	25–30	25–30	Abbasi & Abbasi, (2010)
Newspaper	18–30	25–40	40–55	Howard *et al.* (2003)
Grasses	10–30	25–50	25–40	Malherbe & Cloete (2002)
Wheat straw	16–21	26–32	29–35	McKendry (2002)
Banana waste	14	14.8	13.2	John *et al.* (2006)
Bagasse	23.33	16.52	54.87	Guimarães et al. (2009)
Sponge gourd fibres	15.46	17.44	66.59	Guimarães et al. (2009)

(Iqbal et al., 2013)

Table 1-2 The organic chemical composition of different lignocellulosic biomass

Type	**Chemicals**
Acids	Formic, acetic propanoic, hexanoic, benzoic, etc.
esters	Methyl formate, methyl propionate, butyrolactone, methyl n-butyrate, velerolactone, etc
Alcohols	Methanol, ethanol, 2-propene-1-ol, isobutanol, etc
Ketones	Acetone, 2-butanone, 2-butaone, 2-pentanone, 2-cyclopentanone, 2,3-pentenedione, 2-hexanone, cyclo-hyxanone, etc
Aldehydes	Formaldehyde, acetaldehyde, 2-butenal, pentanal, ethanedial, etc
phenols	Phenol, methyl substituted phenols
alkenes	2-methyl propene, dimethylcyclopentene, alpha-pinene, etc., Aromatics: benzene, toluene, xylenes, nphthalenes, phenanthrene, fluoranthrene, chrysene, etc
Nitrogen compounds	Ammonia, methylamine, pyridine, methylpyridine, etc
Furans	Furan, 2-methyl furan, 2-furanone, furfural, furfural alcohol, etc
guaiacols	2-methoxy phenol, 4-methyl guaiacol, ethyl guaiacol, eugenol, etc
Syringols	Methyl syringol, 4-ethyl syringol, propyl syringol, etc
Sugars	Levoglucosan, glucose, fructose,D-xylose, D-arabinose, etc
Miscellaneous oxygenates	Hydroxyacetaldehyde, hydroxyacetone, dimethyl acetal, acetal, methyl cyclopentenolone, etc

(Goyal et al., 2008)

Table 1-3 Previous study about biological utilization of pyrolytic

Product	Strain	Productivity	Yield	Reference	Material	Detoxification
Ethanol	*Escherichia coli* ACCC 11177	0.93 g/L·h^{-1}	0.41 g ethanol/g glucose	(Chang et al., 2015)	Cotton	Acid hydrolysis $Ba(OH)_2$ neutralization Ethyl acetate extraction
Ethanol	*Saccharomyces pastorianus*	12.12 g/L	0.50 g ethanol/g glucose	(Sukhbaatar et al., 2014)	Clear pinewood particles of 1–3 mm	Solvent extraction/Membrane filtration/Freeze-drying
Ethanol	*Escherichia coli* KO11+ *lgk*	0.65 ± 0.08 g/L	N/A	(Chi et al., 2013)	Softwood	Overliming
Ethanol	*S. cerevisiae* D SM 1334	20 g/L	0.21 g ethanol/g glucose	(Luque et al., 2014)	Pine wood	Fractionation Extraction via cold water and then ethyl acetate Acid hydrolysis $Ba(OH)_2$ neutralization
Ethanol	*Escherichia coli* KO11+ *lgk*	0.9±0.2 g L^{-1}	N/A	(Rover et al., 2014)	Hardwood	Fractionation Water washes Overliming
Ethanol	*S. cerevisiae*	N/A	0.473 g ethanol/g glucose	(Lian et al., 2010)	Acid washed poplar	Solvent extraction Hydrolysis Adsorption on activated carbon
Ethanol	S. pastorianus ATCC 2345	N/A	0.4 g ethanol/g glucose	(Wang et al., 2012)	Loblolly pine particles	Water extraction Acid hydrolysis Air stripping Activated carbon Extraction Microbial digestion Adaptive evolution
Ethanol	*Saccharomyces cerevisiae* T2	N/A	0.45 ± 0.05 g ethanol/g glucose	(Chan & Duff, 2010)	N/A	Water extraction Acid hydrolysis Overliming Extraction with organic solvents Adaptive evolution of yeast
Ethanol	*Saccharomyces cerevisiae* T2	N/A	0.45 ± 0.05 g ethanol/g glucose	(Zhuang et al., 2001)	N/A	Liquid–liquid extraction Acid hydrolysis Adaptive evolution Overliming Extraction with organic solvents Adaptive evolution of yeast
Fatty acids	*Chlamydomonas reinhardtii*	N/A	20 g/g cell dry weight	(Zhao et al., 2013)	Softwood	NaOH treatment
Fatty acids	*Chlamydomonas reinhardtii*	N/A	20 g/g cell dry weight	(Liang et al., 2013)	Softwood	Activated carbon adsorption

Table 1-3 (continued)

Lipids	oleaginous yeasts Rhodosporidium toruloides and Rhodotorula glutinis	0.78 g/L	N/A	(Lian et al., 2013)	Douglas Fir wood	Ethyl acetate extraction Rotary evaporation Activated carbon
Lipids	*Cryptococcus curvatus*	2.2 g/L	N/A	(Lian et al., 2012)	Pelletized wood	Neutralization Distillation Activated carbon adsorption
Lipids	*Cryptococcus curvatus* and *Rhodotorula glutinis*	N/A	0.167 g lipids/g sugar	(Lian et al., 2010)	acid washed poplar	Solvent extraction Hydrolysis Adsorption on activated carbon
PHA (Polyhydroxyalkanoates)	A predominance of *Betaproteobacteria* class and *Amaricoccus genus*	0.27 g/L·d^{-1}	47% cell dry weight	(Moita Fidalgo et al., 2014)	Chicken beds	Distillation
Citric acid	*Aspergillus niger (A. niger) CBX-209*	N/A	0.42 g ethanol/g glucose	(Yang et al., 2014)	Corn stover	Biological treatment: *Phanerochaete chrysosporium (P. chrysosporium)* is desirable to decrease the content of other compounds except levoglucosan
Itaconic acid	*Aspergillus terreus K26*	N/A	Theoretical yield as glucose	(NAKAGAWA et al., 1984)	Cellulose tar	N/A

Table 1-4 Mass distribution of red oak bio-oil fractions

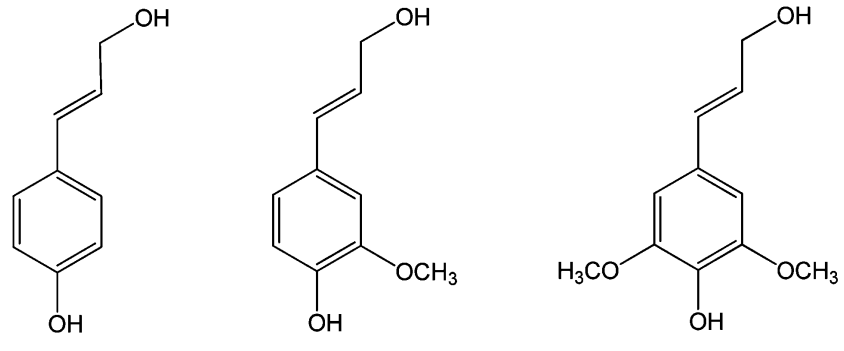
Collected from the fluid bed reactor with the fractionating bio-oil recovery system (Pollard et al., 2012)

Stage fraction (SF)	**Wt %**
SF 1	21
SF 2	26.5
SF 3	5.46
SF 4	11.1
SF 5	36

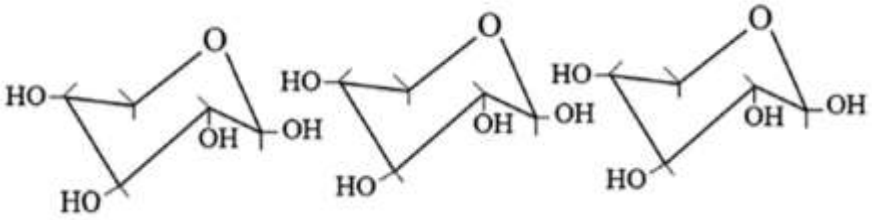
Figure 1-1 Chemical structure of lignocellulosic material.

a) Building blocks/units of lignin; b) Xylose unit of hemicellulose; and c) Cellulose (Iqbal et al., 2013)

a)



b)



c)

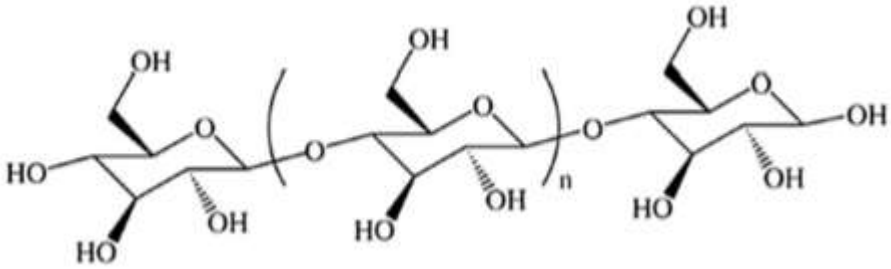
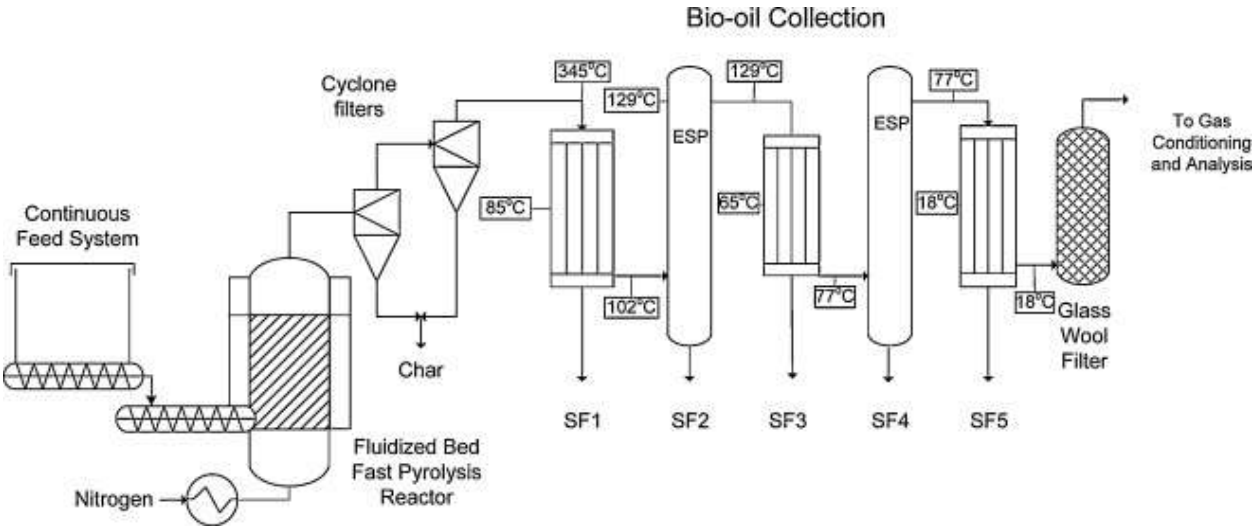


Figure 1-2 Stage fraction system in Iowa State University (Pollard et al., 2012)



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CHAPTER 2 MICROALGAE FERMENTATION OF ACETIC ACID-RICH PYROLYTIC BIO-OIL: REDUCING BIO-OIL TOXICITY BY ALKALI TREATMENT

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Abstract

Bio-oil derived from fast pyrolysis of lignocellulosic biomass contains various substrates that can be fermented to produce fuels and chemicals. The goal of this research was to utilize an acetic acid-rich fraction of bio-oil for the growth and lipid production of microalga *Chlamydomonas reinhardtii*. As various toxic compounds are contained in the bio-oil, the algal cells cannot survive in medium containing this bio-oil fraction even at a low level (0.05 wt%). An alkali-based treatment with sodium hydroxide was used to reduce the toxicity and enhance its fermentability by microalgae. It was found that treating the acetic acid-rich bio-oil fraction by adjusting pH to 10 greatly improved the algal growth. The algae can thrive in medium containing 4 wt% alkali-treated acetic acid-rich bio-oil fraction. When using a metabolic-evolved algal strain with high level of toxicity tolerance, the algae were even capable of growing in medium containing 5.5 wt% alkali-treated bio-oil fraction. At this level, 100% of the acetic acid had been replaced by bio-oil. The algal biomass grown in medium containing alkali-treated bio-oil fraction exhibited fatty acid profilessimilar to those of the control in which pure acetic acid was used, but the total fatty acid content was lower. The benefit of alkali treatment for enhancing algal growth was confirmed to be due, at least partially, to the removal of toxic compounds in bio-oil such as furfural, acetol,phenolics, and 5-hydroxymethylfurfural (HMF). Collectively, the results showed that fast pyrolysis-microalgal fermentation is a viable approach for producing lipid from

lignocellulosic biomass. Moreover, alkali-based treatment is an effective method for reducing bio-oil toxicity, and thereby, greatly enhancing the fermentability of bio-oil by algae.

Keywords: Pyrolysis, Bio-oil, Acetic acid, *Chlamydomonas reinhardtii*, Alkali treatment

2.1 Introduction

In recent years, the production of fuels from microalgae has garnered extensive interest [1]. Most of the research in this area use photoautotrophic cultures, i.e., algal cells grown in open ponds or enclosed photobioreactors using CO_2 and light as carbon and energy sources. Although this culture mode has advantages of CO_2 sequestration and avoidance of competition with arable land, the commercialization of photoautotrophic algal culture is facing challenges such as low biomass productivity and high costs for the harvesting and dewatering of the biomass [2].

Heterotrophic culture, in which organic carbon sources such as sugars or organic acids are used as carbon and energy sources, is an attractive alternative to photoautotrophic growth because it eliminates the requirement for light and offers the possibility of greatly increasing cell density and productivity [3]. A heterotrophic batch culture may be further modified for a high-cell-density culture such as a fed-batch, chemostat, or perfusion culture. The development of high-cell-density cultures for lipid production would also result in lower costs for lipid recovery.

In heterotrophic culture, the use of in-expensive carbon sources is crucial to make the process cost effective. By-products such as crude glycerol from the production of biodiesel are an ideal carbon source for producing high-value niche products such as omega-3 polyunsaturated fatty acids [4, 5]. However, for producing of commodities like fuels, the supply of substrate for algal culture needs to be large and sustainable. Bio-oil from the fast pyrolysis of lignocellulosic biomass pyrolysis represents such a substrate.

Production of fermentable sugars from lignocellulosic biomass has been studied for several decades [6]. The majority of this research has focused on the cellulosic hydrolysis, whereby the biomass is pretreated to disrupt its recalcitrant structure, then further processed with

enzymatic hydrolysis. There are several major challenges for this route, such as high costs for pretreatment and enzyme [7, 8]and under-utilization of lignin in the biomass [9].

An alternative to this biological approach to releasing sugars from lignocellulose is fast pyrolysis, which used heat to deconstruct biomass. Fast pyrolysis occurs at a high temperature (400-500°C) and heating rate producing an energy-rich liquid (bio-oil) as the major product [10]. Bio-oil contains a variety of compounds suitable as fermentation substrates including levoglucosan and acetic acid [11-15]. Compared to enzymatic hydrolysis to produce fermentable substrates, fast pyrolysis has several advantages including feedstock flexibility and the conversion of lignin into useful co-product.

Recently, researchers at Iowa State University have developed a unique system to fractionate bio-oil into five stage fractions (SF) with distinctive physical and chemical characteristics [16]. The first stage fraction (SF1) concentrates the anhydro-sugar levoglucosan, which has been used as substrate for *E. coli* fermentation to ethanol [11, 12]; while the last stage fraction (SF5) concentrates acetic acid and is suitable for the heterotrophic growth of the microalga *Chlamydomonas reinhardtii*as for lipid production [17, 18]. While using these so-called pyrolytic substrates as fermentation substrates is very appealing, one major challenge is that the bio-oil contains fermentation inhibitors such as furfural, furans and phenolic compounds [11]. Furthermore, due to the extreme chemical complexity of bio-oil, those inhibitory compounds are difficult to identify, complicating their removal. To address this limitation, various methods such as activated carbon adsorption [19] have been used to reduce the toxicity by eliminating those inhibitory compounds. Another approach is to increase the microorganism tolerance to those toxicity compounds through metabolic evolution of the strain [19].

The phenomenon of toxicity has also been observed for hydrolysates derived from acid and enzymatic hydrolysis of lignocellulosic biomass. Various methods have been studied for reducing this toxicity [20]. Among those methods, alkali treatment has proved effective, particularly for detoxifying the acid-treated hydrolysate [21]. The aim of this work is to evaluate the effectiveness of alkali treatment for reducing the toxicity of fermentation substrates derived from bio-oil for the production of algae-based lipids.

2.2 Materials and methods

Preparation and storage of acetic-acid-rich bio-oil stage fraction

The bio-oil used in this work was derived from fast pyrolysis of mixed softwood. The acetic acid-rich stage fraction of the bio-oil (SF5) was prepared in the fractionating bio-oil recovery system described in a previous publication [16], and stored in 1-L Nalgene HDPE bottles at 4^{o}C. The samples were mixed by hand shaking prior to use.

Microorganism, medium, and seed culture

The microalga *C. reinhardtii* ST21 was provided by Dr. Martin Spalding at Iowa State University. The strain was maintained on an agar slant at 4^{o}C under 12/12 dark/light cycle, and transferred to 250-mL Erlenmeyer flasks containing 50 mL tris-acetate-phosphate (TAP) medium with 1 mL/L acetic acid [22]for preparation of the seed culture. The pH of the medium was adjusted to 7 prior to autoclaving at 121^{o}C for 15 minutes. The flasks were placed in an orbital shaker (200 rpm) at 25^{o}C. Although cells can grow in complete darkness, the addition of light promotes cell growth, so continuous illumination at 110-120 μmol $s^{-1}m^{-2}$ was used.

Previous research has developed a metabolic evolution approach for enhancing the toxicity tolerance of algae cells through incremental increases in SF5 concentrations in the medium [19]. The evolved strain, here designated as *C. reinhardtii* YL01, has been shown

greatly improved tolerance to bio-oil toxins. In this work, both strains (ST21 and YL01) were used in the evaluation of the effectiveness of alkali treatment of SF5 on algal growth.

Alkali treatment of SF5

SF5 (100 mL) was placed into 150-mL beakers and adjusted to varying pH levels (4.0, 6.0, 8.0 and 10.0) with sodium hydroxide (solid pellets), per experimental set-up. The pH-adjusted solutions were then centrifuged in 50-mL centrifuge tubes at 750 *g* for 5 minutes. The supernatant was transferred to fresh tubes and stored at 4^{o}C prior to being used as acetate substrate for algal culture.

Microalgae culture on alkali-treated SF5

Microalgae were cultured in 24-well multiwell plates. Each well added 1 mL TAP medium containing alkali-treated SF5. Previous research in our laboratory has shown that optimal acetic acid concentration for algal growth in the TAP medium was 4 mL/L (data not shown). Therefore, the acetic-acid-rich SF5 was added to the medium in such a way to bring the total acetic acid concentration to 4 mL/L. The concentration of SF5 in medium was based on wt%. After SF5 addition, the TAP medium was adjusted topH7.0 prior to filtrationthrougha0.45-μm membrane filter. Each well was then inoculated with 0.1 mL algae seed. The plates were placed on an orbital shaker (130 rpm) at 25^{o}C with continuous illumination at 110-120 μmol $s^{-1}m^{-2}$. Algal growth was monitored by measuring the optical density (OD) of the cultureineach well using a BioTek EL×800 microplate reader (Winooski, VT) at 730 nm. The OD of a control well containing cell-free TAP medium (with SF5 addition) was monitored to provide a calibration for a baseline OD reading. Three replicates were made for each culture condition to provide data for statistical analysis.

Analyses

The water content of SF5 was measured as described previously[19]. The chemical compositions of raw and alkali-treated SF5 were identified by gas chromatography/mass spectroscopy using the method published by Pollard et al. [16], and quantified by gas chromatography equipped with a flame ionization detector, as described previously [19]. Formic acid and acetic acid in the SF5 were analyzed by a Dionex ion chromatography ICS 5000 system (Sunnyvale, CA) with a DionexIonPac® ICE-AS1 column (4×250 mm) [19]. The concentration of total phenolic compounds was measured by a colorimetric method as described previously [23]. As one of the most important phenols identified by GC/MS, vanillin was chosen as the calibration standard.

2.3 Results and discussion

Characterization of SF5

As shown in Table 2-1, SF5 contained 63.1%water. Most of the chemicals commonly found in lignocellulosic hydrolysates derived from the pretreatment-hydrolysis process, such as organic acids, methanol, furfural, acetol, HMF, and phenolics, were also found in SF5. About 20% of the compounds in SF5 could not be identifiable by GC/MS, indicating the extreme complexity of bio-oil.

The high acetic acid content of SF5 led us to investigate the feasibility of using this stream as a substrate for the microalga *C. reinhardtii*, which is capable of using acetic acid as a carbon source for heterotrophic culture [24, 25]. *C. reinhardtii* has also been widely studied as a model strain for lipid fuel production due to its fast growth rate and readiness for genetic manipulation [17, 18]. However, since SF5 also contains a variety of known and unknown compounds (Table 2-1) that may inhibit the growth of *C. reinhardtii,* therefore,the cell growth

performance of *C. reinhardtii* with SF5 as the acetic acid source needed to be tested. Both the original strain (ST21) and the metabolic-evolved strain (YL01) [19] were tested for SF5 fermentability.

Feasibility of algal growth on raw SF5

Figure 2-1A shows the cell growth of the strain ST21 on TAP media containing varying levels of SF5. At 0.03% and 0.05% of SF5 loading, the algae reached a maximum cell density similar to that of the control, although they had a longer lag phase and lower growth rate than the control. When 0.1% SF5was added to the medium, however, the algal growth was completely inhibited after 4-5 days. Figure 2-1B shows the cell growth of the strain YL01 in media with varying levels of SF5. As this strain has been adapted to the toxicity of SF5 through metabolic evolution [19], it can grow at SF5 levels up to 0.2% without significant inhibition, although cell growth was mildly inhibited at 0.5% SF5 (Figure 2-1B).

The above results indicate that the bio-oil fraction SF5 had strong inhibitory effects on algal growth, for both cell density and cell growth rate. Even the strain YL01 that had been modified through vigorous metabolic evolution was also inhibited at certain levels of SF5, although overall growth performance of this strain was much better than that of ST21. To better utilize SF5 as an effective acetic acid source, an alkali treatment was developed for removing the inhibitory compounds and the effects of this treatment on algal growth improvement was studied.

Effects of elevated pH treatment of SF5 on algal growth

The effects of alkali treatment of SF5 on algal growth were studied by adjusting the pH of SF5 solution to 4.0, 6.0, 8.0, and 10.0, and growing the algae in medium supplemented with the alkali-treated SF5. The algal growth in medium containing raw SF5 (with original pH 2) was designated as control. At each pH treatment level, the concentration of SF5 was incrementally

increased until it reached a “threshold” level at which the cell growth was completely inhibited with no viable cells existing after 2-3 days of incubation. As shown in Figure 2-2, at pH 2, 4, and 6, the cells grew in medium containing up to 0.05% SF5, and no cell growth occurs when SF5 reaches 0.1%. When SF5 pH was adjusted to 8, however, the cells survived at the 0.1% SF5 level, indicating that higher pH greatly reduces the toxicity of the SF5. The benefit of alkali treatment of SF5 was more appreciable when pH was increased to 10. At this pH level, algae cells were able to grow in medium containing up to 4% SF5 (Figure 2-2).

Figure 2-2 also indicates that at low pH levels (pH= 2, 4, and 6), the maximum cell density remained relatively unchanged when SF5 loading increased from 0.3% to 0.5% (Figure 2-2A). The inhibition of cell growth by SF5 was mainly evidenced in cell growth productivity and specific cell growth rate (Figures 2-2B and 2-2C). At higher pH levels (pH=8 and 10), increasing SF5 loading reduced maximum cell density (Figure 2-2A) along with cell growth productivity and specific cell growth rate (Figures 2-2B and 2-2C).

Improved algal growth on SF5 treated with alkali at pH 10

Figure 2-2 indicates that a significant improvement of cell growth occurred when SF5 was treated by increasing its pH level. In particular, when SF5 was adjusted to pH 10, a value commonly used in the alkali treatment for lignocellulosic hydrolysates [21], the algal cells were able to survive in medium containing up to 4% SF5. Based on the acetic acid content in SF5 (Table 2-1), this represents SF5 providing 75% of the acetic acid found in the growth medium (4 mL/L), the highest replacement achieved for *C. reinhardtii* ST21. To further increase the amount of acetic acid provided by SF5, we grew the strain C. *reinhardtii* YL01, which has been developed with higher tolerance for bio-oil toxicity [19], in medium containing 5.5% of alkali-treated SF5 (pH 10). This represents SF5 providing 100% of the acetic acid in the TAP medium.

Figure 2-3 shows a comparison of cell growth performance of ST21 and YL01 strains at varying SF5 levels. The strain *C. reinhardtii* ST21 can grow in medium containing 1% - 4% of SF5, although maximum cell density and cell growth rate decrease with increasing SF5 level. However, this strain could not survive in medium increased to 5.5%. The strain YL01 shows a much better growth performance than ST21, with similar growth being observed in the medium containing 4% and 5.5% of SF5.

The fatty acid composition of the two algal strains growing in medium with the highest-possible SF5 levels were further analyzed in an effort to evaluate the potential for producing lipid-based biofuel from bio-oil. The fatty acid profile of the strain ST21 growing in SF5-free medium was also evaluated as a control. As shown in Table 2-2, the control culture had higher total fatty acid (TFA) content than the SF5-containing cultures, indicating that the inhibitory effects of SF5 on the growth of C. *reinhardtii* extend beyond growth to impact lipid synthesis as well. Such a low fatty acid content in the SF5-containing medium was also reported in previous study [19], which may be related to certain bio-oil compounds such as acetol. It has been reported that acetol may promote the lipid synthesis although it inhibited growth of the yeast *Lipomycesstarkeyi*[15]. Alkali-treatment removes those lipid-synthesis promoters, which eventually resulted in lower lipid content.

Table 2-2 also shows that the TFA content and fatty acid profile of the two strains, ST21 and YL01, were similar, with palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (18:2), and α-linolenic acid (C18:3) constituting the major fatty acids. The metabolically-evolved strains possess fatty acid synthesis pathways similar to those of the un-evolved strain.

Collectively, the results shown in Figures 2-2 and 2-3 demonstrate that alkali treatment is an effective method for reducing the toxicity and enhancing the fermentability of the bio-oil

fraction SF5. In the following section, the mechanism for alkali detoxification of bio-oil is investigated.

The mechanism of the alkali treatment for detoxification of SF5 and enhanced algal growth

Figure 2-4 shows the concentrations of major compounds contained in SF5 after alkali treatment. The results demonstrate that the compounds respond differently to alkali treatment. For example, alkali treatment didnot significantly (P>0.05) change the levels of acetic acid, formic acid, and methanol in SF5 (Figures 2-4A-4C). The concentrations of furfural and acetol leveled off for pH in the range of 2-8, but dropped significantly (P>0.05) when pH increased to 10 (Figures 2-4D-4E). The concentration of phenolics decreased gradually with pH increasing from 2-10 (Figure 2-4F), and HMF was almost entirely removed at every pH level (Figure 2-4G).

The above results indicate that alkali treatment at pH 10 most effectively removed inhibitory compounds like furfural, acetol, phenolics, and HMF. This may explain why this pH level resulted in the highest improvement in the algal growth shown in Figures 2-2 and 2-3.To further confirm this hypothesis, an additional algal culture experiment was performed. Each potential inhibitor was added back to the culture medium (containing 1% and 4% alkali-treated SF5 at pH 10) and algal growth performance was then evaluated. A mix of these four compounds was also added to the culture in order to test their combined effect on algal growth.

As shown in Figure 2-5, when SF5 concentration was at 1%, addition of HMF, furfural, phenolics, acetol, and their mixture, all reduced the maximum cell density, cell productivity, and specific growth rate although the cells could still survive. Each of the four individual compounds effected similar degrees of inhibition; the mix of the four compounds resulted in a more pronounced decrease of cell growth, indicating the combined inhibitory effect. When the algal cells were incubated in medium containing 4% alkali-treated SF5, further addition of those four

compounds completely inhibited cell growth, with no viable cells observable after 2-3 days of incubation. The 4% SF5 level had already created a more inhibitory condition for algal growth than was present at the 1% SF5 level. Thus, even a slight addition of individual inhibitors would intensify the inhibitory effects and lead to no growth of algae cells. Collectively considering the data in Figures 2-2, 2-4, and 2-5, it is clear that the improvement of algal fermentability upon media containing alkali-treating SF5 (at pH 10) is mainly due to the removal of HMF, furfural, phenolics, and acetolfrom the raw bio-oil fraction SF5.

2.4 Conclusions

In summary, this study demonstrated that alkali treatment, particularly at pH 10, is a feasible and effective method for detoxification of the acetic acid-rich fraction of bio-oil for improved growth of the microalga *C. reinhardtii.* Detoxification was mainly due to removal of HMF, furfural, phenolics, and acetol from the bio-oil fraction. As a result of this detoxification, it was possible to grow the metabolically evolved strain *C. reinhardtii* YL01on media in which the acetic acid was completely derived from the bio-oil fraction. This result suggests a new pathway for fermentative production of biofuels and biobased chemicals through pyrolytic substrates.

2.5 Acknowledgement

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Table 2-1 Chemical characterization of SF5

Chemical compounds	wt%
Water	63.12±1.00
Acetic acid	7.53±0.17
Formic acid	1.22±0.06
Methanol	1.49±1.42
Furfural	0.20±0.02
Acetol	5.06±0.18
5-Hydroxymethylfurfural (HMF)	0.28±0.09
Total phenolics	2.09±0.02
Unknown compounds	21.72±1.03
Mass closure	100

Data are means of three replicates ± standard deviations.

Table 2-2 Fatty acid composition change under different levels of alkali-treated SF5

Fatty acid (%TFA)	ST21 Control	ST21 4% SF5	YL01 5.5% SF5
16:0	30.02±0.13	30.97	31.43±0.93
18:0	3.85±0.11	2.03	3.45±0.40
18:1 (trans)	10.74±0.20	9.93	11.10±1.66
18:1 (cis)	9.82±0.57	7.65	4.82±0.27
18:2	20.50±0.49	13.07	11.45±0.81
18:3	18.99±1.06	28.33	29.13±3.91
20:0	6.08±0.19	8.02	8.61±0.15
TFA (%DW)	20.01±0.55	10.37	9.82±0.54

Data are presented as means of three replicates ± standard deviations.

Fatty acid (%TFA)	ST21 Control	ST21 4% SF5	YL01 5.5% SF5
16:0	30.02±0.13	31.00±0.31	20.63±1.73
18:0	3.85±0.11	3.06±0.02	4.31±0.10
18:1 (trans)	10.74±0.20	8.37±0.09	5.46±0.05
18:1 (cis)	9.82±0.57	9.65±0.31	20.29±1.24
18:2	20.50±0.49	18.94±0.18	23.65±2.54
18:3	18.99±1.06	23.23±0.67	19.51±1.15
20:0	6.08±0.19	5.76±0.12	6.17±0.86
TFA (%DW)	20.01±0.55	9.75±0.32	11.74±1.00

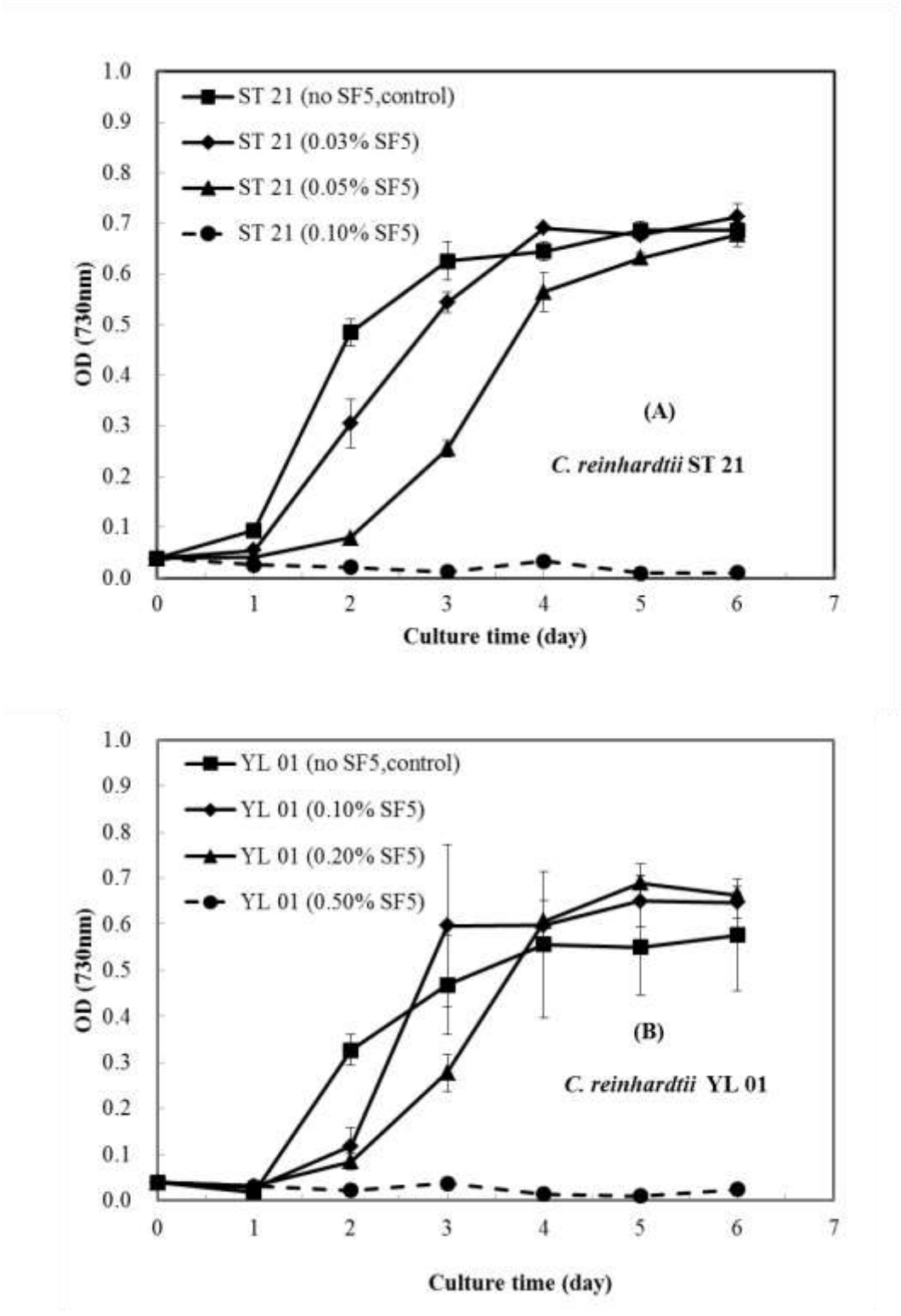

Figure 2-1 Growth of different strain under different level of SF5
C.reinhardtii strain ST21 (A) and strain YL01 (B) in TAP medium containing different level of SF5. Data are means of three replicates and error bars show standard deviations.

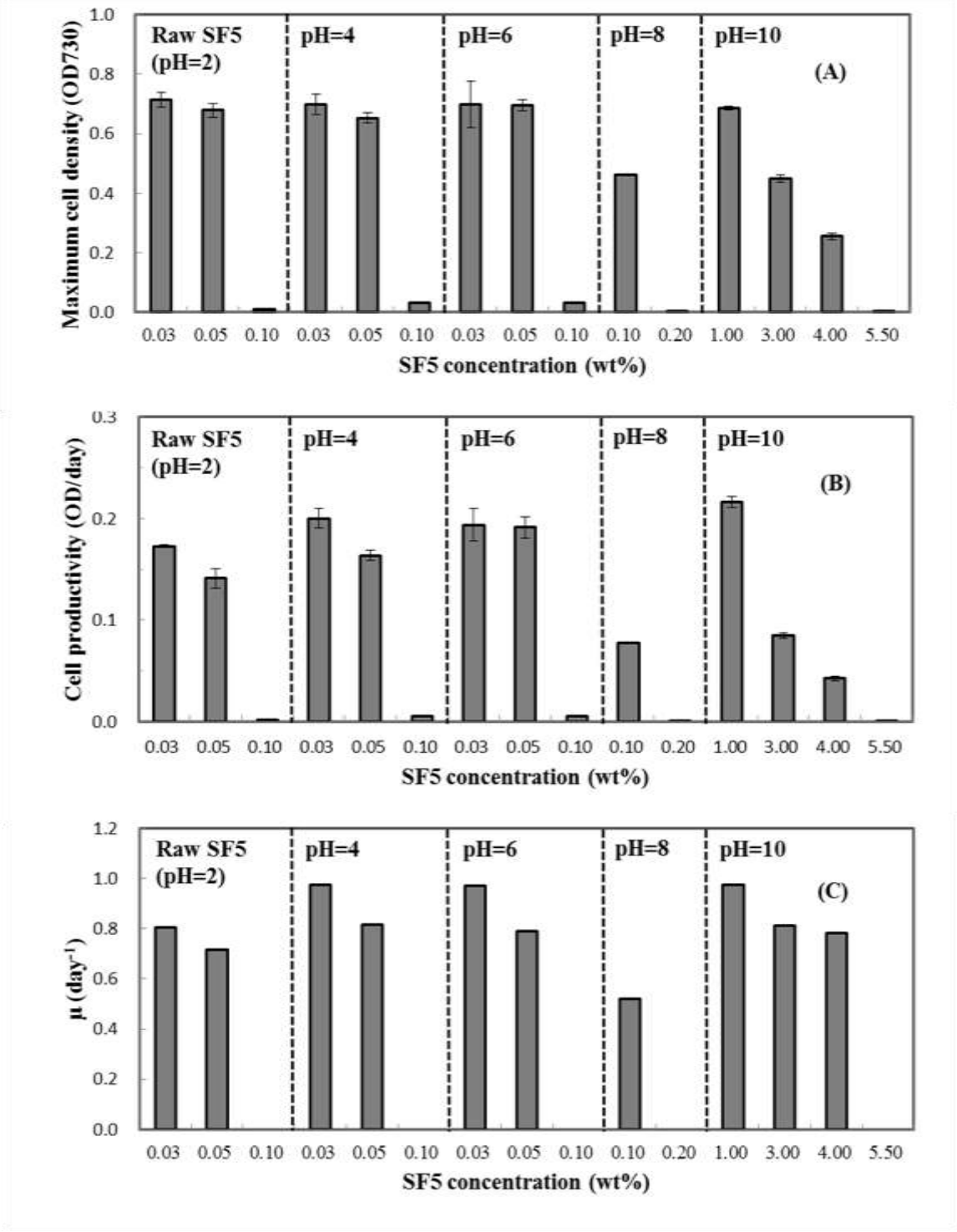

Figure 2-2 Algae growth on medium containing different levels of treated SF5
Strain: *C. reinhardtii* strain ST21 (A): maximum cell density; (B): biomass productivity; (C): specific growth rate (μ). Data are means of three replicates and error bars show standard deviations.

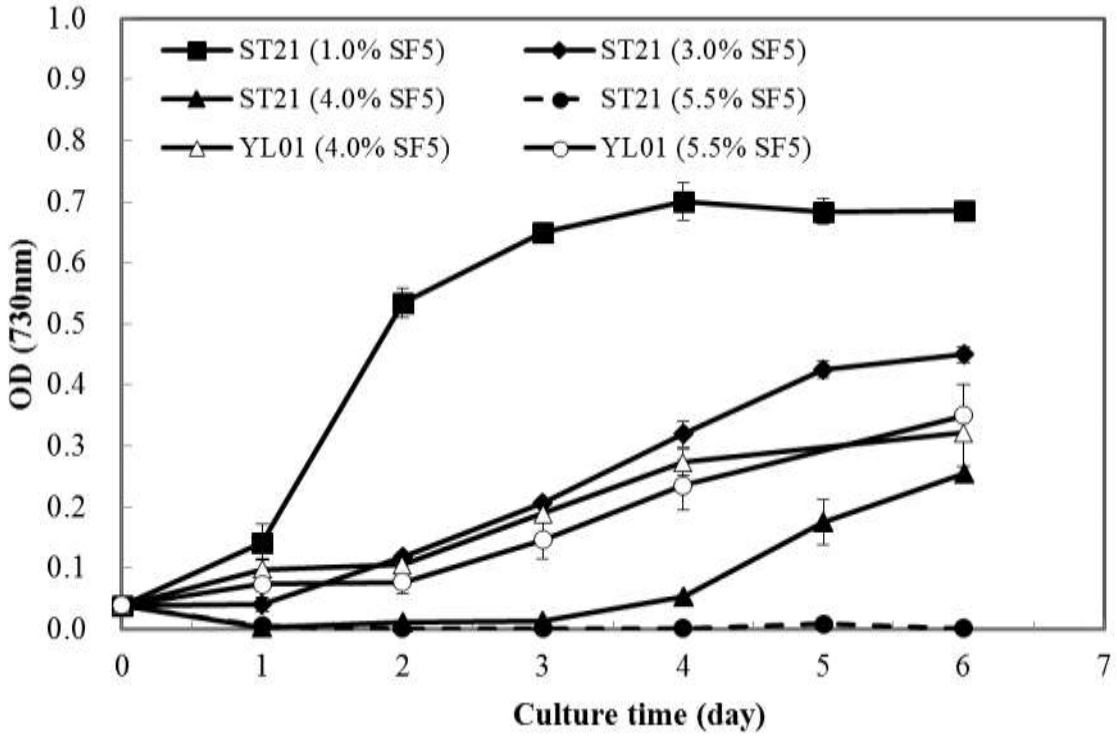

Figure 2-3 Different strain growth under different levels of treated SF5
Data are means of three replicates, and error bars show standard deviations.

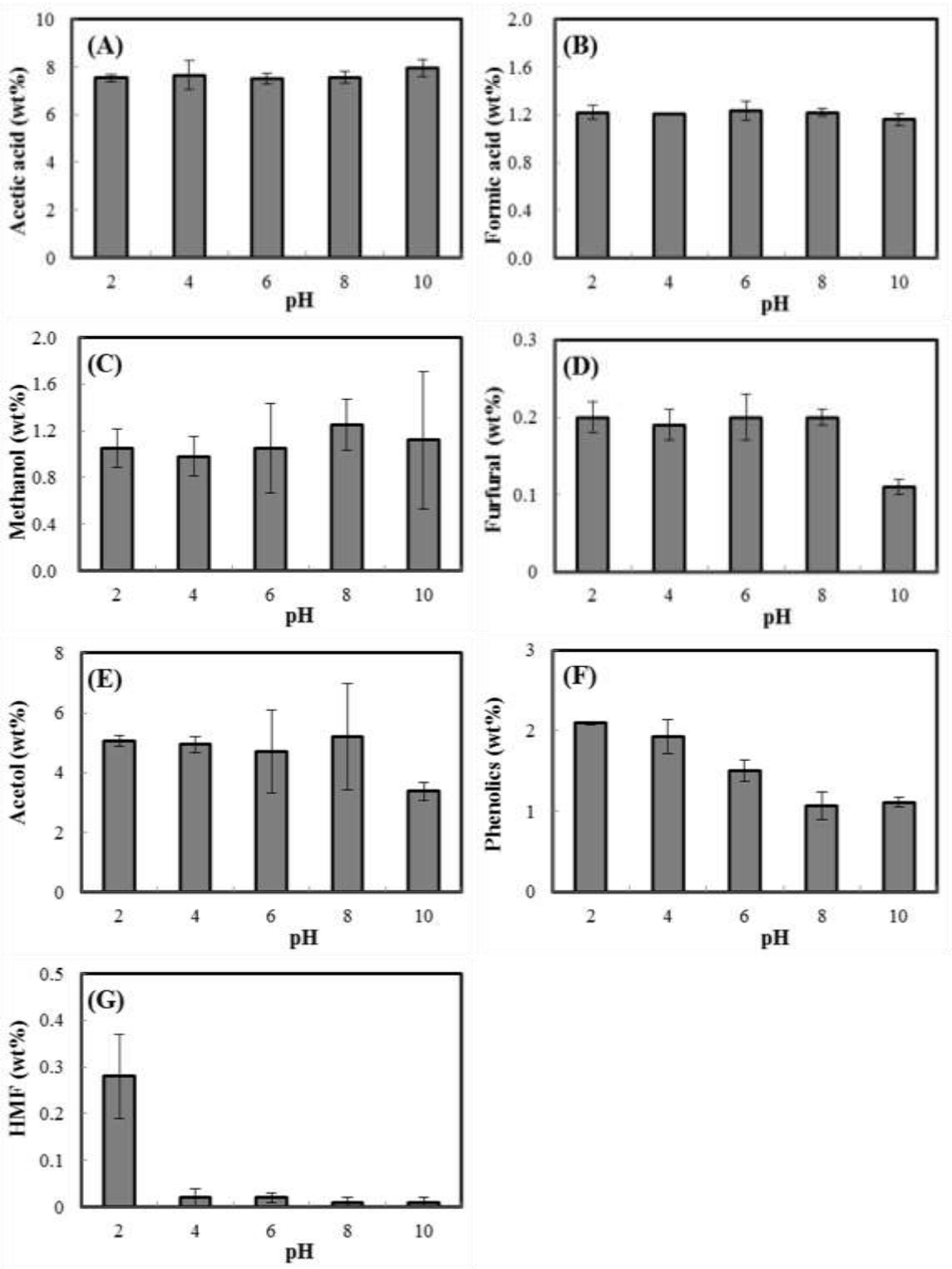

Figure 2-4 Concentration of various compounds in SF5 after treated with alkali at pH 10. Data are means of three replicates and error bars show standard deviations.

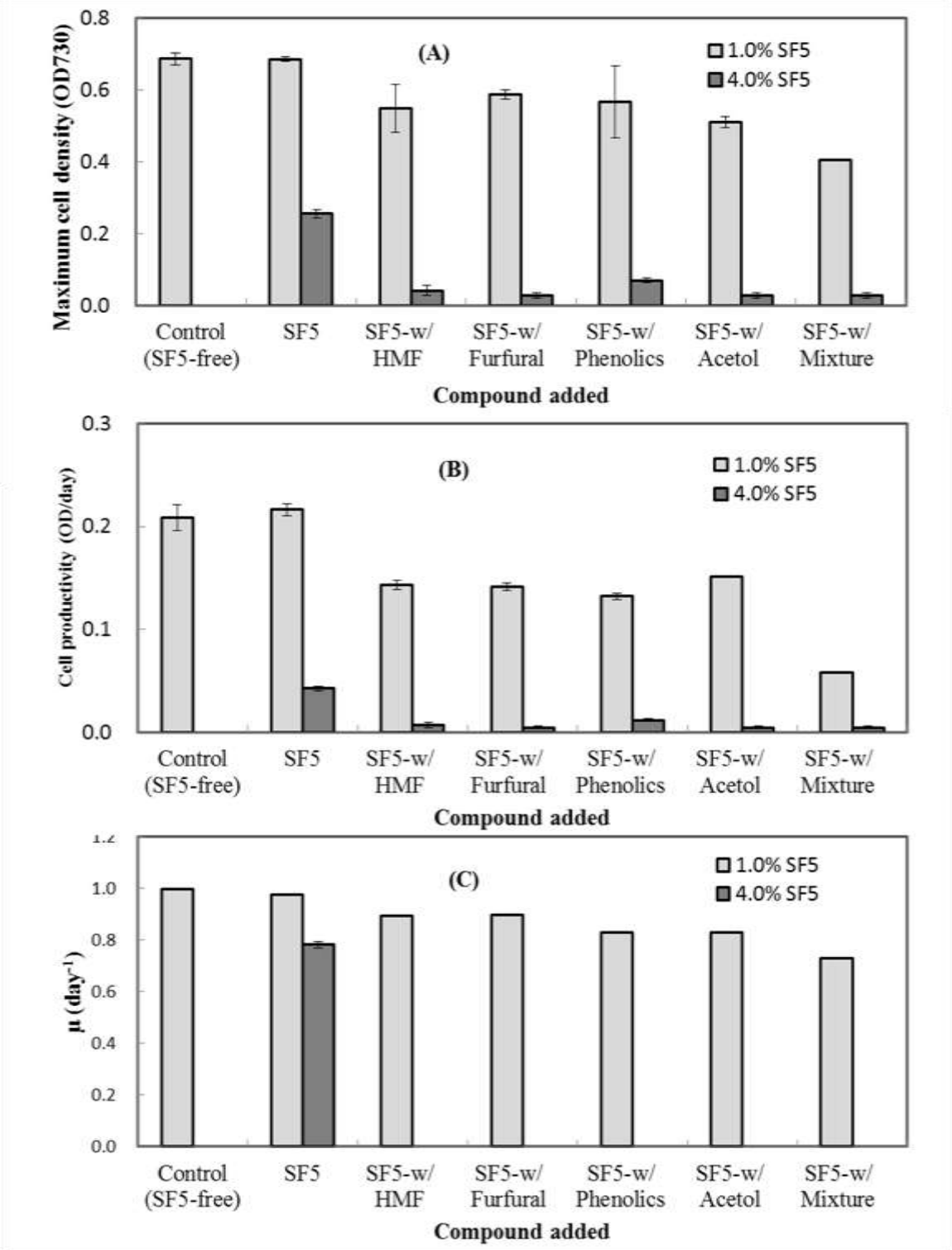

Figure 2-5 Algal growth with treated SF5 and different contaminants
The SF5 level in the medium was 1% and 4%, respectively.
(A): maximum cell density; (B): biomass productivity; (C): specific growth rate (μ). Data are means of three replicates and error bars show standard deviations.

CHAPTER 3 ALKALINE TREATMENT FOR DETOXIFICATION OF ACETIC ACID-RICH PYROLYTIC BIO-OIL FOR MICROALGAE FERMENTATION: EFFECTS OF ALKALINE SPECIES AND THE DETOXIFICATION MECHANISMS

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Abstract

Bio-oil derived from Pyrolysis of lignocellulosic biomass contains appreciable amounts acetic acid, which can be used as substrate for growing microlagae *Chlamydomonas reinhardtii*. However, the toxic compounds in the bio-oil inhibit the cell growth. This work is to develop alkaline treatment methods to reduce the toxicity and improve fermentability of acetic acid rich bio-oil. When growing in raw bio-oil without any detoxification treatment, the algae can only tolerate up to 0.1 wt% of bio-oil. Treatment with KOH, NaOH and $Ca(OH)_2$ significantly reduced the toxicity and consequently improved the fermentability of bio-oil. The bio-oil tolerant level by microalgae depended on the alkali species used. Among the three alkali species, $Ca(OH)_2$ proved the most effective detoxification reagent. Inhibitory compounds such as furans, phenols, ketones, aldehydes, ethers, esters, alcohols were removed by $Ca(OH)_2$ treatment through precipitation. The detoxification mechanisms by the $Ca(OH)_2$ -based treatment were also explored. The synergistic effect of alkaline pH, high temperature, and presence of Ca^{2+} played an important role for the precipitation of those compounds, and the consequent detoxification. Collectively, the results shows alkali, particularly $Ca(OH)_2$-based, treatment is an effective for reducing the toxicity of the pyrolysis derived bio-oil as fermentative substrate for microalgae

growth. The microalgae can tolerant $Ca(OH)_2$-treated bio-oil up to 5.5 wt%, which was 55 times higher than algal tolerance level of untreated bio-oil.

Keywords: Acetic acid, Alkali treatment, Lignocellulosic biomass, Fermentation, Microalgae, Pyrolysis

3.1 Introduction

Producing fuels and chemicals from lignocellulosic biomass is often achieved through biochemical pathways, which are commonly composed of three steps: pretreatment of biomass to break down its recalcitrant structure; enzymatic hydrolysis of pretreated biomass into reducing sugars; and fermentation of the sugars into various desired products [1]. This process is limited by several technical and economic barriers such as the high costs for pretreatment and enzymes, lack of robust microbes capable of fermenting mixed sugars (hexose and pentose), and under-utilization of lignin compounds in the biomass [2,3,4].

Thermochemically-based fast pyrolysis is another method converting lignocellulosic biomass into fuels. Fast pyrolysis is the thermal decomposition of biomass in the absence of oxygen, the process can convert biomass into an energy rich liquid (bio-oil), a flammable gas mix (syngas) and a carbon- and nutrient-rich solid (biochar) [5]. Raw bio-oil is an extremely complex mix of chemical compounds. The phenolic oligomers contained in bio-oil can be upgraded into hydrocarbon, which can be further refined into drop-in fuels by using existing petroleum refining technology and infrastructure [6], while other compounds such as levoglucosan and acetic acid, can also be used as fermentative substrates to produce various fuels and chemicals [7,8,9,10,11].

To simplify the bio-oil composition and facilitate further bio-oil refinery, a fractionation system has been developed in Iowa State University to separate raw bio-oil into different stage fractions (SFs) with distinct chemical and physical properties [12]. For example, the stage fraction 1 (SF1) contains the majority of levoglucosan and phenolic oligomers; while stage fraction 5 (SF5) contains the majority of water and acetic acid [12]. The acetic acid contained in the bio-oil is often undesirable due to its high corrosiveness and low heating value [13].

Researchers have attempted to use bio-oil derived acetic acid as road deicers [14]. As a fermentative substrate, the bio-oil derived acetic acid has also been used for microorganism fermentation. For example, Lian et al. (2012) explored yeast fermentation using pyrolytic acetic acid. Our research team also reported the use of acetic acid contained in bio-oil fraction SF5 for the growth of microalga *Chlamydomonas reinhardtii* [10,11]. The purpose of using *C. reinhardtii* as model organism in these studies is that the algal species is amenable for genetic manipulations [15,16,17], therefore, it is possible to elucidate the genomic response of the algal cells to this unique substrate. Such an underlying mechanism can then be used as guidance for developing genetic manipulation strategies for other microorganisms to improve the utilization efficiency of the pyrolysis-derived acetic acid.

Use of acetic acid rich bio-oil fraction (SF5) as pyrolytic substrates for microorganism growth, however, still faces a major challenge; i.e., the inhibition of cell growth by the toxic compounds contained in the bio-oil [10,11,18]. Some identified compounds in bio-oil such as phenols and furfural have proven inhibition for microorganism growth, while other compounds are difficult to identify and may also be inhibitory to microorganism growth.

In the near term, the practical approach for efficient utilization of SF5 is to develop various treatment methods for reducing its toxicity. Our previous research has shown that various treatment methods such as activated carbon adsorption [10] and sodium hydroxide (NaOH) can effectively reduce the toxicity of raw SF5 for the alga *C. reinhardtii* fermentation [11]. However, development of an appropriate detoxification method should also consider the process economics. For example, treatment of SF5 by NaOH is very efficient in terms of removing toxic compounds such as furfural, phenols, acetol, and HFM [11]. From an economic production point of view, however, NaOH is a relatively expensive alkali species, and thus, will entail a significant process

cost. To develop cost effective treatment methods, it is appealing to explore less expensive alkali species while achieving the similar removing efficiency. In addition, the underlying mechanism of those alkali-based treatments for reducing the toxicity should also be explored. The main objective of this study is to evaluate the various alkaline species for reducing the toxicity while improving fermentability of SF5. The mechanism of detoxification with each alkali, particularly $Ca(OH)_2$, was also investigated.

3.2 Materials and methods

Microalgae subculture and the effects of acetic acid concentration on cell growth

Microalga *C. reinhardtii* ST21 strain was provided by Dr. Martin Spalding from Iowa State University. The strain was used due to its high lipid content and thus, the potential of being used as biofuel feedstock [10,11]. The strain was stored on an agar slant at 4^{o}C under 12/12 light/dark cycle. To prepare the seed culture, the cells were transferred to 250-mL Erlenmeyer flasks containing 50-mL tris-acetate-phosphate (TAP) medium containing 1 g/L acetic acid. Here, we approximate 1 mL pure glacial acetic acid as 1g of acetic acid. The medium pH was adjusted to 7 before autoclaving at 121^{o}C for 15 min. The flasks were placed in an orbital shaker (200 rpm) at 25^{o}C with continuous illumination at 110–120 μmol s^{-1} m^{-2}.

Initial work was performed to test the effects of initial acetic acid concentration on the microalgae growth. TAP medium containing acetic acid was adjusted to pH 7 via NaOH solution and then filtered via 0.22 μm membrane for sterilization. Cells were grown in 24-well plates. Under aseptic conditions, each well in the plate was added with 1 mL medium containing different levels of acetic acid and 0.1 ml algae seed. The plates were placed on an orbital shaker with a speed of 130 rpm. The temperature was set at 25^{o}C with continuous illumination at 110-120 μmol $s^{-1}m^{-2}$. The optical density of the culture at 730 nm (OD_{730}) were measured via a

BioTek EL×800 microplate reader (Winooski, VT) on daily basis. The OD_{730} value was then converted into biomass yield (cell dry weight concentration, g/L) through a correlation curve. All the experiment was performed in triplicates.

Preparation of raw bio-oil and acetic-acid-rich bio-oil stage fraction SF5

The pyrolysis and bio-oil fractionation system for preparing acetic acid-rich stage fraction 5 (SF5) were described previously [12]. The pyrolysis feedstock was a mix of softwood including pine, aspen, poplar, birch, and maple. The SF5 was collected in 1-L Nalgene HDPE bottles and stored at 4^{o}C for later use.

Alkaline treatment of SF5

SF5 solution was transferred to a 100-ml beaker. Different alkali species including NaOH, KOH and $Ca(OH)_2$ (in the form of dry power) were respectively added to the solution to reach pH 10. Approximately 140 g of each base was needed for treating 1 L of SF5 solution. The solution temperature increased to around 80^{o}C shortly after alkali addition (within 10 minutes). The solution was stirred with a magnetic stir bar for about 1 hour until the temperature reduced and stabilized at room temperature. The treated SF5 solution was then centrifuged at 750 g for 5 minutes to remove the precipitants. The supernatants were then neutralized to pH 7.0 with HCl solutions and stored at 4^{o}C prior to use. This stock solution contained 7.3 wt% of acetic acid.

Microalgae culture on acetic acid-rich SF5

The algal growth in acetic acid-rich SF5 solution was also performed in 24-well plates. To prepare medium containing different levels of pyrolytic acetic acid, a certain volume of alkali-treated SF5 stock solution was mixed with 0.1 mL acetic acid-free concentrated (10x) TAP salts solution. Additional reagent grade acetic acid was added to ensure the total acetic acid in the final culture medium was maintained at the same level (4 g/L). Distilled water was further

added to make the final volume of the medium to 1mL in each well. The solutions were filtered through 0.22 μm membrane before added to the well. The final medium pH was 7.0. Each well in the plate was inoculated with 0.1 ml algae seed.

Chemical compounds analysis

Water content of SF5 was measured by Karl Fischer titration (Karl Fischer MKS-500). Major chemical compounds contained in FS5 were analyzed by either a gas chromatography (for acetol, methanol, furfural, furfural alcohol and 5-hydroxymethylfurfural) or an ion chromatography (for acetic acid and formic acid). The detailed procedures for the above analyses were described previously [10].

Further identification and quantification of trace amount of compounds were conducted with an Agilent 6890 GC coupled with a Micromass® GCT mass spectrometer (Waters Corporation, Milford, MA, USA). The separation of chemicals was realized by an Agilent DB-5 non-polar capillary column (27.5m × 0.25 mm × 0.25 μm). The methods for chemical separation, measurement and analysis were reported previously [19].

The concentration of total phenolic compounds was evaluated using a colorimetric method [10]. As one of the most important phenols identified by GC-MS, vanillin was chosen as the calibration standard.

3.3 Results and discussion

Effects of acetic acid concentration on cell growth

In order to maximize use of acetic acid obtained from bio-oil as a substrate for the heterotrophic growth of *Chlamydomonas reinhardtii*, it is desirable that the addition of SF5 to TAP medium can provide sufficient (but not inhibitory) amount of acetic acid. Therefore, the initial study in this work is to evaluate the effects of acetic acid concentration on the growth the

C. reinhardtii so that the addition of SF5 to the medium can be optimized. The acetic acid concentration in a typical TAP medium was 1 g/L. In this work, the acetic acid concentration was evaluated within a wide spectrum ranging from 0.5 to 12 g/L. An autotrophic control culture without acetic acid was also performed to confirm the acetic acid was indeed used by the algae as a carbon source. However, as light illumination was applied for this control culture, all the other acetic acid-containing culture was also illuminated with the same intensity of the duration of the light so that a same baseline comparison can be achieved.

Figure 3-1A shows that the autotrophic control culture reached to the maximum cell density at day 5. When acetic acid was added to the medium, the cell growth performance was greatly improved, acetic acid ranging from 1-4 g/L resulted in a similar exponential growth pattern, but the acetic acid concentration above 4 g/L resulted in cell growth inhibition. For example, at 6 g/L acetic acid, the cells exhibited a slower exponential phase although the maximum cell density still reached to a comparable level as 1-4 g/L acetic acid cases. Acetic acid at 12 g/L completely inhibited the cells growth. Figure 3-1B shows the time course of acetic acid consumption. Acetic acid was completely consumed within the range of 0.5-4 g/L of initial acetic acid, indicating the acetic acid was the limiting substrates. When initial acetic acid concentration exceeded 4 g/L, however, significant amount of residual acetic acid was observed at the end of culture.

The cell growth kinetics in terms of specific growth rate and maximum cell density was summarized in Figure 3-2. It clearly shows that 4 g/L is the optimal concentration of acetic acid for the growth *C. reinhardtii* in TAP medium. In the following work, 4 g/L was chosen as the acetic acid concentration in the culture medium.

Effects of alkaline treatment on the reduction of major inhibitory compounds in SF5 and the subsequent improvement of fermentability

Multiple compounds contained in SF5, such as acetol, furfural, phenolic compounds, and 5-hydroxymethylfurfural (HMF), have been proven to be inhibitory to the growth of *C. reinhardtii* [10,11]. The inhibition was exhibited by either the individual compound or the combined effects of different compounds [11]. Based on the previous finding that treatment of SF5 by sodium hydroxide reduced the toxicity of raw SF5 and enhanced its fermentability for *C. reinhardtii* [11]. we expanded the scope of work to the treatment of SF5 by different alkaline species (NaOH, KOH, and $Ca(OH)_2$).

To evaluate the effects of alkali treatment on the reduction of toxic compounds contained in SF5, the concentrations of major compounds in SF5 including acetol, phenols, furfural, formic acid, acetic acid, and methanol before and after treatment were presented. As shown in Figure 3-3, acetol, phenolics and furfural were significantly removed from SF5 with the three alkali treatments, with $Ca(OH)_2$ resulting in the most reduction of acetol and phenols. However, the alkaline treatment did not reduce the content of acetic acid, formic acid, and methanol in SF5, and even caused increases of certain compounds such as formic acid and methanol (Figure 3-3). This may be due to the decomposition of methyl esters and/or ethers [20,21]. Fortunately, as these three compounds have not shown significant inhibition to the algal growth [10,11], alkaline treatment can still be regarded as an effective way for detoxification of SF5. It should be noted that HMF in the raw SF5 was not detected after treatment.

The effect of alkali treatment on the improvement of the fermentability of SF5 was further evaluated. For each type of alkali treatment, different levels of alkali-treated-SF5 were added to the medium to determine the highest levels that algae cells can tolerate. At each SF5

level, the specific growth rate and maximum cell density were determined as the evaluation of the cell growth performance. It is worth noting that as alkali-treated SF5 contained a fixed amount of acetic acid (7.3 wt%, see Section 2.3), different levels of SF5 would contribute different levels of acetic acid in the medium. To avoid the possible cell growth fluctuation caused by different levels of acetic acid in medium, additional reagent grade acetic acid was added to the medium to ensure the total acetic acid concentration in each culture was maintained the same (4 g/L). This practice will ensure that the algal growth will only represent the effect of alkali-based detoxification on the cell growth.

As shown in Figure 3-4A, when untreated SF5 was added to the culture medium, the algal cells can only tolerate SF5 level up to 0.1wt%. The cell growth was completely inhibited at 0.2 wt% SF5. For KOH treated SF5, the cells could grow in medium with up to 2.0 wt% although the specific growth rate reduced to a certain degree at this level (Figure 3-4B). Figure 3-4C shows that SF5 treated by NaOH resulted in a better fermentability as the cells tolerated up to 4.0wt% SF5 although the specific growth rate and cell density reduced at this level. When SF5 was treated by $Ca(OH)_2$, its fermentability was further improved compared to NaOH treatment, as higher cell density and specific growth rate were achieved at 2% and 4% SF5, respectively (Figure 3-4D); the algal cells could even survive in medium containing 5.5 wt% SF5 (Figure 3-4D), at which level all the acetic acid (4 g/L) in the TAP medium was provided from SF5.

Collectively, the above results indicate that alkaline treatment of SF5 greatly improved algal fermentability (Figure 3-4). This improvement is thought to be due to the decrease in major toxic compounds in SF5 by alkaline treatment (Figure 3-3). Indeed, our previous research has confirmed that compounds in SF5 such as phenols, furfural and acetol were inhibitory for the

algal growth [11]. However, the inhibition reported in the previous studies was rather qualitative, without quantitative correlation between the cell growth and the toxic compound concentration [11]. In this work, we quantitatively correlated the maximum cell density and specific growth rate as functions of the residual concentrations of the major toxic compounds including acetol, phenols, and furfural (Figure 3-5). Here, the concentrations of residual toxic compound in the medium were obtained by considering the levels of SF5 added to the medium and the toxic compounds in the SF5 after alkaline treatment. As shown in Figure 3-4, both the maximum cell density and specific growth rate show a negative relationship with the residual concentrations of acetol, furfural, and phenols. The *P* values for the slope for each sub-figure are less than 0.05, further confirming the inhibitory effects of those compounds on the algal growth.

Comprehensive analysis of chemical compounds in SF5 before and after $Ca(OH)_2$ treatment

The results shown in Figures 3-3~3-5 show that several major compounds in SF5 inhibited the algal growth, while alkaline treatment reduced this inhibition. However, there are still many other compounds contained in SF5 not identified but may also play a role in the cell growth inhibition. Therefore, a thorough analysis of the chemical composition of SF5 before and after alkaline treatment was performed. Here, we focused on $Ca(OH)_2$-based treatment as it is the most effective method among the three alkaline treatments.

As shown in Table 3-1, the untreated SF5 contained various types of organic compounds, such as organic acids, furans, ketones, phenols, aldehydes, ethers, esters, and alcohols. Many of these organic compounds have been reported to inhibit microorganism growth. Organic acids such as formic acid, acetic acid, propionic acid and glyceric acid present in raw SF5 have been reported to lead to the excessively low pH and thus, inhibit cell growth [22,23]. Furans and ketones have also shown inhibition in various microorganism fermentation including yeast, *E.*

coli, and microalgae [24,25]. Furfural is converted to furfural alcohol via microbial transformation and thus inhibits yeast respiration [26]. Furanone can affect the bioluminescence of *E. coli* by replacing furanone-like compounds from the receptor. As a matter of fact, furanone has been widely used to inhibit the formation of *E. coli* biofilm [25]. Phenolics can damage the integrity of cell membranes, resulting in the lysis of microorganism [24], while aldehydes inhibit microorganism growth through their chemical reactivity causing plasma membrane leakage [23]. The compounds presented in Table 3-1 have also been reported to have synergistic inhibitory effects on microorganism growth. For example, furans and aldehydes can aggregate the toxicity of other compounds [23,24].

After treatment by $Ca(OH)_2$, a majority of the compounds in SF5 were partially or completely removed except acetoin and ethyl acetate (Table 3-1). Compared to the untreated SF5, treatment by $Ca(OH)_2$ resulted in a higher concentration of acetoin. Ethyl acetate did not exist in the untreated SF5 but generated during the treatment. The reason for the elevated levels of acetoin and ethyl acetate may be due to the specific chemical reactions that occurred during treatment, resulting in the production of these two compounds. Overall, the results in Table 3-1 confirms that $Ca(OH)_2$ treatment is effective in reducing several inhibitory compounds in SF5 and increasing its fermentability.

Mechanisms of improved detoxification effect by $Ca(OH)_2$-treatment

The above results show that alkaline treatment, particularly $Ca(OH)_2$ treatment, is an effective method to remove the inhibitory compounds and improve fermentability of SF5. It was noted that a significant precipitation occurred during alkaline treatment of SF5. The precipitation of the inhibitory compounds was considered the major mechanism for alkali-based detoxification [11]. Similar results were also observed in the biochemical pathway for cellulosic ethanol

production, where a significant precipitation of aromatic and aliphatic carboxylic acid were formed when dilute-acid treated hydrolysate was neutralized by $Ca(OH)_2$ [27].

Compared to NaOH- and KOH-treatment, $Ca(OH)_2$ treatment resulted in more precipitants and better SF5fermentability. Here, we hypothesize that the $Ca(OH)_2$-induced precipitation was caused not only by the high pH effect, but also by the temperature effect and Ca^{2+} effect. During the treatment, we found that the temperature of the solution increased sharply from room temperature (~ 25ºC) to 80ºC. Such as a high temperature can facilitate precipitation reactions with high activation energy requirement [28]. It has also been reported that calcium ion can react with certain inhibitory compounds to form insoluble precipitants such as the calcium half-salt, PhO-Ca-OH [29].

A set of experiments was conducted to test the above hypothesis. As shown in Table 3-2, to evaluate the pH effect, $Ca(OH)_2$ was replaced by NaOH to avoid the interference of Ca^{2+}, while temperature was maintained around room temperature (25ºC) by slowly adding a small amount of alkali and quickly dissipating the heat generated through a water batch heat exchanger. The treated SF5 solution was then used for algal culture; the maximum tolerant concentration (MTC) of SF5 by algal cells was used to as indicator to evaluate the detoxification effect. As shown in Table 3-2, the MTC value for the pH treatment greatly increased compared to the control, indicating significant role of alkaline effect during the SF5 detoxification.

The temperature effect on the detoxification of SF5 was performed by increasing SF5 solution to 80ºC without pH adjustment or Ca^{2+} inclusion. This treatment did not reduce toxicity of the SF5 as the MTC values were almost unchanged compared to the control (Table 3-2). To create the calcium effect, $CaCl_2$, instead of $Ca(OH)_2$, was added to the SF5 solution at 25ºC.

Again, no precipitation in SF5 or MTC improvement was observed (Table 3-2), indicating that Ca^{2+} ion alone does not contribute the detoxification of SF5.

The above results show either temperature or calcium alone did not contribute the detoxification. The synergistic effects of pH with temperature and/or calcium were further studied. As shown in Table 3-2, the pH-temperature treatment greatly increased the MTC value to 4.0, but the pH-calcium treatment did not improve the MTC as compared to the pH effect only. However, the synergistic effect on detoxification became more significant when all the three factors (pH, temperature, and calcium) were combined (Table 3-2), which explains why $Ca(OH)_2$-based treatment resulted in a better detoxification effect than the other two alkaline species.

Collectively, the results in Table 3-2 indicate that the pH effect is the ultimate mechanism for $Ca(OH)_2$-based detoxification for SF5, while high temperature and presence of calcium also played roles in detoxification. These factors synergistically enhanced the precipitation of inhibitory compounds in SF5. Indeed, as an effective flocculation reagent, $Ca(OH)_2$ has been used in wastewater treatment for removing suspended solid and organics. The mechanism behind this flocculation is that $Ca(OH)_2$ effectively destabilizes colloidal materials, leading to the consequent agglomeration and settlement of small particles [30]. In the biochemical conversion of lignocellulosic biomass to biofuel, $Ca(OH)_2$ has also been used as an effective reagent to reduce the toxicity of the enzymatic hydrolysates [31,32,33]. The present study reveals that $Ca(OH)_2$ is also an effective reagent for detoxification of pyrolysis-derived bio-oil fractions.

Fatty acid analyses

To further evaluate the potential for producing lipid-based biofuel, the fatty acid composition of *C. reinhardtiias* grown in medium containing 5.5% SF5 treated by $Ca(OH)_2$ were

analyzed. The fatty acid profile of the algae grown in SF5-free medium was determined as a control. As shown in Table 3-3, the percentage of total fatty acid in algal cells grown in $Ca(OH)_2$-treated SF5 was approximately half of that in the control culture, indicating that the inhibitory effects of SF5 on the growth of *C. reinhardtii* extended beyond growth to lipid synthesis as well. Such a low fatty acid content derived from the SF5-containing medium was also reported in our previous studies [10,11]. However, the fatty acid profiles of the cells grown in different media were similar, with palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and α-linolenic acid (C18:3) being the major fatty acids. In conclusion, alkaline treatment decreased the concentration of total fatty acid in *C. reinhardtii* without notably changing the fatty acid profile.

3.4 Conclusions

This work showed that alkali-, particularly $Ca(OH)_2$-, treatment can greatly reduce the toxicity and improve fermentability of SF5. When SF5 was treated by $Ca(OH)_2$, its fermentability was improved to the greatest level and algal cells could grow in medium containing up to 5.5 wt% SF5, where all the acetic acid in the medium was replaced by SF5. The detoxification effect by $Ca(OH)_2$ was due to the removal of various compounds including furans, phenols, ketones, aldehydes, ethers, esters, and alcohols. The synergistic effects of alkaline pH, high temperature, and presence of Ca^{2+} contribute to the high effectiveness of detoxification by $Ca(OH)_2$.

3.5 Acknowledgement

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3.6 Reference

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Table 3-1 GC-MS analysis of chemical compounds in SF5 [a]

Retention Time (min)	Compound identified	CAS #	Peak Area in Untreated SF5	Peak Area in Treated SF5
2.30	Acetic acid	64-19-7	4686±1591	6782±82
2.67	Acetol	116-09-6	3423±332	606±304
3.29	Acetoin	513-86-0	107±14	210±32
3.57	n-Propyl acetate	109-60-4	671±92	
3.66	2-Butanone, 4-hydroxy-3-methyl-	3393-64-4	1968±396	
3.86	unknown	NA	94±22	
4.59	Alpha-monopropionin	624-47-5	2604±1111	
4.70	2-Methyl[1,3,4]oxadiazole	3451-51-2	87±79	
5.01	Succindialdehyde	638-37-9	202±80	
5.18	Methylolacetone	68648-26-0	108±38	
5.31	1,2-Cyclopentanediol, trans-	5057-99-8	135±33	
5.43	Methyl 2-methoxypropenoate	80-62-6	75±52	
5.48	unknown	NA	97±26	
6.87	Furfural	98-01-1	2463±742	
7.61	Ethane, 1,1,1-trimethoxy-	1445-45-0	399±78	
7.99	Butanal, 2-ethyl-	97-96-1	139±55	
8.49	Glycidyl methyl ether	930-37-0	57±26	
8.59	Acetol acetate	592-20-1	198±25	
9.05	Furan, tetrahydro-2,5-dimethoxy-	696-59-3	271±123	
9.68	1,2-Epoxy-3-propyl acetate	6387-89-9	96±27	
9.80	Furan, tetrahydro-2,5-dimethoxy-	696-59-3	234±75	
10.23	unknown	NA	386±112	
10.35	2(5H)-Furanone	497-23-4	1189±151	
10.45	Ketone, 2-furyl methyl	1192-62-7	198±7	
10.64	unknown	NA	152±90	
10.85	2-Cyclohexen-1-ol	822-67-3	65±30	
11.06	Pentanal, 2,4-dimethyl-	32749-94-3	277±124	
11.20	1,2-Cyclopentanedione	3008-40-0	92±79	
11.78	2(5H)-Furanone, 5-methyl-	591-11-7	190±31	
12.65	1,2-Ethanediol, monoacetate	542-59-6	115±37	
12.86	unknown	NA	805±199	
13.15	unknown	NA	148±94	
13.32	unknown	NA	169±26	
13.68	2(5H)-Furanone, 3-methyl-	22122-36-7	196±33	
14.30	Phenol	108-95-2	124±49	
14.61	unknown	NA	66±30	
14.78	Ethyl Acetate	141-78-6		746±48
15.11	Silane, ethoxytrimethyl-	1825-62-3	75±40	

Table 3-1 (continued)

Retention Time (min)	Compound identified	CAS #	Peak Area in Untreated SF5	Peak Area in Treated SF5
16.37	2-Cyclopenten-1-one, 2-hydroxy-3-methyl-	80-71-7	125±141	
16.93	2-Cyclopenten-1-one, 2,3-dimethyl-	1121-05-7	64±33	
17.13	4-Methyl-5H-furan-2-one	6124-79-4	102±21	
17.43	Hexanal dimethyl acetal	1599-47-9	82±25	
17.90	2-Deoxy-D-galactose	1949-89-9	99±49	
18.08	Phenol, 2-methyl-	95-48-7	101±69	
19.60	Phenol, 2-methoxy-	90-05-1	283±277	
20.68	2,6-Dimethyl-1,6-heptadien-4-ol acetate	70187-91-6	137±71	

[a] Data are mean ± standard deviation of three replicates

Table 3-2 Evaluation of parameter effect on the detoxification of SF5 during overliming

Effect(s)	Treatment parameters			MTC [a]
	pH	Chemical used	Temperature	(wt%)
Control	2	None	25°C	0.1
pH effect	10	NaOH	25°C	2.0
Temperature effect	2	None	80°C	0.1
Calcium effect	2	$CaCl_2$	25°C	0.1
pH + Temperature effects	10	NaOH	80°C	4.0
pH + Calcium effect	10	$Ca(OH)_2$	25°C	2.0
pH + Temperature + Calcium effects	10	$Ca(OH)_2$	80°C	5.5

[a] MTC: Maximum tolerant concentration, the highest concentration of SF5 added to the TAP medium that the algal cells can grow without significant inhibition.

Table 3-3 Fatty acid composition and total fatty acid (TFA) content of *C. reinhardtii*

Grown in TAP medium supplemented with reagent grade of acetic acid (4 ml/L) (control) and the $Ca(OH)_2$-treated SF5 (5.5wt%) with the final acetic acid concentration at 4 ml/L.

	Algal fatty acid composition (%) [a]	
Fatty acid	**Control**	**Treated SF5**
16:0	30.02±0.13	28.13±2.53
18:0	3.85±0.11	3.89±0.41
18:1 (trans)	10.74±0.20	5.12±1.19
18:1 (cis)	9.82±0.57	12.71±2.22
18:2	20.50±0.49	25.84±1.13
18:3	18.99±1.06	16.27±0.12
20:0	6.08±0.19	5.12±1.19
TFA content (%DW)	20.01±0.55	9.31±2.54

[a] Data are mean ± standard deviation of three replicates

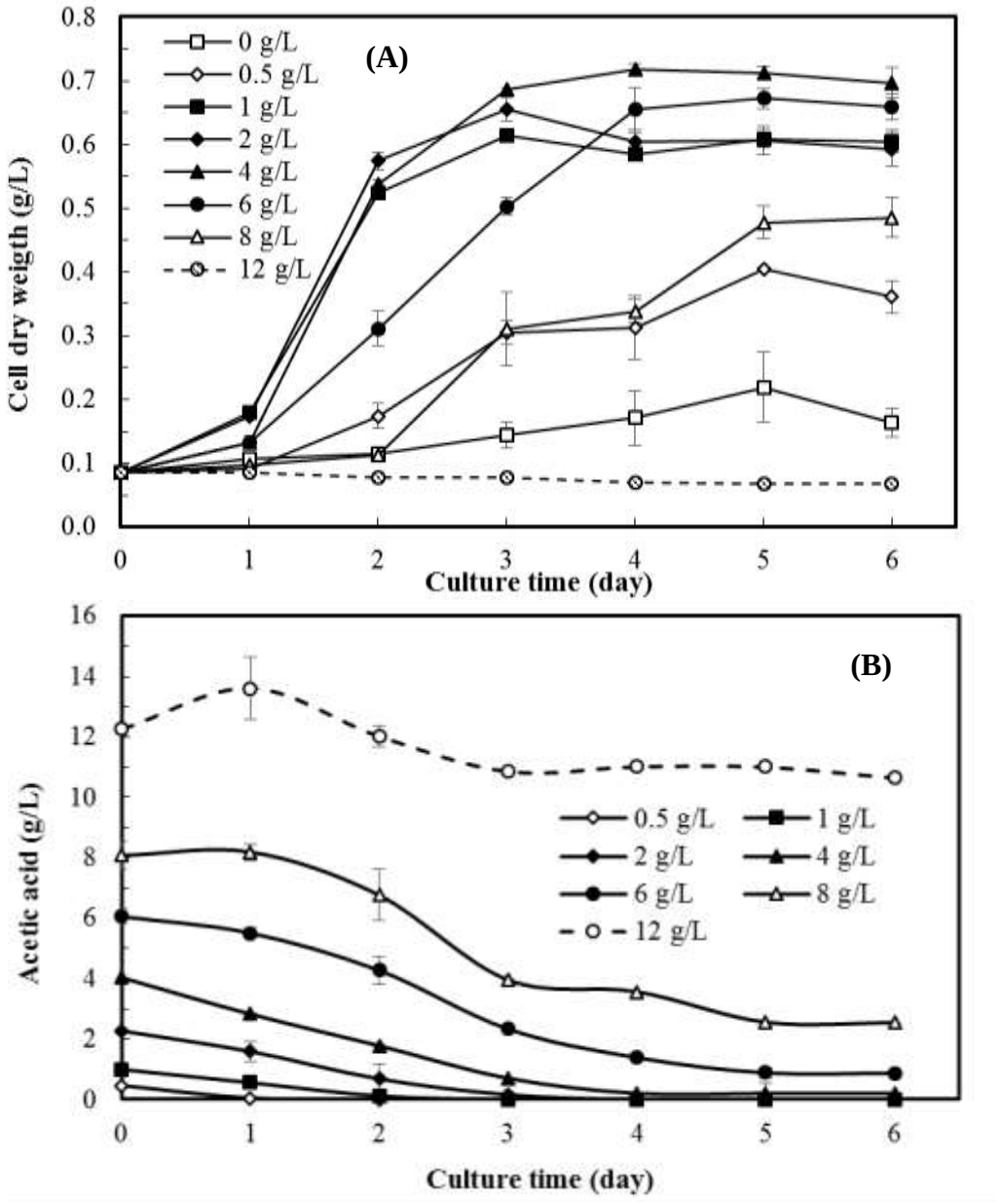

Figure 3-1 Cell performance with differetn initial aceitic acind concentraitons

Data are means of three replicates and error bars show standard deviations. Cell grwoth (**A**) and acetic acid consumption (**B**)

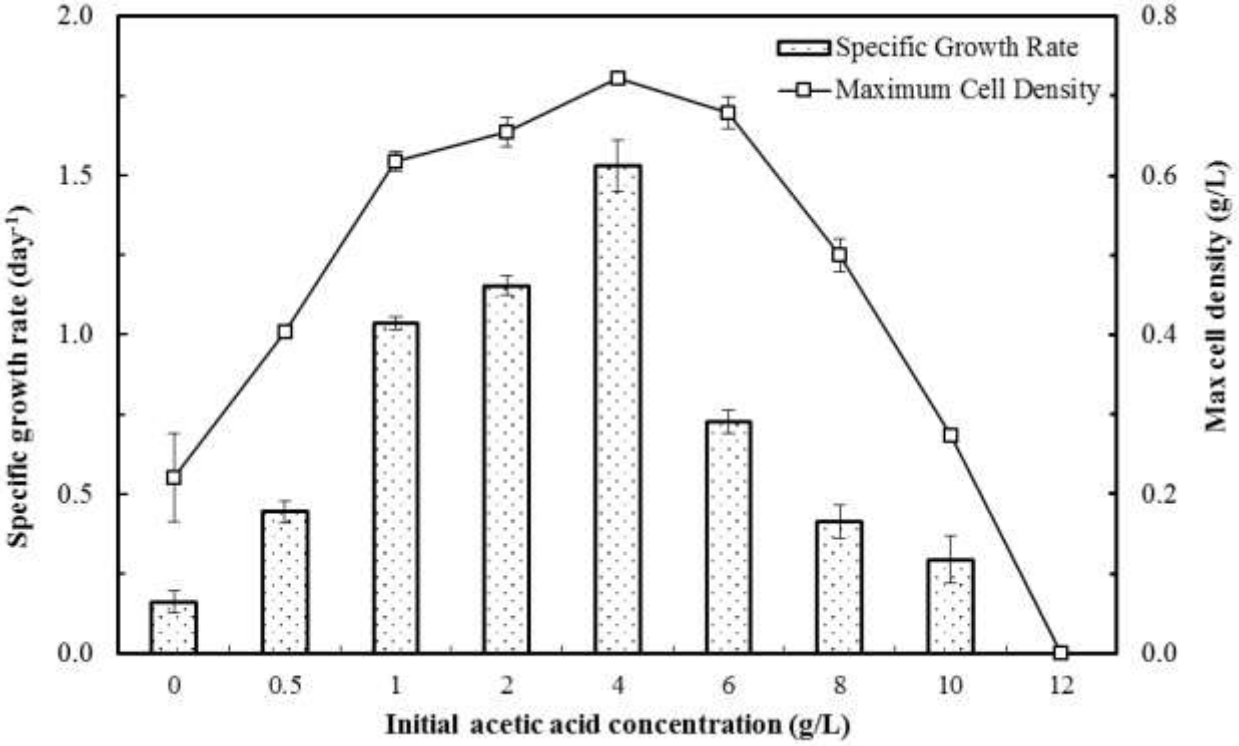

Figure 3-2 Effects of initial acetic acid concentration

Specific growth rate and maximum cell density of *C. reinhardtii* included.

Data are means of three replicates and error bars show standard deviations.

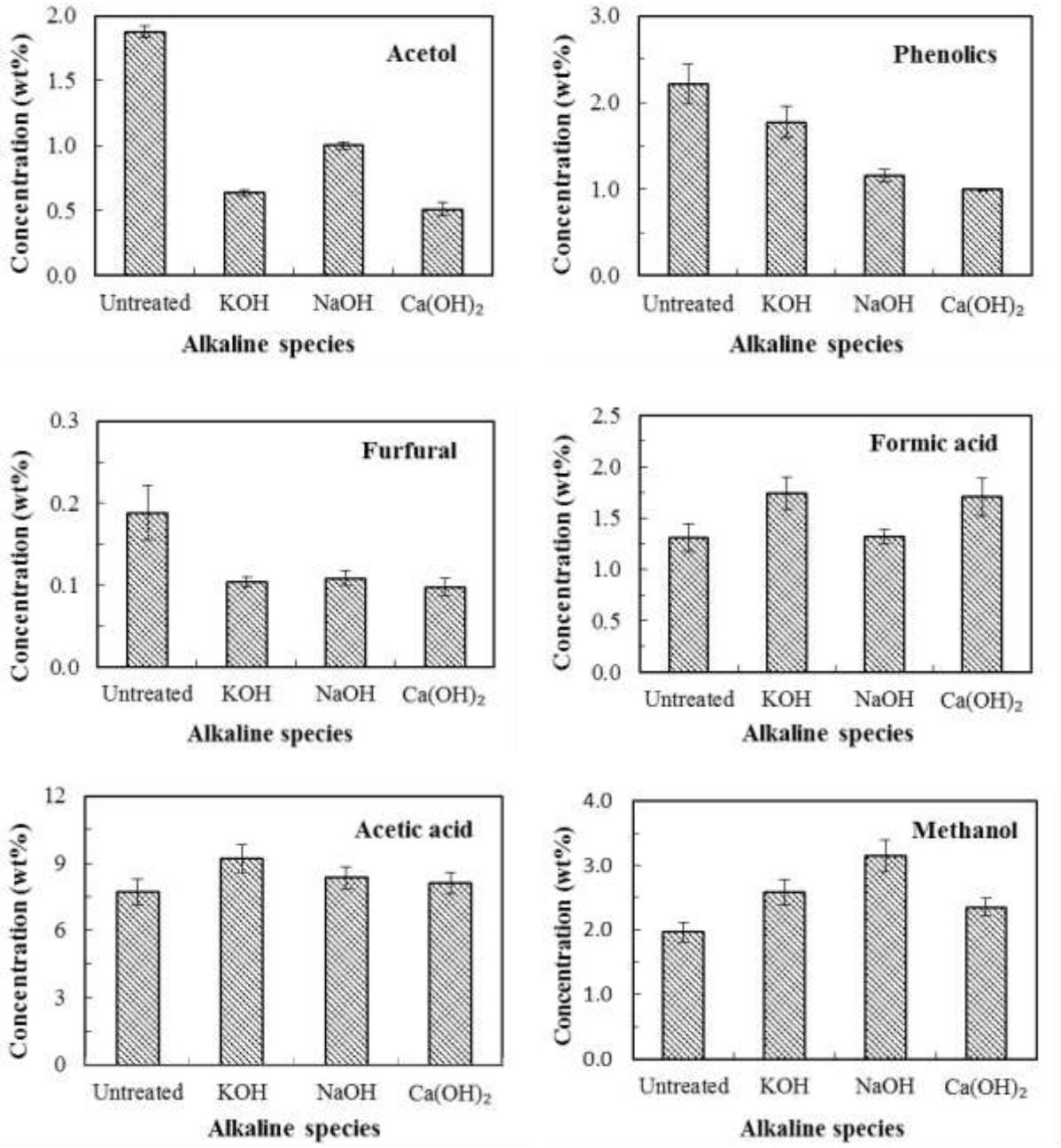

Figure 3-3 The concentration of major compounds in SF5 after alkaline treatment

Data are means of three replicates and error bars show standard deviations.

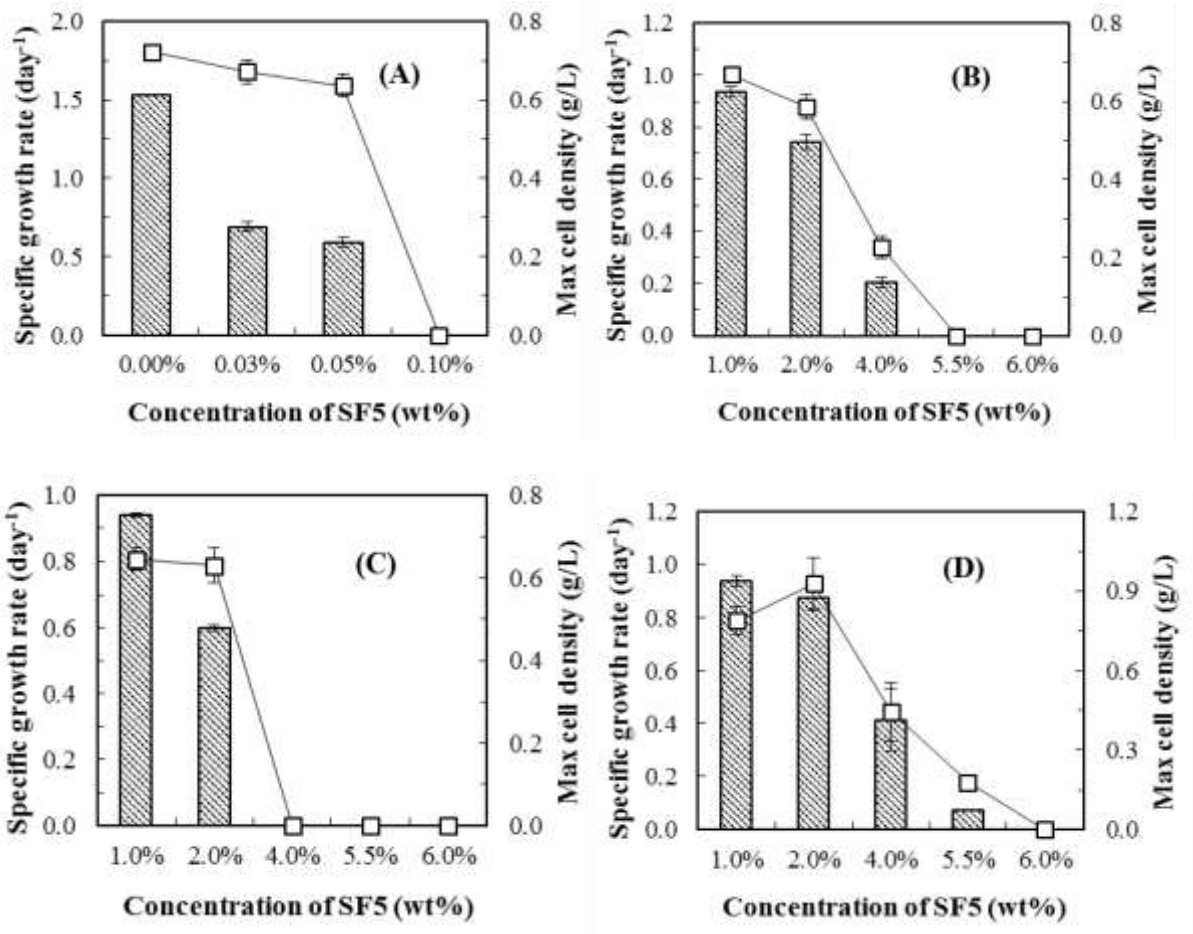

Figure 3-4 Algae growth performance under different concentrations of treated SF5

(A) untreated SF5; (B) SF5 treated by KOH; (C) SF5 treated by NaOH; (D) SF5 treated by Ca $(OH)_2$. Bars: specific growth rate; Lines: maximum cell density. Data are means of three replicates and error bars show standard deviations.

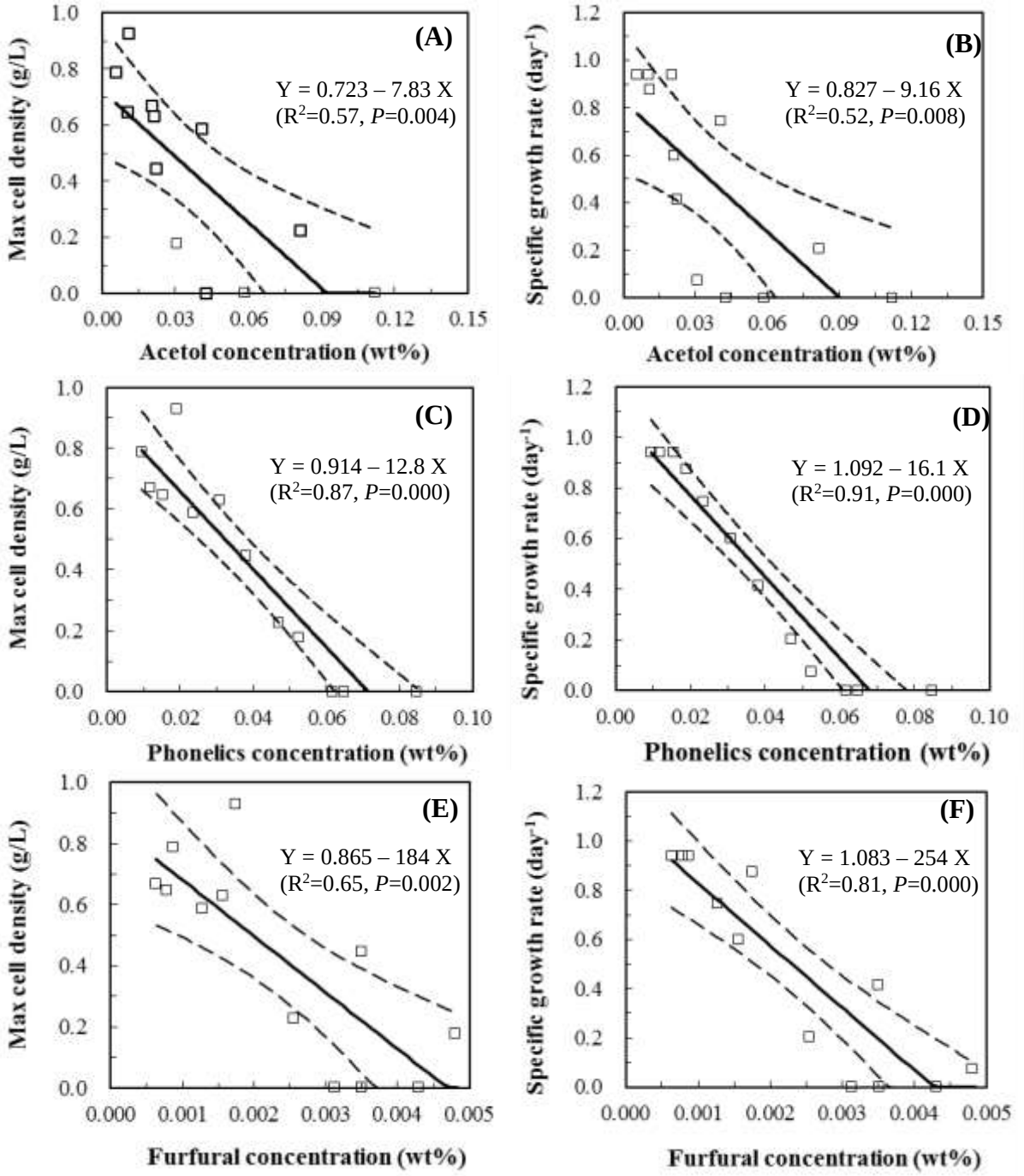

Figure 3-5 Cell growth as functions of the concentration of potential inhibitors
(Maximum cell density and specific growth rate included)
(A) and (B): Acetol. (C) and (D): Phenolic compounds; (E) and (F): Furfural. The scattered points are the experimental data; the solid lines are the regression line; the dash lines: boundary with 95% confident interval.

CHAPTER 4 PYROLYTIC BIO-OIL DE-TOXICITY FOR LIPIDS PRODUCTION: IMPROVEMENT OF ALGAL FERMENTABILITY OF BIO-OIL BY DIFFERENT OXIDATIVE PROCESSES

Abstract

Pyrolytic acetate derived from lingo-cellulosic biomass is a promising substrate for microalgae fermentation of lipids production. However, pyrolytic-acetate-containing bio-oil has an extremely complex composition and high inhibition on microorganism fermentation. In this study, three different oxidation processes, bleach oxidation, ozone oxidation and Fenton's process, were used to remove the contaminants in pyrolytic bio-oil. Based on microalgal fermentation test, ozone oxidation was proved to be the best methods for further optimization. With 3 hour ozone treatment under pH 10, the fermentability of pyrolytic-acetate-rich Stage Fractionation of bio-oil (SF5) was increased from 0.05 wt% to 5.5 wt%. Chemical identification and quantification for untreated and treated SF5 was done to investigate the mechanism of ozone oxidation. Potential inhibitive compounds, like aldehydes, ketones and phenolics, were proved to be removed partially or completely via ozone oxidation under basic pH.

4.1 Introduction

Lignocellulosic biomass represents a potential feedstock for biofuel production [1-4], which can also aid in reduction of greenhouse gas emission and mitigation of climate change [5]. Among different types of lignocellulose processing, fast pyrolysis shows a promising process for biomass-to-biofuel conversion [6, 7]. Under moderate temperature and high heating rate, biomass is decomposed in the absence of oxygen, while syngas (13~25 wt%), bio-oil (60~70 wt%) and biochar (12~25 wt%) are produced [8, 9]. Bio-oil has potential as a fuel substitute without additional processing, but can also be upgraded to higher value fuel such as biodiesel [9] Additionally higher value chemicals can be extracted from bio-oi [9, 10]. However, the utilization of pyrolytic bio-oil faces a major challenge due to its unstable physical properties and complex chemical composition [11].

Raw bio-oil contains a variety of anhydrosugars and carboxylic acids, which can serve as carbon substrates for microorganism fermentation [12-16]. To better study and utilize fast pyrolysis bio-oil, a unique fractionation system has been developed at Iowa State University [17]. This system can fractionate raw bio-oil into five stage fractions, each with different chemical compositions and fermentabilities [17]. One of the most promising fermentable stage fractions is stage fraction 5 which is rich in acetic acid. This acetic acid rich fraction can be utilized for biolipid production through microalgae fermentation [15-17]. Microalga *Chlamydomonas reinhardtii* (*C. reinhardtii*), has been previously heterotrophically grown on acetic acid [18] and demonstrated overproduction of lipids as a result of genetic modification [19-22]. Thus it is a prospective candidate to ferment SF5.

Although SF5 shows promise as a fermentable substrate, a major hurdle is its complexity. It is composed of hundreds of chemicals, some of which result in high inhibition on microalgae

fermentation [12, 15, 16]. In previous research, a fermentability test of SF5 was established growing *C. reinhardtii* in flask-scale. The microalga could only survive under 0.05 wt% of SF5 and it was determined that acetol, furfural, hydroxymethylfurfural and phenolic compounds present in SF5 were toxic and caused inhibition of microalgal growth [15, 16]. Due to the complexity of bio-oil, ~30 wt% of chemical compounds are still unknown and the inhibition of SF5 cannot be completely explained based on previous research [15, 16].

Methods to promote better fermentability in bio-oil are to either modification of microbial genotypes or to detoxify the bio-oil by different chemical or physical methods. Directed evolution improved the tolerance of *C. reinhardtii* to facilitate marginal improvements in bio-oil fermentability from 0.05 wt% to 0.5 wt% [15, 16]. However detoxification has shown to considerably improve bio-oil fermentability in comparison to genetic modification. For example, activated carbon adsorption decreased the concentration of several inhibitors in SF5 and 1.0 wt% of SF5 can be tolerated by cells. [15] and NaOH treatment increased the maximum tolerable concentration (MTC) of SF5 to 4.0 wt% [16]. It should be noted that the best reported fermentability of SF5 occurred when an evolved strain was cultured in alkaline treated SF5, which resulted in a MTC of 5.5 wt%. In this research we investigate the use of oxidation as a new alternative treatment method to detoxify bio-oil.

It is known that various inhibitors contained in the acetic acid rich bio-oil fraction such as phenols are highly reductive compounds. Oxidation of those reducing substances into less-toxic compounds can therefore, provide a possible effective detoxification method for bio-oil. As a matter of fact, oxidation with various chemical oxidants has been widely applied in wastewater treatment [23-25] to remove compounds such as carboxylic acids, chlorides and sulfides as well as aromatic compounds. Indeed some of the same compounds that are removed in wastewater are

also present in pyrolytic bio-oil. The goal of this work was to evaluate oxidizing agents such as, hydrogen peroxide, bleach and ozone, to detoxify bio-oil. The optimization and mechanism of the oxidative treatment was also explored.

4.2 Materials and methods

Microalgae culture and monitoring

The microalga *C. reinhardtii* ST 21 strain used in this work was obtained from Dr. Martin Spalding's laboratory at Iowa State University. Seed culture was established in in 250-mL Erlenmeyer flasks containing 50-mL tris-acetate-phosphate (TAP) medium under previously described procedure [16]. *C. reinhardtii* growth test was performed in 24-well plates with 1 ml medium and 0.1 ml algal seed in each well. The multi-well plates were placed at an orbital shaker with a speed of 130 rpm. Ambient temperature was set at 25^{o}C. Continuous illumination at 110-120 μmol $s^{-1}m^{-2}$ was provided. The optical density of each well at 730 nm (OD_{730}) were measured via a BioTek EL×800 microplate reader (Winooski, VT) on daily basis. Each test was performed in triplicates for statistical analysis.

SF5 preparation and storage

The fast pyrolysis and bio-oil stage fractionation were done at Central Iowa Research and Demonstration Farms by Dr Robert C. Brown's group. [17] The acetic-acid-rich SF5 was collected in 1-L Nalgene HDPE bottles. It was stored at 4^{o}C and would be shaken up before each use.

Oxidation methods for treating bio-oil fraction

SF5 was completely mixed and divided into three bottles equally. SF5 in the first bottle was kept at pH 2, while solid NaOH powders were added to bottle 2 and 3 to adjust the pH to 7

and 10, respectively. Then each bottle of SF5 was centrifuged at 750 g for 5 minutes to remove sediments.

4.5 ml of SF5 (pH 2, 7 or 10) was transferred to 15 ml centrifuge tube for treatment by liquid oxidants, Fenton's reagent or bleach. 1.5 g/L of H_2O_2 and 0.75 g/L $FeSO_4$ were achieved for Fenton's reagent treatment. While the concentration of NaClO in bleach treatment is set as 0.5%. Extra DI water was added to each centrifuge tube to make the final volume as 5 ml. Then the centrifuge tubes were put at water bathes at 40^{o}C during 3-hour treatment. Triplicates were made for statistical analysis. As to gaseous oxidants, which is ozone in this work, 100 ml SF5 was transferred to 1-L glass bottle for oxidation. Then 0.1 ml antifoam was added into the reactor to restrain bubble production. 10 CFH of Air was sent to ozone generator. The gas from the outlet of ozone generator contains 30% O_3 and 70% air. The mix of O_3 and air were sent to glass bottle for SF5 oxidation. The treatment of SF5 continued for 8 hours for each bench. At 0, 0.5, 1, 3, 5, 8 h, the loss of the reaction bulk was replenished by DI-water prior to triple sample collection. All of the samples were stored at 4^{o}C before the measurement and fermentation.

Chemical Analyses

The water content of SF5 was measured by previously described Karl Fisher analysis. Some compounds were quantified by gas chromatography with published method. Carboxylic acids were analyzed by a Dionex ion chromatography ICS 5000 system (Sunnyvale, CA) equipped with a DionexIonPac® ICE-AS1 column (4×250 mm) [26]. The concentrations of total phenolics were measured by a published colorimetric method with vanillin as the calibration standard. Further investigation was done by Gas chromatography/Mass spectroscopy analysis [14].

4.3 Results and discussion

Characterization of chemical compounds in SF5

SF5 is the aqueous stage fractionation of fast-pyrolysis bio-oil. It is reported that SF5 is composed of ~60% of water and many other chemicals with different properties [15, 16]. In order for better detoxification and fermentability of SF5, tentative identification via GC-MS was done in this work (Table 4-1). About forty compounds was identified, most of which are esters, acids, aldehydes, ketones and phenolics. Some aldehydes, ketones and phenolics were identified at previous bio-oil study too [14, 17, 27-31]. What's more, some of these compounds were reported as inhibitive compounds for microorganism fermentation [32-35]. Removal of these compounds might be helpful to improve the fermentability of SF5.

Remarkable unsaturated bonds, like double bands (-C=C-), aldehyde group (-CHO), ketone group (-C=O) and hydroxyl group (R-OH, Ar-OH) can be found from structures of identified compounds, which implies the possibility of bio-oil detoxification via oxidation. For example, phenolic compounds are present in fast pyrolysis bio-oil from decomposition of lignincellulosic biomass. They were implicated as causing compounds of solution taste and odor [36] as well as the inhibition on microorganism fermentation [15, 16, 34]. Phenolic compounds were as well known for being vulnerable under oxidative reagents. For example, ozone is reported to efficiently oxidize aqueous phenolics through hydroxylation, degradation and oxidative coupling pathways [36]. In conclusion, the toxicity of SF5 may come from aldehydes, ketones and phenolic compounds, and oxidative methods are promising for bio-oil detoxification.

Effectiveness of different oxidative treatments on SF5 fermentability enhancement

In electrochemistry, standard electrode potential (E^0) is used to indicate the strength of oxidizing and reducing agent. Chemicals with high standard electrode potential are active

oxidants, which were potentially effective to remove the aldehydes, ketones and phenolic compounds. With apparently high standard electrode potential, Fenton's reagent, bleach and ozone were applied to SF5 treatment in this work. For these oxidants, the working pH of the solution would affect the reactant type and oxidation efficiency significantly. The reaction and standard electrode potential for each oxidant under different solution pH were listed at Table 4-2.

SF5 under acidic pH (2), neutral pH (7) or basic pH (10) were treated by Bleach, Ozone and Fenton's reagent, respectively. Because of the complexity of bio-oil, the concentration of one or several compounds cannot represent the comprehensive toxicity of SF5. Thus in order to investigate the detoxification efficiency of each treatment, the MTC (maximum concentration of SF5 in medium which can be tolerated by microalgae) of all treated SF5 were tested. Higher MTC indicates higher fermentability and less toxicity of the media, and is corresponding to better treatment of SF5. The MTC of each sample were shown on Figure 4-2.

Sodium hypochlorite (NaClO) is the major compound of household bleach. Under acidic and basic pH, the major oxidative reactant is HClO and ClO^- respectively. The two oxidizing agents show different standard electrode potentials. HClO, the oxidant appeared at acidic solution has higher oxidation abilities than ClO-, which works at basic solution (Table 4-2). However, the MTC of SF5 with bleach treatment under acidic, neutral and basic pH are 0.1%, 0.2% and 4%, respectively (Figure 4-2). Then the bleach treatment efficiency cannot be explained only by the work of sodium hypochlorite, the contribution of the pH adjustment via solid NaOH powder to the detoxification of SF5 is unignorable.

As to ozone treatment, two oxidizing actions might happen during ozonation procedure: direct reaction between molecular O_3 and inhibitants, and radical way between hydroxyl radicals, which were generated via O_3 decomposition, and inhibitive compounds. Hydroxyl radicals,

rapidly generated from molecular ozone under basic pH, is very reactive oxidizing agent and can improve the ozone oxidation of contaminants significantly. In this work, better SF5 detoxification results for ozonation was obtained under alkaline pH, which improves the fermentability of SF5 from 0.10% to 5.5%. As a result, the radical oxidation coupled with increased pH via solid NaOH powder is promising for SF5 detoxification.

As to Fenton's reagent, the presence of Fe^{2+} at acidic solution gives rise to the sustained formation of .OH and .HO_2, leading to high oxidation efficiency (Guido, 2008) of H_2O_2. However, the best detoxification of SF5 via Fenton's reagent happened under basic pH. SF5 with pH 10 treated via Fenton's reagent can improve the MTC of SF5 to 4.0%. Therefore the detoxification efficiency of each treatment is decided both by both oxidation and pH change of SF5 via solid NaOH powder.

Under acidic pH (pH 2), all of the treatments increased the fermentability of SF5 to 0.1 wt%. Under neutral pH (pH 7), 0.2 wt% or more of treated SF5 can be tolerated by microalgae cells. SF5 treated by Fenton's reagent shows the highest fermentability due to the formation of intermediate hydroxyl radical and hydroperoxyl radical during oxidation of SF5. While oxidated under basic pH (pH10), higher improved fermentibility of SF5 was approached. After the treatment of Bleach, Ozone and Fenton's reagent, 4.0, 5.5 and 2,0 wt% of SF5 can be tolerated in microalgal culture, respectively. In conclusion, all of these three oxidants were proved as an efficient oxidant for SF5 treatment under basic pH and ozone is the most efficient one.

There are several factors to be considered before the application of these oxidants, such as the environmental effect. Bleach treatment has hidden dangers caused by the vitalization of gaseous Cl_2. For Fenton's reagent, it is an environmentally-safe oxidant but there are problems such as stoichiometric excess of hydrogen peroxide and the need of disposal significant

quantities of ferric salts. Ozone is a reliable and environmental-friendly oxidants technically applied (73,Guido 2008) and it shows the best improvement on the fermentability of SF5 under basic pH. Based on the comprehensive comparison of detoxification efficiency, environmental influence and effect on subsequent medium preparation, ozone was chosen as the best oxidants in SF5 treatment. The further optimization and mechanism investigate of ozone treatment were shown in following paragraph.

Optimization of ozone-based oxidative treatment conditions

The fermentation of ST 21 at TAP medium with addition of raw SF5 (pH 2) was set as the control. Due to the toxicity of raw SF5, there is no microalga growth at control group when the addition of SF5 is 0.1 wt% (Figure 4-2a). While the dosage of ozone was set as the maximum ozone production of the instrument, the optimization of ozone treatment focused on treatment time under different solution pH. The initial solution pH was set as 2, 4, 6, 7, 8 and 10. Sample would be taken at t=0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8 hr. The fermentability of these samples was tested and shown at Figure 4-3.

pH adjustment of SF5 was done 5 min, during which the temperature of SF5 could increase from 25 ℃ (pH=2) to as high as 80 ℃ (pH=10). After pH adjustment, SF5 samples would stand for a while until cool down to room temperature. The pH change with the addition of NaOH was recorded and shown on Figure 4-4. The pH of SF5 were quite stable at pH 4~6 and pH 10, which might be due to the existence of formic acid (pKa=3.75), acetic acid (pKa=4.75), Propanoic acid (pKa=4.87), and phenol (pKa=9.80), m-cresol (pKa=10.09) and other derivatives.

For SF5 with initial solution pH 2, no solid NaOH was needed to adjust pH. After ozone oxidation, the pH of SF5 was decreased to 1.45. However, the fermentability of SF5 is no better than the control group (Figure 4-3a). Similar fermentation result was found for pH 4 and 6. To

get neutral solution, ~70 g solid NaOH was added to adjust raw SF5 pH to 7, which had no benefit to the fermentability of SF5. While after ozone oxidation of neutralized SF5, no matter how long the oxidation is, ST 21 can survive at medium with 0.1% of SF5 (Figure 4-3b-1) but not at medium with 0.2% of SF5 (Figure 4-3b-2).

Significant improvement on fermentability were found in basic SF5, which needs about 80 or 140 g NaOH per 1L raw SF5 to adjust pH from 2 to 8 or 10, respectively. The fermentability of SF5 pH 8 with ozone treatment was 1.0 wt%. While 5.5 wt% of basic SF5 (pH=10) without ozone treatment can be tolerated by microalgae. The benefit might come from the precipitation of inhibitive compounds under basic pH. Based on Figure 4-6c, it was shown that ozone oxidation of basic SF5 within 3 hours can significantly further improve the microalgal growth performance (both maximum cell density and specific growth rate, $P<0.05$) but no increase on MTC of SF5 (Figure 4-3b).

Mechanism of ozone-based oxidative treatment

Concentration change of compounds in ozone-based oxidative treatment

GC-MS analysis was done for both untreated SF5 and SF5 with pH 10 and 3 hours ozone oxidation (Table 4-1). The result shows that most of the compounds can be partially and completely removed via this treatment. To explore the efficiency of each effect on inhibitors removal, the concentration change of major compounds during ozone treatment were further quantified to investigate the toxicity change of SF5. Compounds identified in SF5 were mainly ketones, aldehydes and phenolics compounds, which were reported as fermentation inhibitors. Acetol and furfural, mostly identified compounds and proved to have inhibition on ST 21 fermentation, were used to represent ketones and aldehydes. The weight percentage of these two compounds was obtained by calibration on GC. The total phenolics compounds, which was

reported to disarrange microalgal membrane fluidity, was obtained by spectrophotometer with vanillin as the calibration standard.

The concentrations of inhibitors in different SF5 solutions were shown at Figure 4-5. Adjusting pH of SF5 from 2 to 10 via NaOH can remove 42 wt% of acetol, 65 wt% of furfural and 21 wt% of total phenolics. While ozone oxidation can increase the removal of acetol, furfural and total phenolics to 67 wt%, 100 wt% and 70 wt%. Based on the concentration change, the conclusion can be drawn that pH adjustment can significantly remove all of these three types of compounds, while the main effect of ozone oxidation is significantly improve of total phenolics removal efficiency.

Phenols removal via ozone-based oxidative treatment

The concentration change of phenols under ozone-based oxidative treatment was shown on Figure 4-7. pH increase can significantly decrease the total phenols concentration. With pH adjusted to 4, 6, 7, 8, 10, the removal rate of phenols is 14.9, 28.7, 39.6, 40.0, 49.0 wt%, respectively. With ozone treatment, phenols was mostly removed with the first 3 hour and then achieve stable phase. The aqueous phenolics in this work might be degradated to smaller molecular carboxylic acid such as acetic acid, for the acetate concentration is increasing with the ozone oxidation (Figure 4-7d).

4.4 Conclusions

This study demonstrated that ozone treatment is the most feasible oxidative method for detoxification of fast pyrolysis bio-oil. With 3 hour ozone treatment under pH 10, the fermentability of Stage Fractionation 5 of bio-oil was increased from 0.05 wt% to 4.0 wt%. Based on the GC-MS identification, the inhibition of SF5 is mainly from ketones, aldehydes and

phenolics. Adjusting pH from 2 to 10 can significantly remove these inhibitors and improve the fermentability SF5. Ozone treatment under basic pH mainly works on further oxidation of phenolics compound to ameliorate the maximum cell density and algal productivity. In summary, this result shows an effective oxidative method to improve the fermentative production of biofuels and biobased chemicals through pyrolytic substrates.

4.5 Acknowledgement

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4.6 Reference

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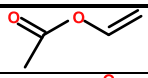
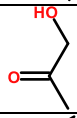
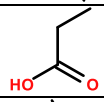
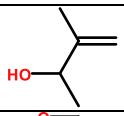
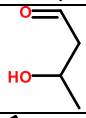
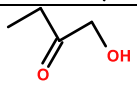
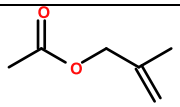
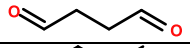
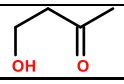
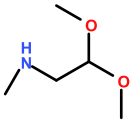
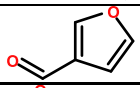
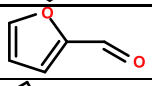
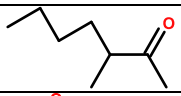
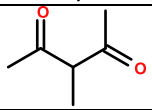
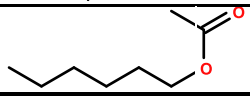
Table 4-1 Tentative identification of SF5 compounds by GC-MS

Retention Time (min)	Compound identified	CAS #	Structure	Reference	Concentration (raw SF5 pH 2, no ozone treatment)	Concentration (treated SF5 pH 10, Ozone treatment =3 hr)	Percentage of residual to original
2.43	Acetic acid ethenyl ester	108-05-4		6	2921±353	86±3	2.96%*
2.82	Acetic acid	69-14-7		1,3,4,5,6	2822±470	3851±449	136.47%
3.21	Acetol	116-609-6		2,3,4	8499±456	15749±6357	53.97%
3.62	Propanoic acid	79-09-4			190±70		0*
3.83	Acetoin	513-86-0		5,6	180±34	385±140	214.53%
4.64	Butanal, 3-hydroxy-	203-530-2			97±17		0*
5.13	2-Butanone, 1-hydroxy-	5077-67-8			224±36	246±12	109.78%
5.41	Methallyl acetate	820-71-3			490±112		0*
5.54	Succindialdehyde	638-37-9		2,6	622±17		0*
5.68	Methylolacetone	68648-26-0		6	382±31	325±13	84.90%
5.99	Acetaldehyde, (methylamino)-, dimethyl acetal	122-07-6			180±60		0*
6.81	3-Furaldehyde	498-60-2			4953±91		0*
7.22	Furfural	98-01-1		1,3,4,5,6	316±42	150±63	47.54%
8.38	2-Heptanone, 3-methyl-	2371-19-9			144±4		0*
8.94	Methylacetylacetone	815-57-6			155±12		0*
9.97	Acetic acid, hexyl ester	88230-35-7			861±326		0*

Samples were 50 times diluted; peaks with area <85 were neglected

*means the difference between original and residual is significant

Table 4-1 (continued)

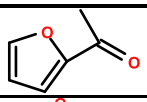
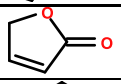
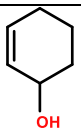
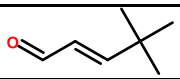
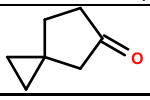
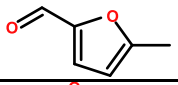
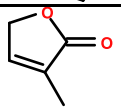
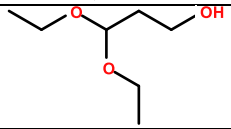
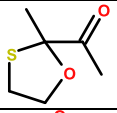
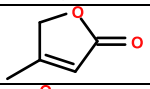
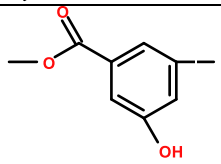
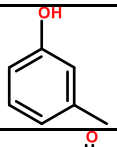
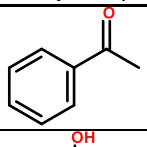
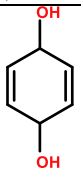
Retention Time (min)	Compound identified	CAS #	Structure	Reference	Concentration (raw SF5 pH 2, no ozone treatment)	Concentration (treated SF5 pH 10, Ozone treatment =3 hr)	Percentage of residual to original
10.42	Ethanone, 1-(2-furanyl)-	1192-62-7		6	130±32		0*
10.63	2(5H)-Furanone	497-23-4		2,4,6	1927±224	422±2	21.87%*
11.08	2-Cyclohexen-1-ol	822-67-3		6	135±15		0*
11.93	4,4-Dimethylpent-2-enal	22597-46-2			110±16		0*
13.01	Spiro[2.4]heptan-4-one	5771-32-4			132±30		0*
13.18	2-Furaldehyde, 5-methyl-	620-02-0		1,5	154±39		0*
13.88	2(5H)-Furanone, 3-methyl-	22122-36-7		5,6	116±5		0*
14.75	3,3-Diethoxy-1-propanol	16777-87-0			313±35	377±39	120.58%
15.24	Ketone, methyl 2-methyl-1,3-oxothiolan-2-yl	33266-06-7			314±38		0*
17.24	2(5H)-Furanone, 4-methyl-	6124-79-4		2,6	230±4		0*
17.88	Hexanoic acid, 3-hydroxy-5-methyl-, methyl ester	2615-71-6			190±22		0*
18.03	Phenol, 3-methyl- (m-cresol)	108-39-4		4,5	479±55	0*	
18.56	Acetophenone	98-86-2			218±112	0*	
19.13	2,5-Cyclohexadiene-1,4-diol	63453-92-9			131±25		

Table 4-1 (continued)

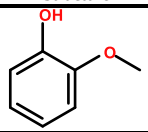
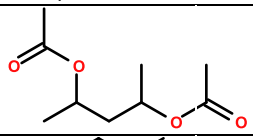
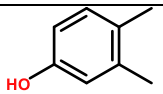
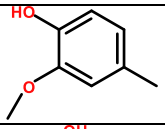
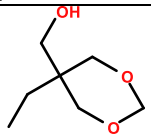
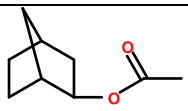
Retention Time (min)	Compound identified	CAS #	Structure	Reference	Concentration (raw SF5 pH 2, no ozone treatment)	Concentration (treated SF5 pH 10, Ozone treatment =3 hr)	Percentage of residual to original
19.61	Phenol, 2-methoxy-	90-05-1		4,5,6,8	921±88	0*	
20.74	2,4-Diacetoxypentane	7371-86-0			248±56	0*	
22.89	Phenol, 3,4-dimethyl-	95-65-8		4,5	120±13	0*	
24.81	Phenol, 2-methoxy-4-methyl-	93-51-6		4,5	155±32	0*	
25.30	1,3-Dioxane-5-methanol, 5-ethyl-	5187-23-5			106±11	0*	
28.99	exo-Norborneol, acetate	34640-76-1			117±3	0*	

Table 4-2 Standard reduction potentials of major oxidants at different pH

Species	pH	Electrode reaction	Standard potential value
NaClO	Acidic	$ClO^- + 2H^+ + 2e^- \rightarrow Cl^- + H_2O$	1.698
	Alkaline	$ClO^- + H_2O + 2e^- \rightarrow Cl^- + 2OH^-$	0.841
O_3	Acidic	$O_3 + 2H^+ + 2e^- \rightarrow O_2 + H_2O$	2.076
	Alkaline	$O_3 + H_2O + 2e^- \rightarrow O_2 + 2OH^-$	1.240
		$.HO + e^- \rightarrow OH^-$	2.310
H_2O_2	Acidic	$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O$	1.776
		$.HO_2 + H^+ + e^- \rightarrow H_2O_2$	1.495
		$.HO + e^- \rightarrow OH^-$	2.310
	Alkaline	$HO_2^- + H_2O + 2e^- \rightarrow 3OH^-$	0.878

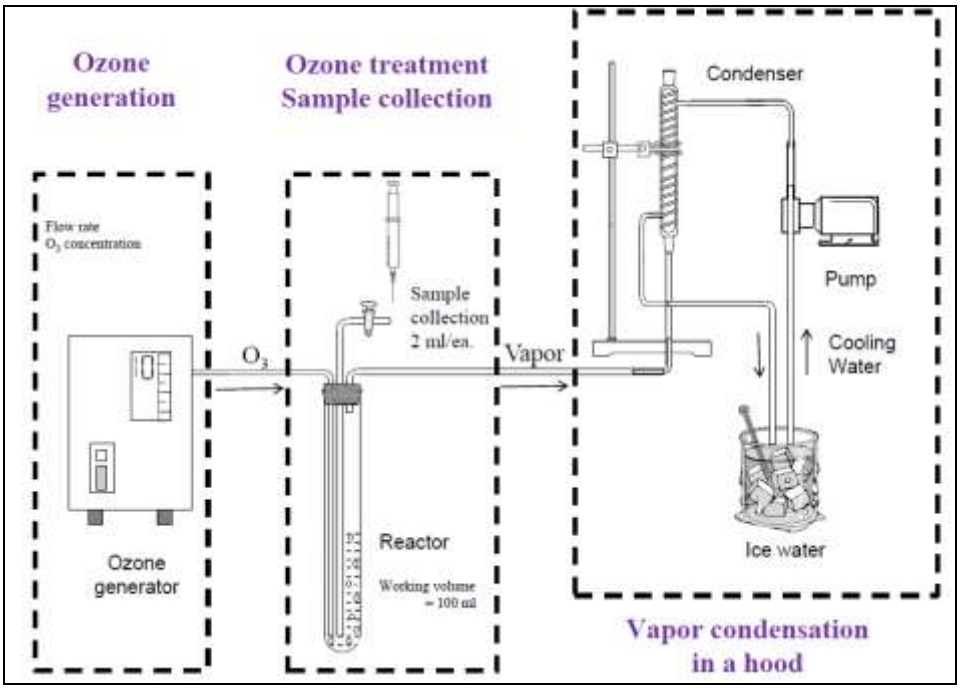


Figure 4-1 Sketch of ozone treatment

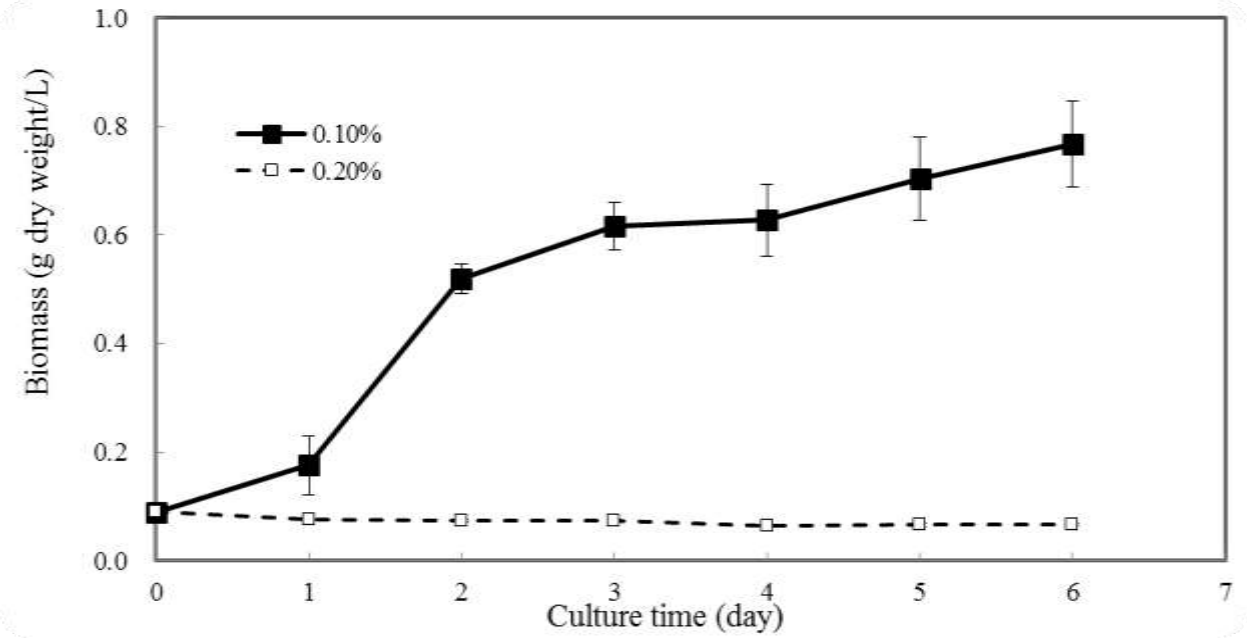


Figure 4-2 Microalgae growth under untreated SF5

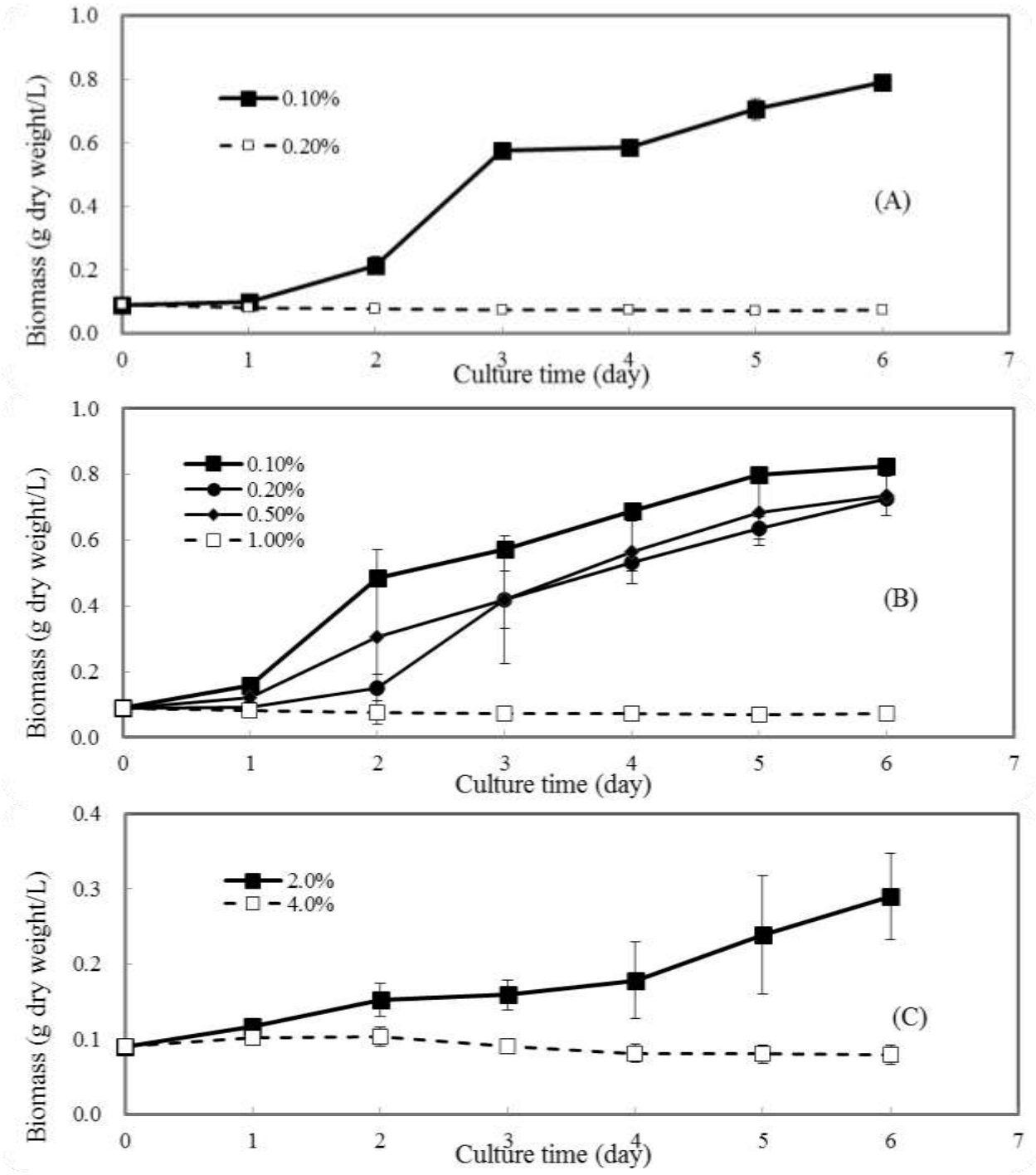


Figure 4-3 Microalgae growth under H_2O_2 treated SF5

(A) Initial pH=2; (B) Initial pH=7; (C) Initial pH=10

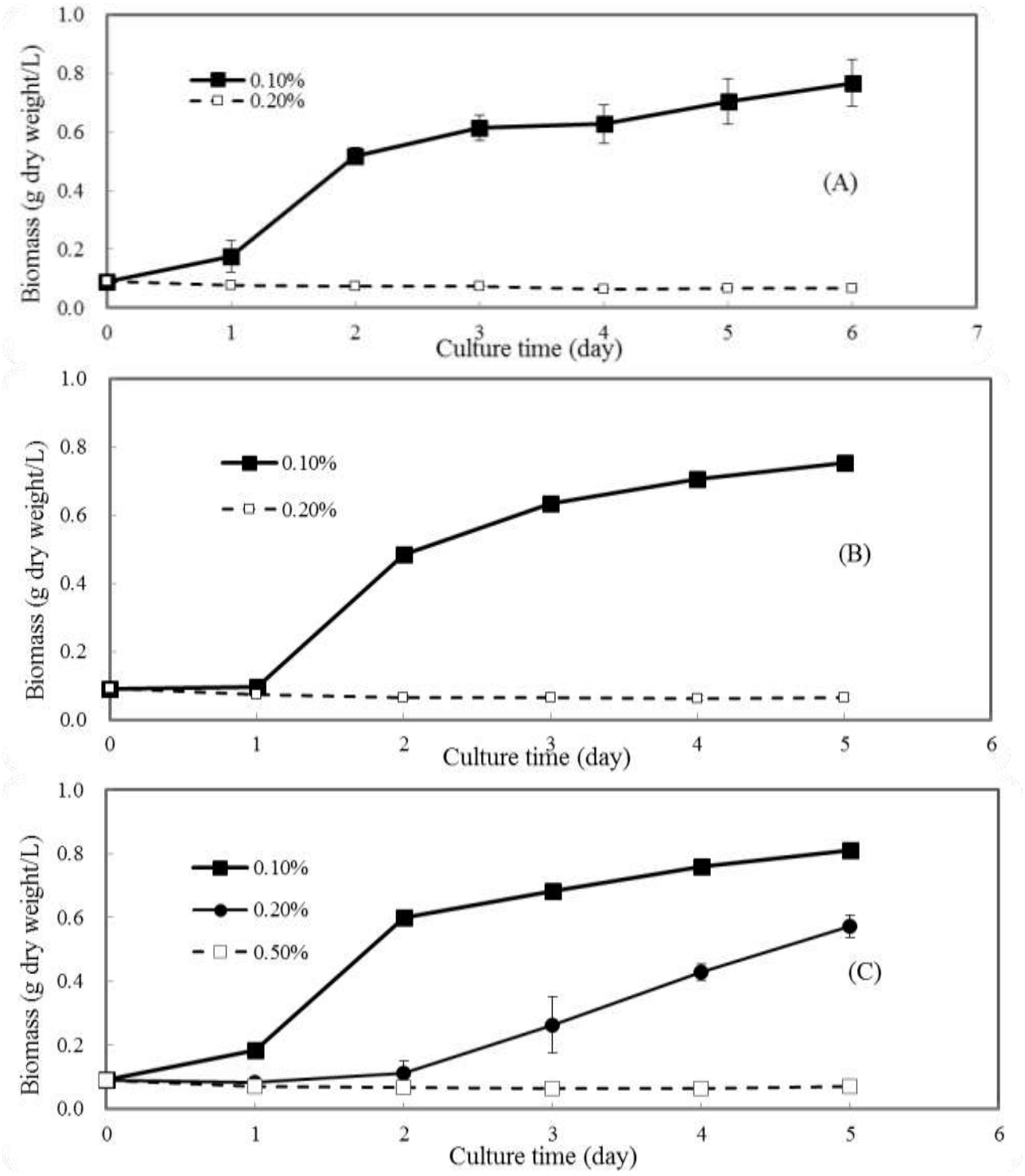


Figure 4-4 Microalgae growth under Bleach treated SF5
(A) Initial pH=2; (B) Initial pH=7; (C) Initial pH=10

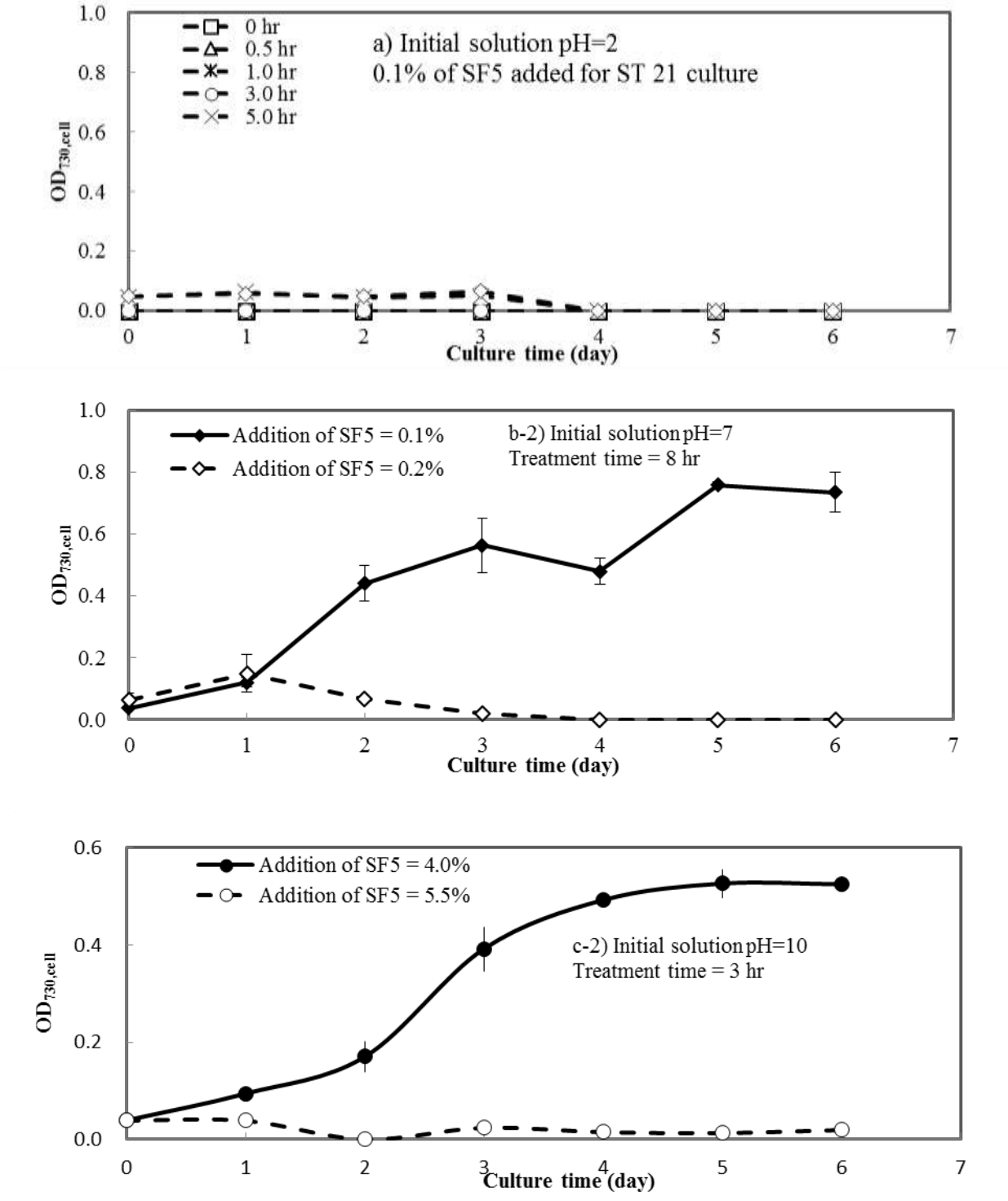


Figure 4-5 SF5 fermentation result for optimization of ozone treatment

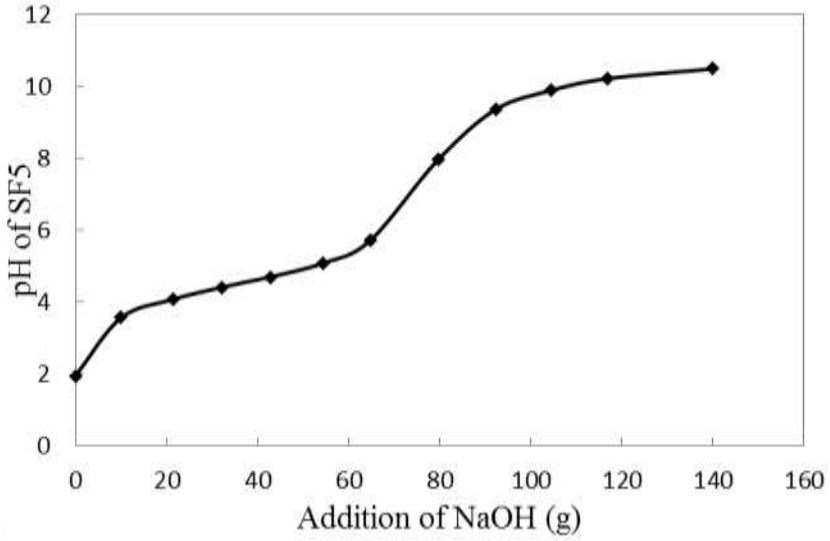


Figure 4-6 SF5 pH change with the addition of solid NaOH

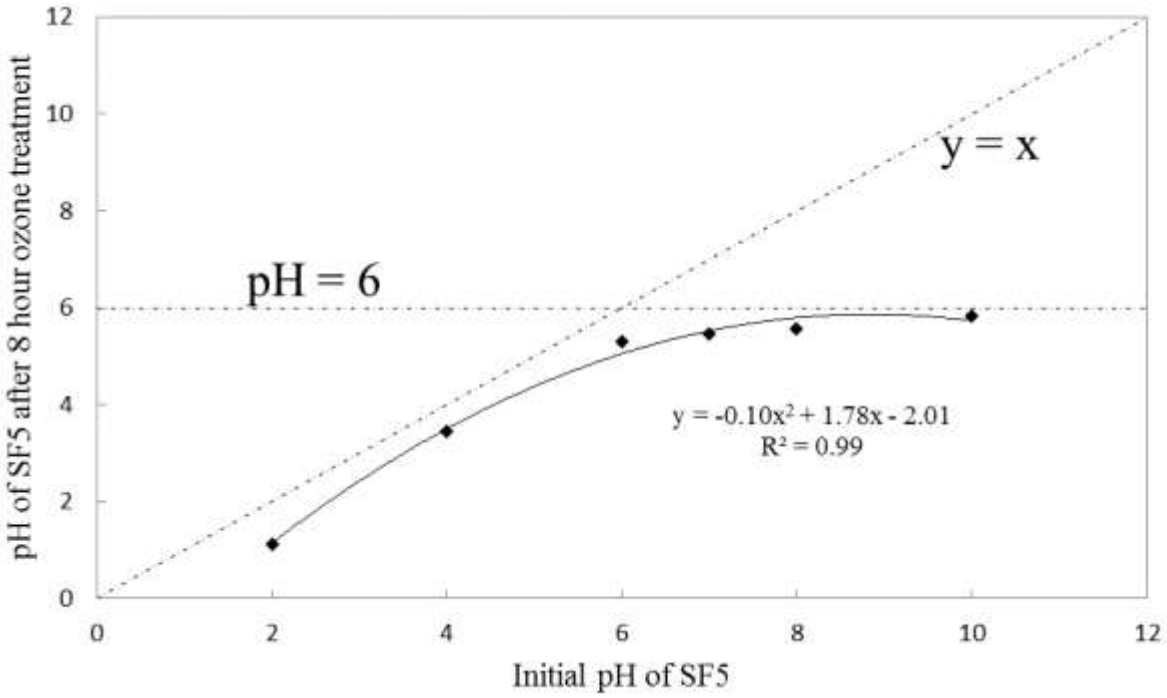


Figure 4-7 SF5 pH change after 8 hour ozone treatment

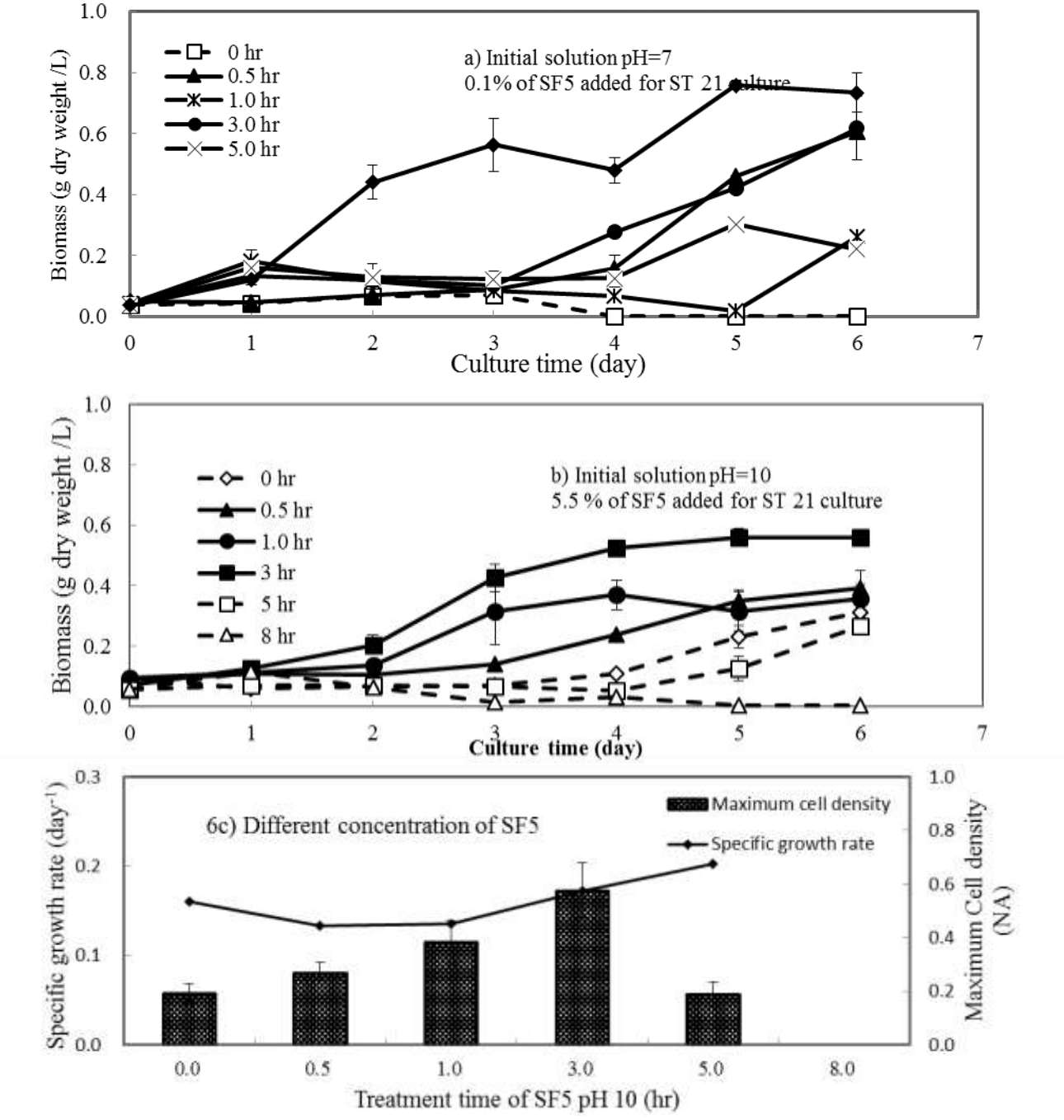


Figure 4-8 Compare of the fermentability of SF5 under different treatment time

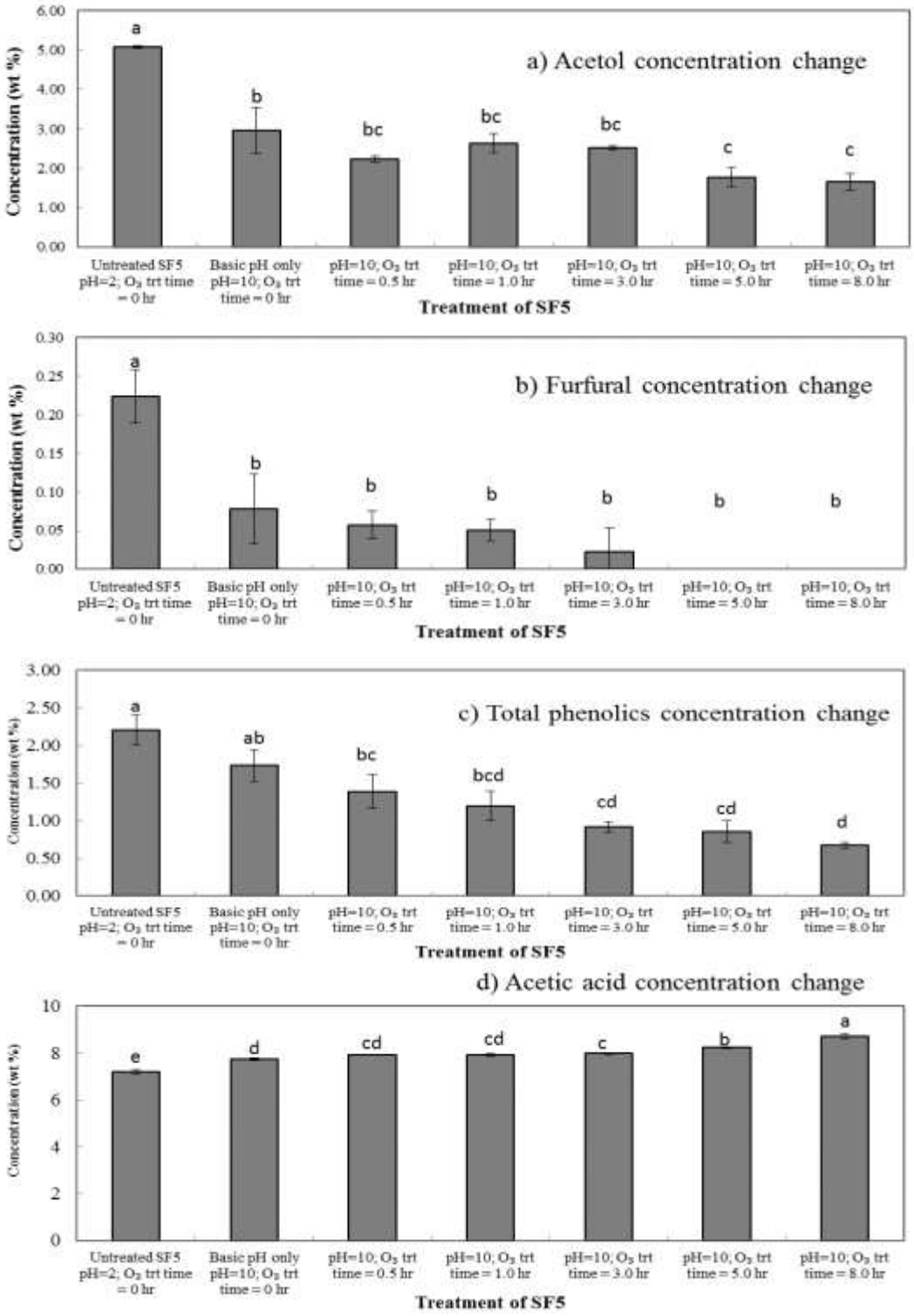


Figure 4-9 Concentration change of major inhibitors in SF5

When concentration is zero, the number was represented as 0.01wt% in statistical test.

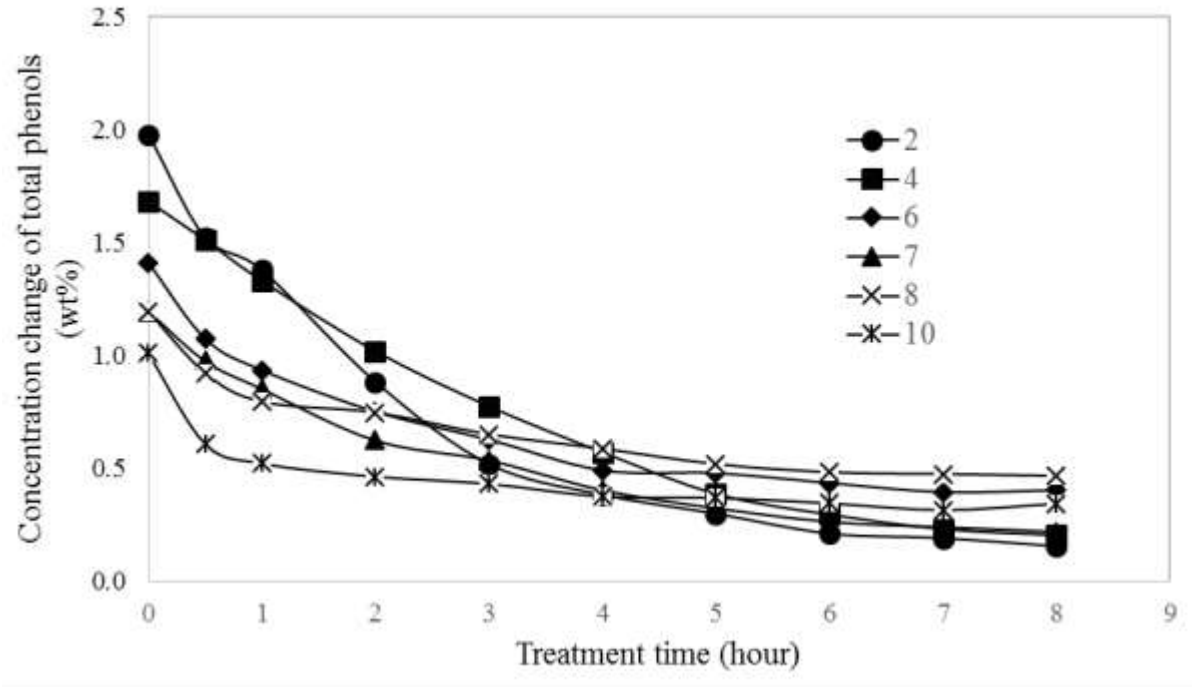


Figure 4-10 Concentration change of phenols in SF5

CHAPTER 5 TOXIC EFFECT OF PYROLYTIC BIO-OIL ON CELL GROWTH AND MEMBRANE PROPERTY IN MICROALGAE *CHLAMYDOMONAS REINHARDTII*

Abstract

Acetic acid derived from fast pyrolysis of lignocellulosic biomass is a promising substrate for microalgae fermentation for producing lipid-rich biomass. However, crude pyrolytic acetic acid solution has an extremely complex composition and contains various toxic compounds inhibiting algal growth. In this work, the inhibitory mechanisms caused by pyrolytic acetic acid, and methods of protect cells from such an inhibition was studied. It was found that acetic acid itself served a carbon source for boosting algal cell growth, but also caused cell membrane leakage, and thus inhibit the cell growth. The optimal acetic acid concentration for highest cell density was 4g/L. Overliming treatment of crude pyrolytic acetic acid stream increase the algal growth which corresponding reduce the cell membrane leakage. Direct evolution of algal strain also increase the cells' tolerance to the pyrolytic acetic acid solution with enhance cell membrane integrity. Statistical analysis shows that there was a correlation between the cell growth performance and the membrane integrity (leakage) but not membrane fluidity. The addition of the cytoprectactant such as Pluronic F68 and Pluronic F127 can enhance the cell membrane integrity and thus enhance the cell growth when growing in pyrolytic acetic acid substrate Collectively, this work indicate that the cell membrane is one major reason for the toxicity of pyrolytic acetic acid when being used for algal culture. To better use this promising pyrolytic substrate, a cell membrane of microorganism need to be strengthened though either strain improvement or addition of membrane protectant reagents.

5.1 Introduction

Conversion lignocellulosic biomass to fuels and chemicals has been traditionally achieved through biochemical or thermochemical pathways. In biochemical process, biomass is converted to reducing sugars through pretreatment and enzymatic hydrolysis followed by microbial fermentation into fuels. In thermochemical process, biomass is treated by pyrolysis or gasification to produce intermediates such as bio-oil or syngas, which are further upgraded into drop-in fuels. As an alternative to these conventional processes, a sequential thermochemical-biochemical processes creates a carbon-efficient pathway for producing fuel products [1]. This new process can be a fast pyrolysis of biomass into pyrolytic substrates followed by microbial fermentation, or a gasification of biomass into syngas followed by syngas fermentation.

Pyrolysis-pyrolytic substrates fermentation represent a promising hybrid process. The process has several advantages including feedstock flexibility, utilization of both the carbohydrates and lignin in biomass, opportunities for distributed processing and promising economic analysis [2]. Various pyrolysis derived compounds have been explored as potential fermentation substrates. For example, anhydrosugars such as levoglucosan has been used for bioethanol production through yeast [3-5] and *E. coli* [6-10], acetic acid have been utilized in yeast [11] or microalgae fermentation for producing lipid-rich biomass [12-14].

Currently, the major challenge in the pyrolysis-fermentation hybrid process is the toxicity of the contaminant compounds in crude pyrolytic substrate [10, 13, 14]. Raw crude pyrolytic substrate stream is an extremely complicated system containing compounds such as acetol, furfural, phenolics, 5- hydroxymethylfurfural (5-HMF), and many unidentified compounds [4]. Some of those compounds have been reported to readily impede the microbial growth. For example, 5-HMF and furfural reacts with NADH in microbial cells and reduced to furfural

alcohols, which decreased the reductive power (i.e. NADH) and growth of microorganisms such as hydrogen producing bacteria [15] and *S. cerevisiae* [16]. Furanic compounds are known for deactivating cell replication, inducing DNA damage, and inhibiting the key enzymes for central carbon metabolism [16]. Phenolic compounds cause the inhibition by altering the permeability of the cell membrane and/or generating reactive oxygen species [16, 17].

Various approaches have been developed to mitigate the inhibitory effects of pyrolytic substrates. For example, crude pyrolytic substrates solution can be fractionated to enrich specific substrates with reduced inhibitory compounds [18]. Inhibitory compounds can be extracted through solvent extraction [7, 19] and adsorbed via activated carbon or biochar [7, 14, 20]. Air stripping was used to remove volatile compounds such as short chain organic acids [7], while overliming was used to mitigate the toxicity of pyrolytic sugars [10] and acetic acid [13] through precipitation of the inhibitory compounds. In addition to those treatment methods, researchers has also developed directed evolution approach to obtain robust strains tolerant high level of inhibitory compounds contained in pyrolytic substrates [13].

Although practical approaches reducing the toxicity of crude pyrolytic substrates have been well developed, the mechanism of the toxicity is still poorly understood. Previous studies on elucidating inhibitory mechanisms have been focusing specific compound with chemical defined medium, the mechanism of inhibition caused by a complex system like crude pyrolytic substrate has not been reported. It has been reported that membrane fluidity and integrity change is one of the important reasons causing the inhibition of many microbial cells ([21-23]). Therefore, the aim of this work is provide a deep insight of the inhibitory mechanism of crude pyrolytic substrate by evaluating the cell membrane property change. The microalga

(*Chlamydomonas reinhardtii*) fermentation using pyrolytic acetic acid as substrate was used as a mode system [24-27].

5.2. Materials and Methods

Microalgae culture

The microalga strain *C. reinhardtii* ST 21 was obtained from Dr. Martin Spalding's laboratory at Iowa State University. The metabolic evolved strain, *C. reinhardtii* YL01, with enhanced tolerance to the toxicity of the pyrolytic acetic acid solution, was developed in our previous research [28]. Both of the two strains were maintained in tris-acetate-phosphate (TAP) medium under the conditions described previously [29].

To evaluate the cell membrane properties of the algal strains, the seed was inoculated into medium containing pyrolytic acetic acid solution. The cells were grown in 250-mL Erlenmeyer flasks containing 50 ml medium. The flasks were incubated in an orbital shaker (130 rpm) at 25^{o}C. A continuous illumination at 110-120 μmol s^{-1} m^{-2} was also provided create a mixotrophic condition in order to further promote the cell growth. The cell density was determined at 730 nm (OD_{730}) using spectrophotometer. Each test was performed in triplicates for statistical analysis.

Preparation and detoxification of acetic acid rich pyrolytic substrate

A fast pyrolysis and stage fractionation unit was used to pyrolyze softwood and prepare acetic acid rich pyrolytic substrate stream [18]. The unit pyrolyze the biomass and fraction the crude bio-oil into 5 distinct stage fraction (SF). Stage fraction #5 (SF5) was rich in acetic acid. Crude SF5 solution was collected in 1-L Nalgene HDPE bottles and stored at 4^{o}C prior use.

Crude SF5 was treated by overliming operation to reduce its toxicity [13]. In brief, SF5 solution was transferred to a 100-ml beaker at room temperature (25^{o}C). Approximate 140 g dry powder of $Ca(OH)_2$ was added to the solution to reach pH10. The temperature of the solution

increased to 80^{o}C shortly after $Ca(OH)_2$ addition. The solution was then stirred for about 1 hour until the temperature returned to room temperature, and then centrifuged at 750 *g* for 5 minutes to remove the precipitants. The supernatants designated as "Treated SF5" were transferred into fresh 50-ml tubes and stored at 4^{o}C prior to use.

Measurement of cell brane leakage and fluidity

The membrane properties of algal cells including leakage and fluidity were measured in this work. A SYTOX Green Nucleic Acid stain method [30] was used to evaluate cell membrane leakage. In brief, algal cells grown at mid-exponential phase were harvested by centrifugation at 4800 *g* for 5 min and rinsed with PBS buffer (pH 7.0) for three times to reach a final cell density of OD_{730}=0.4. The cell suspension was then mixed with 0.5 µM SYTOX Green dye (Invitrogen) and incubated at room temperature for 30 min under a dark condition. The cells were then measured using a Becton Dickinson FACS Vantage flow cytometer equipped with 488 nm excitation. As SYTOX Green dye can only pass through damaged cell membrane and emits green fluorescence after embedded in the nucleic acids, the SYTOX positive cells indicate the cell membrane-damaged cells, which can be distinguished the intact cells (without cell membrane damage).

Mid-log phase cells were also harvested for measuring cell membrane fluidity based on the method described previously [30]. . The broth were centrifuged and cell pellets were washed three time by PBS (pH=7.4) at 4 °C. The density of the PBS-washed cell samples were adjusted to OD_{730}=0.4. 1,6-diphenyl-1,3,5-hexatriene (DPH) dye was dissolved in tetrahydrofuran (THF) with a final concentration of 1 mM to prepare DPH-THF stock solution. 20 µL of DPH-TMA solution was added to 1 mL sample to make a final concentration of DPH to $2x10^{-7}$ M. Then samples were loaded in 96-well plate analyzed with aSynergy 2.0 Microplate reader, where IVH

(horizontal position, Ex=360 nm) and IVV (vertical position Em=450nm) were measured. The membrane polarization ratio can be calculated by P=(IVV - IVHG)/(IVV + IVHG), where G=1 in this case [31].

Transmission Electron Microscopy (TEM) of algal cells.

Algal cells were collected and incubated with 2% glutaraldehyde (w/v) and 2% paraformaldehyde (w/v) in 0.1M cacodylate buffer, pH 7.2 for 48 hours at 4°C. After incubation, cells were pelleted and re-suspended in 1% agarose to concentrate cells together. The agarose was chilled at 4°C for 15 min to solidify. The firm agar was cut into 1mm×1mm cubes which were then treated as follows. Samples were rinsed 3 times in 0.1M cacodylate buffer and then post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at room temperature. The samples were rinsed in deionized distilled water and stained with 2% aqueous uranyl acetate for 1 hour, then dehydrated in a graded ethanol series, cleared with ultra-pure acetone, infiltrated and embedded using EPON epoxy resin (Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 hours at 65°C. Thick and ultrathin sections were made using a Leica UC6 ultramicrotome (Mager Scientific, Dexter, MI). Ultrathin sections were collected onto carbon film copper grids and images were captured using a JEOL 2100 scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA).

Statistical Analysis

All the tests were performed in triplicates, with the results being presented as the mean ± SD. Three-way analysis of variance (ANOVA) and T test were done by the software R (V3.0.2). A *p-value* less than 0.05 was considered significant.

5.3. Result and discussion

Cell membrane properties under different acetic acid concentrations

C. reinhardtii was capable of growing heterotrophically using acetic acid as a carbon source. To investigate the effect of toxic compounds on the cell membrane properties, it is necessary to understand the cell membrane properties as a function of acetic acid. Indeed, high concentration of acetic acid has shown inhibitory effect on the culture *C. reinhardtii* [13].

As shown in Figure 1, *C. reinhardtii* was grown in medium containing acetic acid ranging from 0-12 g/L. The maximum cell density increased with the acetic acid concentration from 0 to 4 g/L, and decreased with further increase of the acetic acid concentration. The biomass yield kept decreasing with the increase of reagent acetic acid concentration. The SYTOX results show that the percentage of the SYTOX positive cells increased with acetic acid, indicating that the cell membrane become vulnerable with the addition of acetic acid. At 8 and 12 g/L acetic acid concentration levels, most of the cells are permeable to SYTOX green, indicating the cell membrane integrity was severely damaged. Correspondingly, the cell growth was also significantly inhibited, presenting significantly decreased maximum cell density and biomass yield. The cell membrane fluidity did not change significantly ($p>0.05$) under different concentration of reagent acetic acid.

Figure 1 shows that 4 g/L acetic acid concentration resulted in the best cell growth performance. This concentration was used through the result of studies in this work. In cased of different levels SF5 (containing 8 wt% acetic acid) was added to the culture, additional reagent grade acetic acid was supplemented to ensure the total acetic acid concentration was fixed at 4/L.

Effects of detoxification treatment of the pyrolytic acetic acid on cell membrane properties

One of major challenges in utilization of pyrolytic acetic acid is the inhibitory residual compounds contained in crude substrate solution, which significant inhibited cell growth [12-14].

Figure 2 shows the growth and the SYTOX results of *C. reinhardtii* ST 21 strain grown in TAP medium containing different levels raw and treated SF5 solution. As shown in the figure, addition of 0.1% of raw SF5 resulted in a 43% decrease of mid log phase cell density, which was associated with increase in SYTOX positive cells from 37% to 53%. When raw SF5 concentration increased to 0.4%, the cell growth was completely inhibited and the SYTOX positive cells reached to 100%.

Our previous research has shown that over-liming ($Ca(OH)_2$) treatment is an effective way to reduce the toxicity of raw SF5 through removing toxic compounds such as furfural, 5-HMF and phenols and the fermentability of SF5 was increased from 0.1 wt% to 5.5 wt% [13]. In this work, the cell growth improvement and the cell membrane property change as a result of over-liming treatment were correlated. As shown in Figure 2, when algal cells were grown in over-liming treated SF5, the cells can tolerate much higher levels of SF5 (up to 4.0 wt%) compared to the raw SF5. For example, 0.4 wt% of raw SF5 leads to completely cell membrane integrity broken while the cells grown under 0.4 wt% of treated SF5 shows similar growth performance and the membrane property as control (P>0.05). The correlation between the cell growth and the SYTOX data indicate that the cell growth inhibition can be explained by the disruption of cell membrane properties.

However, high percentage of treated SF5 can still bring adverse effect on cell growth performance and cell membrane integrity. When the concentration of treated SF5 is increased to 1.0 wt%, even though the maximum cell density can still keep the same as control, the increase of SYTOX positive cell percentage leads to 70% decrease of biomass yield. When the concentration of treated SF5 achieved 4.0 wt%, more than 80% of cells are SYTOX positive,

which is corresponding to significantly decreased maximum cell density, and 95% decrease of biomass yield. Other strategies are needed to enhance cell membrane for better cell growth.

Effects of metabolic evolution of the algal strain to on the cell membrane property

The algal strain ST21 was further evolved in order to enhance its tolerance to the raw SF5 solution [12, 14]. The cell growth and the membrane property of the wild strain ST21 and the evolved strain YL01 were respectively compared under two conditions: without SF5 addition (4 g/L reagent grade acetic acid being added instead); and with SF5 addition (0.4 wt% SF5 plus additional 3.71g/L reagent acetic acid to reach a total final acetic acid concentration as 4 g/L).

Figure 3 shows the cell growth and cell membrane properties changes of ST21 and YL01 strains when reagent grade acetic acid was used. The evolved strain had an overall higher cell density and biomass yield than the wild strain (Figure 3A and 3B). Figure 3C shows that the percentage of SYTOX positive cells of the evolved strain YL01 was less than that of the wild strain ST21. No significant difference was found on cell fluidity among alive cells under different culture conditions.

Figure 4 the cell growth performance and membrane properties of the wild strain and evolved strain when 0.4% SF5 was added. Overall, the trends of these parameters were similar trends as those when growing in reagent grade acetic acid (Figure 3), except that the growth of wild strain was completely inhibited with SYTOX positive cells reached to almost 100%.

Improvement of cell membrane integrity by polymeric cyto-pretectant

The above results indicate that the inhibition of algal growth by the crude pyrolytic acetic acid stream is associated with membrane property of algal cells. To confirm this mechanism, we further tested the use of cell membrane protectant to protect the cell from damaging, and thus increase the cell growth performance in the SF5-containing medium. Some polymeric protectants,

such Pluronic F68 and Pluronic F127 have been used as effective protectants in mammalian cell culture by interacting with cell membrane without adversely affecting cell growth [32]. In this work, these pluronic polymers were used to protect algal cell from membrane damage caused by the inhibitory compounds in pyrolytic substrates.

As shown in Figure 5A, when the cells were grow in medium without SF5 addition (control), i.e., only regent grade acetic acid was used, the cell growth from the protectant-free medium was not significantly different from those growing protectant-containing culture. However, when 0.2% raw SF5 was added to the medium, the growth of the cells without protectant was significantly inhibited, while the cells added with Pluronic F68 and Pluronic F127 resulted in the similar growth performance as those in the control culture. For the evolved algal strain added with 4.0 wt% treated SF5, cells could keep alive even without protectant addition. However, the cell growth is poor compare to those addition with Pluronic F68 and Pluronic F127. The biomass yield showed similar trend as cell growth. Figure 5C shows that the percentage of the SYTOX positive cells reduced significantly ($P<0.05$) when Pluronic F68 and Pluronic F127 were added to the medium throughout all the experimental conditions tested. The data clearly indicate that the two Pluronic polymers protected cells via avoiding cell membrane leakage, which resulted in an improved cell growth. Overall, the protective effect of the two Pluronic acids were similar when raw SF5 was used in the medium, while at 4% treated SF5, the Pluronic F127 had a better protection than Pluronic F68 which is evident from the lower STYOX positive cells when this protectant was used (Figure 5C). Figure 5D shows that there are no significant change of the membrane fluidity of algal cell treated with different types of protectants.

Correlation of the cell growth and membrane properties under different conditions

Previously results showed that the cell growth inhibition is related to the change of cell membrane properties such as membrane leakage and fluidity. However, studies on quantitative analysis of the relationship between cell growth and the membrane properties is very limited. In this work, the correlation between cell growth (represented by maximum cell density and biomass yield) and cell membrane property (represented by cell membrane leakage and cell membrane fluidity) were statistically analyzed and the results are presented in Figure 6. As shown in the Figure, based on the linear regression, the cell growth is significantly ($P<0.05$) correlated with the damage on cell membrane integrity, but is insignificant ($P>0.05$) with cell membrane fluidity. As a matter of fact, the results presented in previous sections indicate that the cell membrane fluidity from samples are very similar among different culture conditions investigated. The result shows that algal cells growth under the presence of pyrolytic acetate can be disrupted by the broken of cell membrane; however, as long as algal cells are alive, the cell membrane would keep similar fluidity.

Cell membrane visualization

The above SYTOX results indicate that the inhibitory effect of SF5 on the algal growth was mainly due to the cell membrane damage caused by the toxic compounds contained in SF5. We further used transmission electron microscopy (TEM) to visualize the cell membrane change. As shown in Figure 7A, the wild strain cells growing in SF5-free medium showed a cohesive morphology with minimal cell membrane damage. With the addition of the 0.2% raw SF5, the cells were not viable with severe damage was observed in the membrane and organelle (Figure 7B). Under the same concentration of raw SF5, however, the addition of protectant Pluronic F68 significantly improved the cell integrity even though some damage of cell membrane and loss of

part of cytoplasm were still observed (Figure 7C). Figure 7D indicated that the evolved strain (YL01) basically kept its integrity even treated with a higher SF5 concentrations (4%), indicating the significant gain of tolerance to crude pyrolytic substrate solution.

5.4 Discussion

Use of pyrolytic substrate for microbial fermentation is a promising technology for producing fuels and chemicals from lignocellulosic biomass. However, this technology is currently impeded by the toxicity of the crude pyrolytic substrate steam, due to various contaminant compounds resulted from the biomass pyrolysis [1]. The investigation of the microbial growth inhibition mechanism is necessary to improve the effectiveness of hybrid processing. Previous study mostly focused on using the model contaminant compounds to their study the inhibitory effect on bacteria or yeast growth, and most of these model compounds were the derivatives during biochemical processing of lignocellulosic biomass or syngas fermentation. In this work, we used the “real” mix of the inhibitory compounds derived from the fast pyrolysis of lignocellulosic bio mass, which is more presentative.

It is reported that cell growth inhibition is related to cell membrane property change under chemical stress. For example, various phenolic compounds were reported to have adverse effect on bacteria, fungi and microalgal cell membrane integrity. The degree of inhibition depends on the media pH, and lipophilicity, degree of ionization and the specific chemical structure of phenolic compounds [33].

The mechanisms how phenolic compounds inhibit yeast cell growth is investigated with thymol as the model compound [34]. The inhibition can be carried out through decrease cell membrane structural compounds or activating specific signaling pathways in yeast cells. As to cell membrane lesion, pyrolytic compounds can inhibit the biosynthesis of ergosterol or binding

with ergosterol, a key structural component in cell membrane. Based on this mechanism, the decrease of cell membrane integrity resulted from phenolic compounds may be associated membrane fluidity change because ergosterol regulates membrane fluidity. Thus it is quite possible that pyrolytic acetate (with 2 wt% phenolic compounds) can significantly affect the cell membrane property and inhibit cell growth.

When evaluating the damage of pyrolytic substrate on cell membrane, the cell membrane-damaging effect from acetic acid also needs to be considered, although this compound was also serving as the carbon source for algal cells. Our result (Figure 1) shows that for the culture of *C. reinhardtii*, acetic acid served as both a cell growth "booster" (as a carbon source) and cell growth inhibitor (as damaging the cell membrane integrity) Within the range of 1~4 g/L acetic acid, the cell growth increase may overcome the damage of cell membrane, as a result, the maximum cell density increased with the acetic acid concentration. When the concentration of acetic acid exceeded 4 g/L, the cell membrane integrity damage become severe and thus, the cell density appeared to reduce.

When studying the toxic effects of pyrolytic acetate solution SF5 on the cell membrane properties, SF5 was blended with reagent acetic acid to reach a 4g/L total acetic acid concentration in the medium. It was found that 0.2 wt% of raw SF5 can severely damage the cell membrane integrity and lead to completely death of algal cells (Figure 5). Through overliming treatment, a large amount of contaminant compounds in SF5 were removed [13], thus overliming-treated SF5 significantly decreased damage on cell membrane integrity (Figure 2). In another word, the SF5 concentration that cells can tolerate increased.

Compared to wild strain direct evolution algal strain showed significantly decrease in cell membrane leakage when treating same concentration of pyrolytic acetate. The maximum SF5

concentration that the evolved algal strain can tolerate also increased as compared to the wild strain. However, the evolved strain shows no significant change of cell membrane fluidity (P>0.05). Previous results have shown that evolved strain can tolerate higher concentration of SF5 but without providing the mechanism behind such an enhanced tolerance [14] . The results obtained in this work demonstrated that the directed evolution enable the algal strain the ability to strengthen cell membrane. Regardless the leakage is resulted from pyrolytic acetic acid or pure acetic acid, and this ability can be passed on to next generation during the continuous evolution process [35]. The result indicates that during direct evolution *C. reinhardtii* cells obtained some changes on its membrane property and these changes are associated with metabolic events happened under the toxic stress. The ability of evolved strain obtained from direct evolution under a specific type of toxic stress might be applied to increase tolerance to another type of toxic stress, as the inhibition mechanism is the fundamental mechanism for those toxic effects.

Our results also shows that cytoprotectant also benefit membrane integrity of algal cells. Some cytoprotectants have been reported to strengthen cell membrane under stress. For example, Pluronic F68, a polymeric substance containing polyol group, was used as an effective protectant in animal cell culture by generating a layer of a Pluronic polyol on the cell surface without adverse effect on cell growth [32]. The protective effect is hypothesized to be associated with the polyols interaction with cell membrane [36]. In this work, it was found that pluronic substances such as Pluronic F68 and Pluronic F127 were capable of protecting membrane damage of algal cell as well. A small amount of cytoprotectant significantly improved the algal cell membrane integrity under different stressful conditions caused by crude pyrolytic acetic acid solution (Figure 5). Such a protective effect led to improved growth performance of algal cell

with presence of pyrolytic acetate. It should be noted that such a protective effect varies based on the protectant types, chemical stresses conditions and culture time.

5.5 Conclusions

This study demonstrated that the growth performance and ability to uptake of acetic acid as carbon source of algal cells disruption by pyrolytic acetate might be realized through the broken of cell membrane. Overliming treatment of SF5, direct evolution and addition of organic protectants are possible methods, which can significantly protect cell membrane integrity and improve the cells tolerance of SF5 to 40 g/L, 4 g/L and 2 g/L, respectively. The mechanism behind each methods are quite different and need further investigation.

5.6 Acknowledgement

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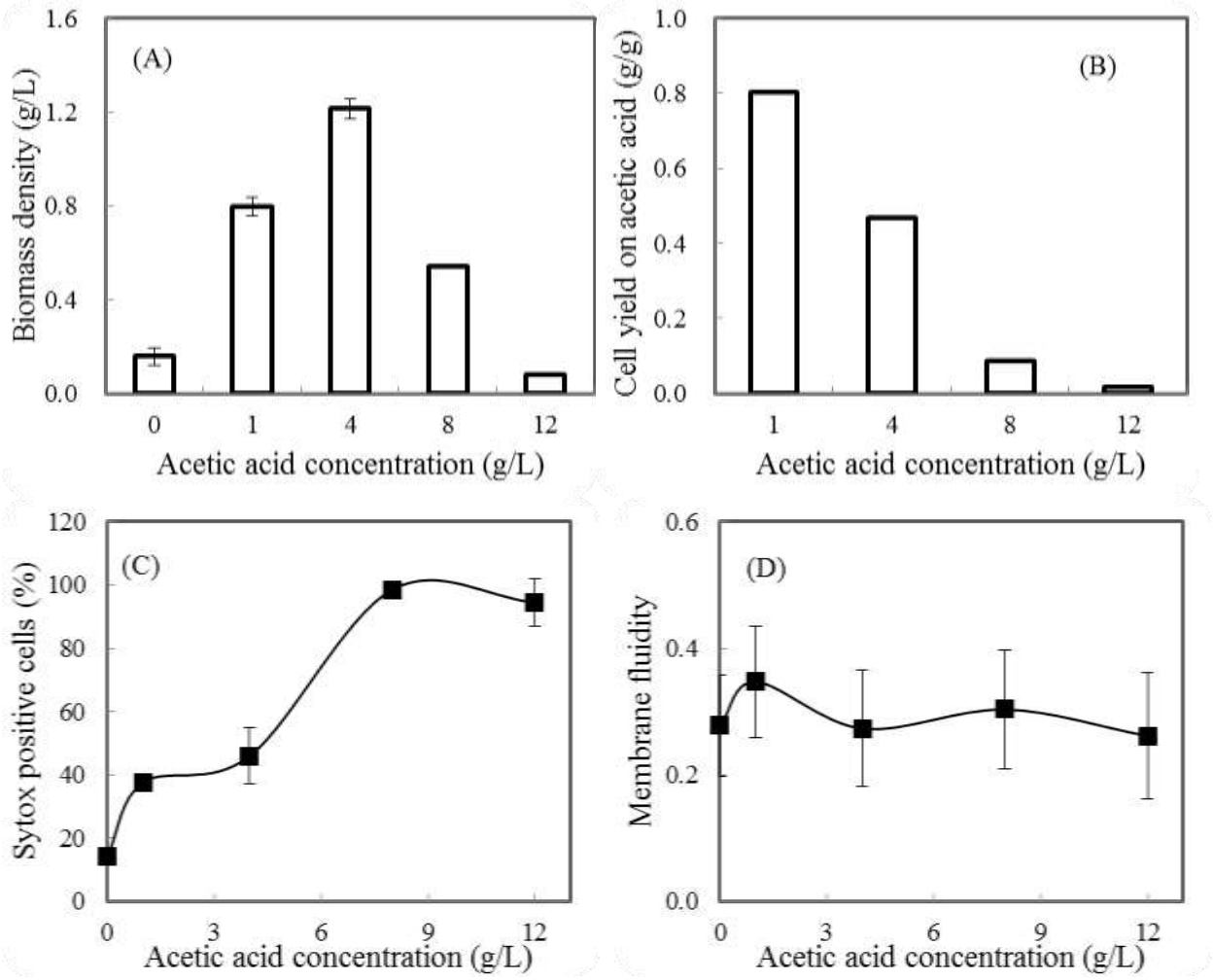

Figure 5-1 Comparison of wild type under different concentrations of acetic acid

A) Biomass; B) Yield; C) Membrane leakage; D) Membrane fluidity

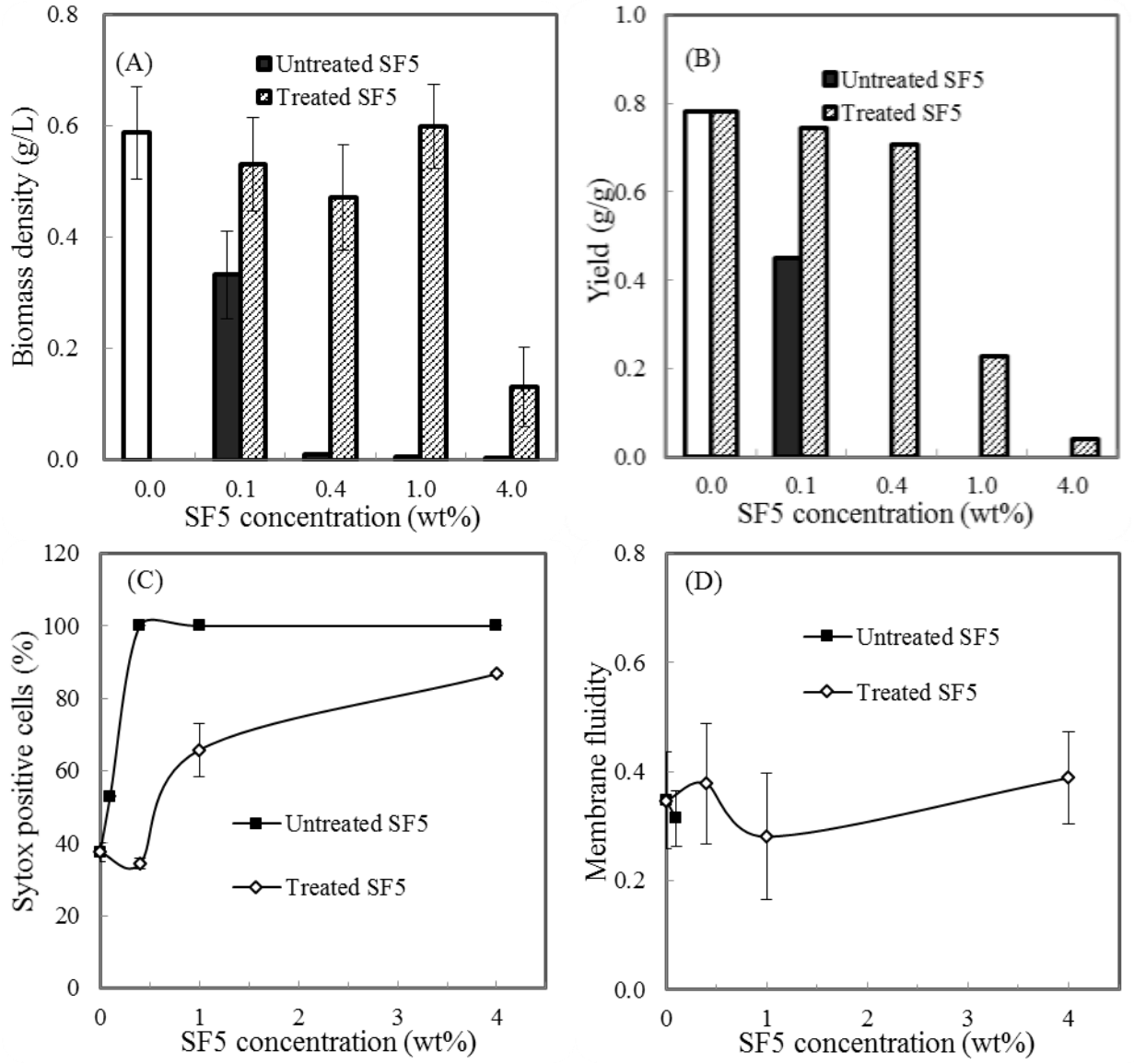

Figure 5-2 Comparison of cell performance under raw/treated SF5

A) Biomass; B) Yield; C) Membrane leakage; D) Membrane fluidity

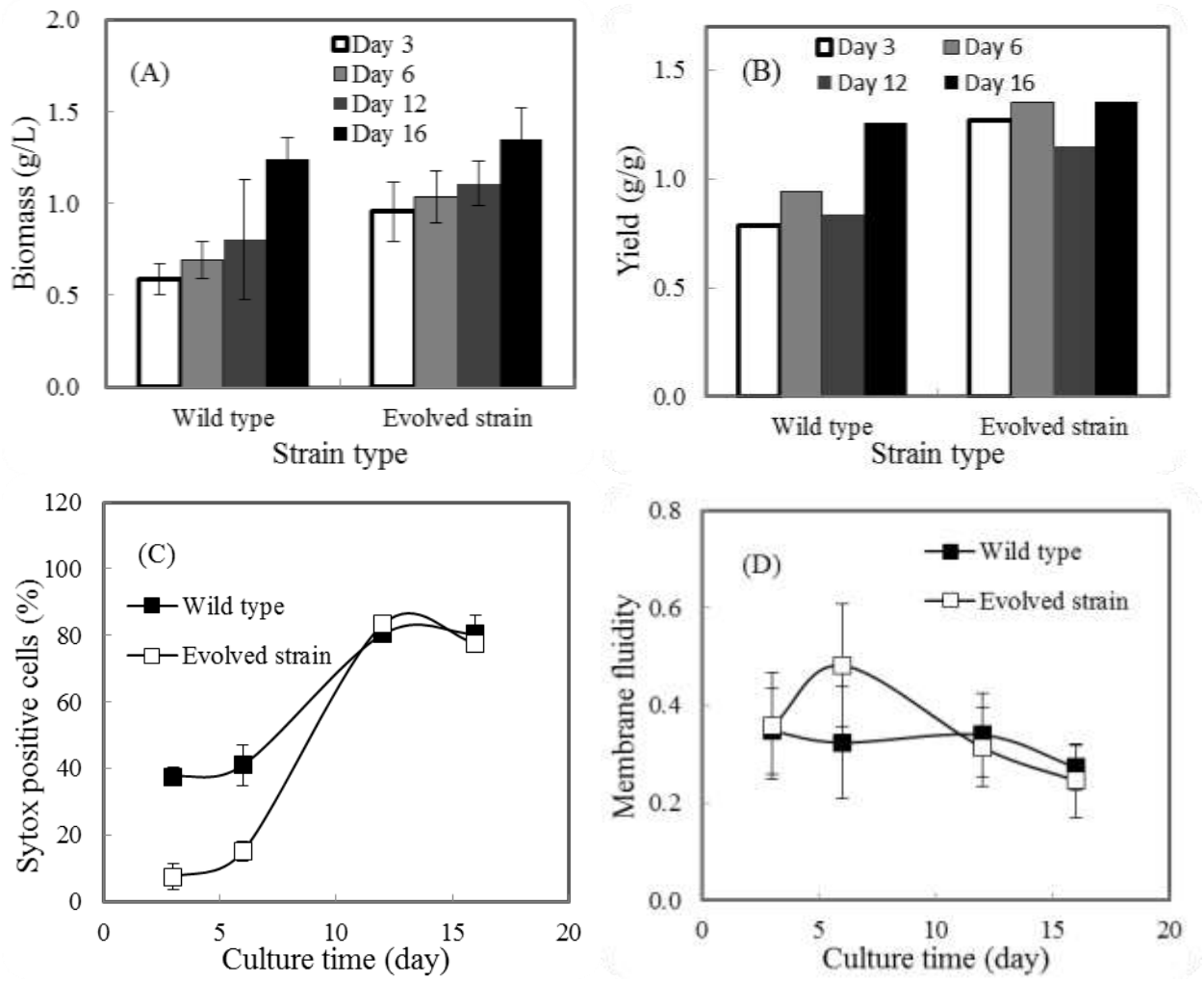

Figure 5-3 Comparison of wild type and evolved strain under TAP medium.

A) Biomass; B) Yield; C) Membrane leakage; D) Membrane fluidity

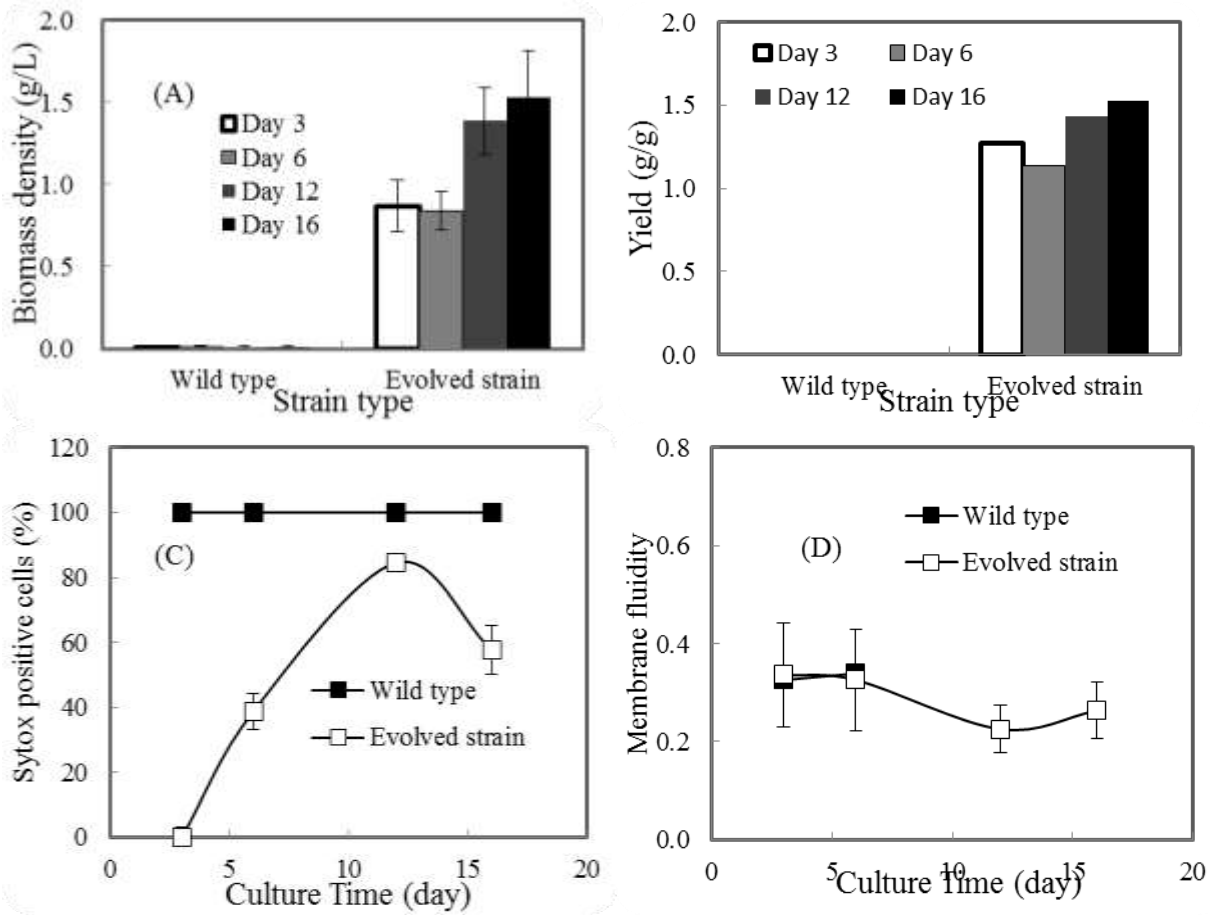

Figure 5-4 Comparison of wild type and evolved strain under 0.4 wt% of raw SF5

A) Biomass; B) Yield; C) Membrane leakage; D) Membrane fluidity

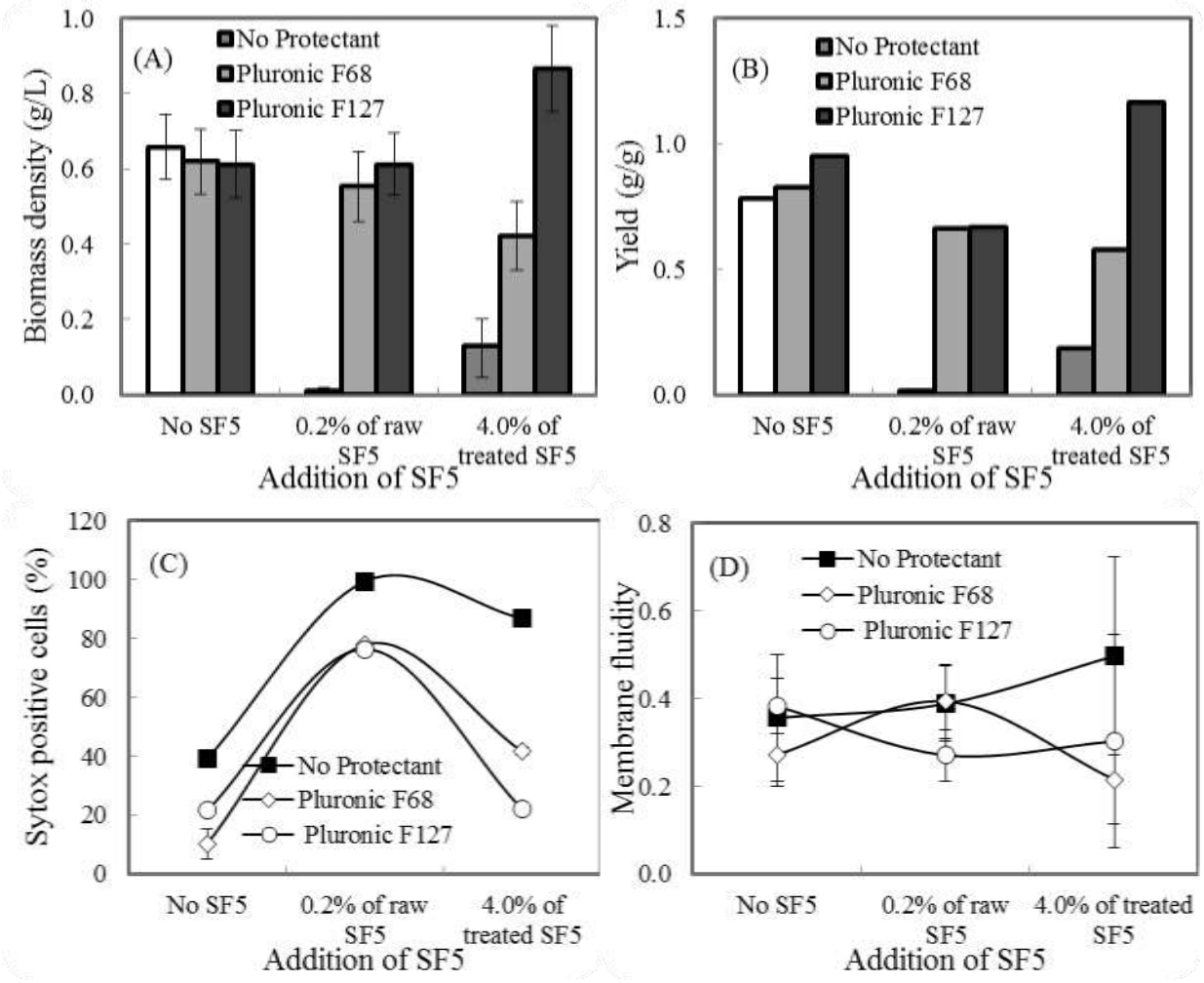

Figure 5-5 Comparison of cells under different types of protectants

5a) Growth 5b) Yield 5c) Leakage 5d) Fluidity

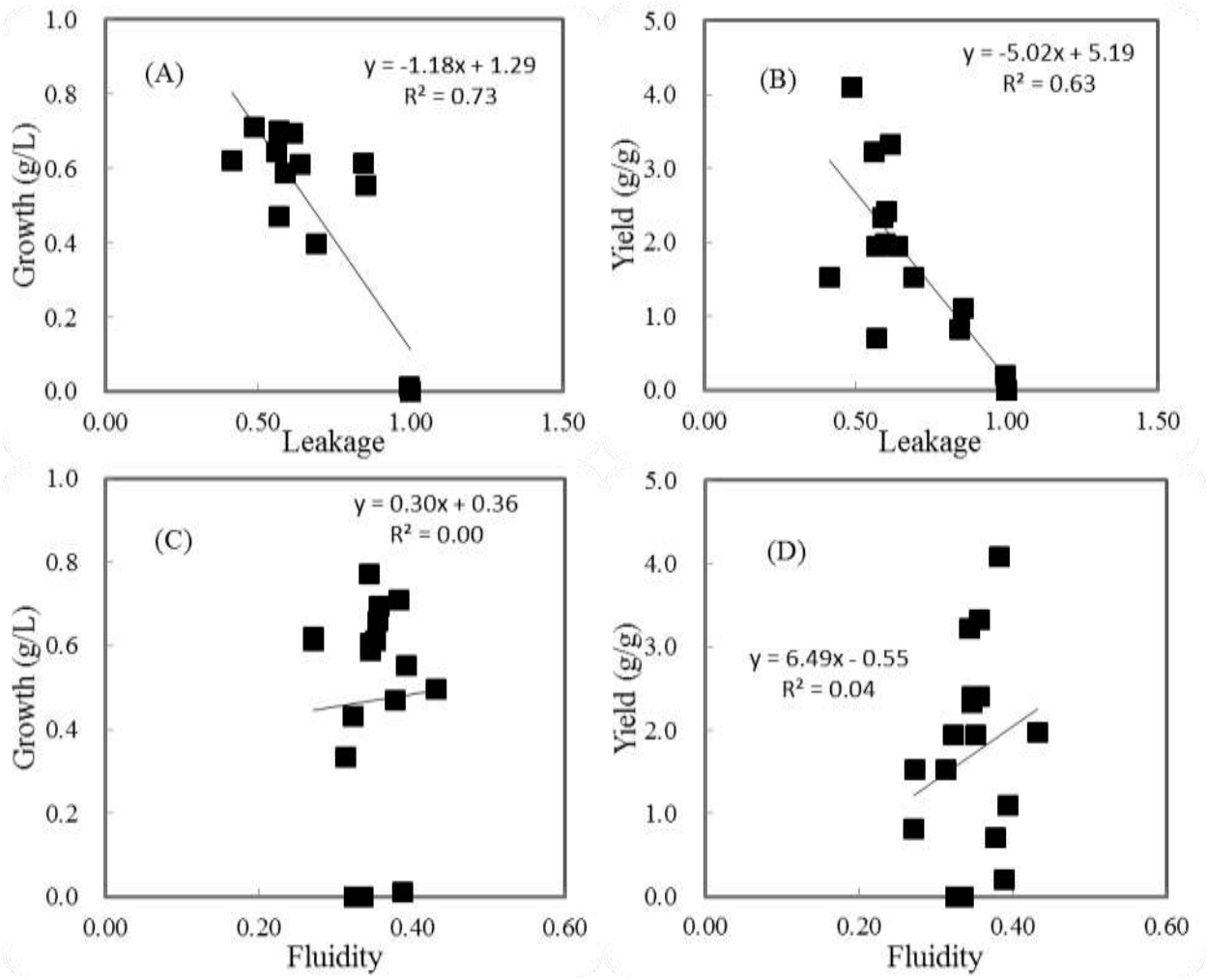

Figure 5-6 Relationship between Growth and membrane property of algae cells

(wild type; culture day <10; acetate concentration is 4 g/L)

6a) growth ~leakage 6b) yield ~ leakage 6c) growth ~fluidity 6d) yield ~fluidity

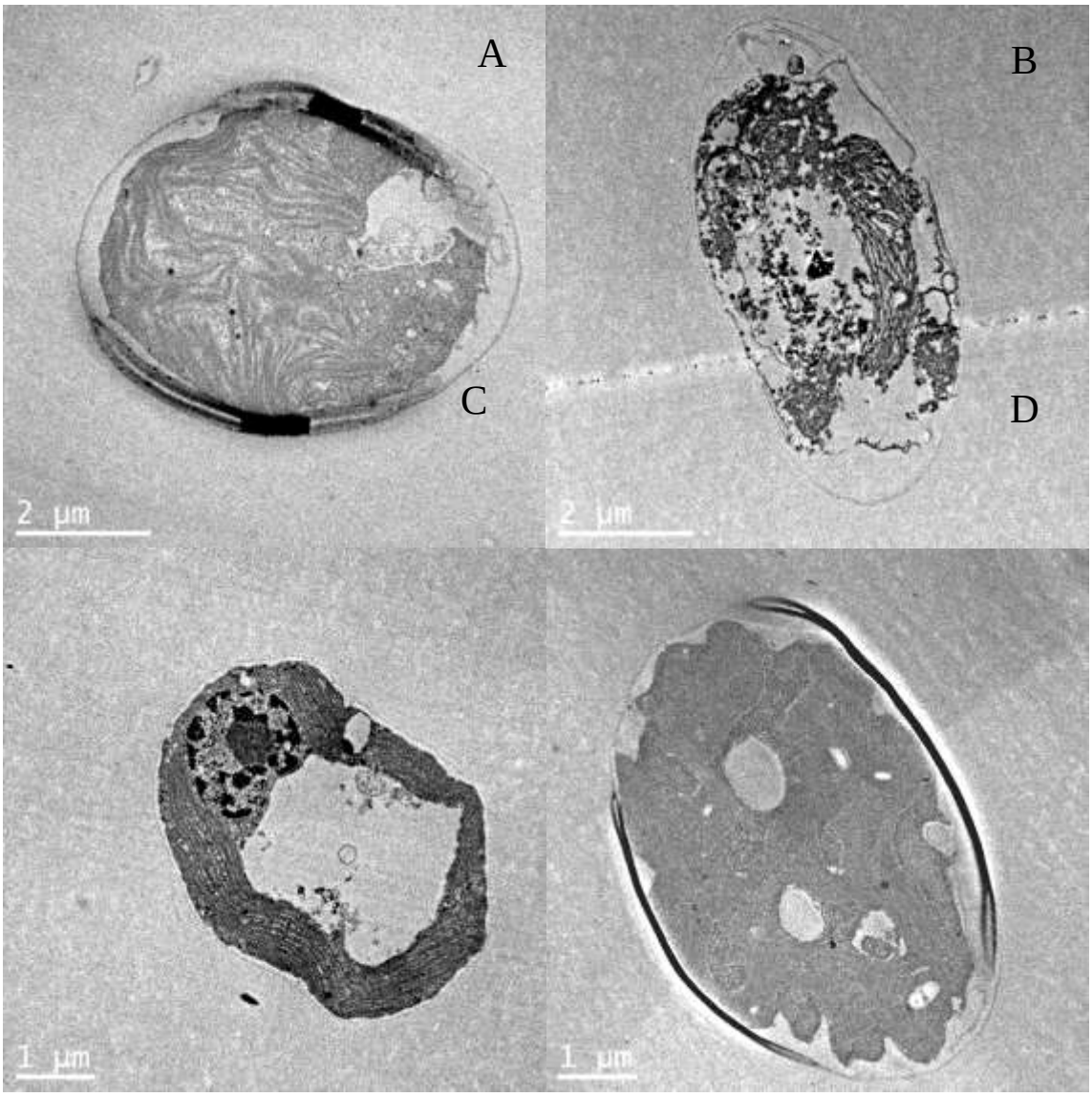

Figure 5-7 Visualization of cell membrane integrity by transmission electron microscopy

A: control (wild type under pure TAP medium)
B: wild type under 2 g/L of untreated SF5
C: wild type under 2 g/L of untreated SF5 with 0.2 wt% of Pluronic F68 addition
D: evolved strain under 4 g/L of untreated SF5

CHAPTER 6 PERFUSION STRATEGY TO IMPROVE MICROALGAE FERMENTATION OF DETOXIFIED PYROLYTIC BIO-OIL

Abstract

Pyrolytic acetate derived from lingo-cellulosic biomass is a promising substrate for microalgae fermentation of lipids production. However, pyrolytic-acetate-containing bio-oil has an extremely complex composition and high inhibition on microorganism fermentation. This study demonstrated different types of cultivation in microalgal mixotrophic growth for utilization of fast pyrolysis bio-oil, like two stage fed batch, continuous cultivation perfusion strategy with cell retention by hollow fiber membrane and the combination of all these cultivation strategies. As a result, 2.05 g/day/L, as 10 times high cell productivity as batch culture can be achieved. In summary, this result shows an effective cultivation method to improve the fermentative production of biofuels and biobased chemicals through microalgal fermentation with pyrolytic substrates.

6.1 Introduction

Bioenergy and biochemical production from lignocellulosic biomass gains more and more attention recently (Neha Srivastava, 2015; Prashant Katiyar2015; Na Wei, 2015 Gholamreza Salehi Jouzani 2015; Davide Dionisi, 2015 Furkan 2015). In 2014, the global production of bioethanol and biodiesel increased by 6.0% and 10.3%, respectively (BP 2015). The production of fatty acid, lactic acid, PHA and PHB, and biolipid and so on from biorenewable biomass, drawn considerable interest as potential substitute as that produced by chemical manufacturing. Lignocellulosic biomass, as waste residues from agriculture, forestry and energy crop systems, is an abundant worldwide biorenewable resource for production of second generation biofuel [Sonil Nanda , 2015; 26]. With significant improvement in conversion technologies and efficiency, lignocellulosic biomass can provide a major proportion of the future energy supply (250–500 EJ per year by 2050) [27].

Hybrid processing, converting lignocellulosic biomass to syngas or bio-oil by thermochemical processing, followed by bioenergy and biochemical production through micro-organism fermentation, is a promising way to utilize lignocellulosic biomass. For example, through fast pyrolysis, at moderate temperature, absence of oxygen and high heating rate, lignocellulosic biomass can be converted to syngas (13~25 wt%), bio-oil (60~70 wt%) and biochar (12~25 wt%) [8, 9]. During syngas fermentation, microorganism catalyst can produce ethanol, acetic acid and other byproducts such as butanol and butyrate through acetyl-CoA pathway.

Inhibition of bio-oil on fermentation is unavoidable. Bio-oil can be fractionated into different portions suitable for different fermentation goals, such as antisugar rich portion

fermentation for bioethanol production and acetic acid rich portion fermentation for fatty acid production.

However, there is a main challenge for biochemical processing of syngas or bio-oil, which is their high inhibition on cell growth. As to acetic acid portion of bio-oil, it contains hundreds of ketones, aldehydes, and phenols as well as acids, which might be the inhibitors to limit cell growth through broking cell membrane integrity. Due to its complexity, even after adsorption/oxidative/alkaline treatment, only a small amount of bio-oil, or its portions can be used as the carbon resource during fermentation. Further strategy is needed for better utilization of bio-oil and higher productivity of bioenergy and biochemical.

Cell retention fermenters, with decoupling of hydraulic retention time (HRT) and solid retention time (SRT) might be potential to increase to achieve the goal listed in last paragraph. It is reported that cells can be entrapped in calcium alginate beads, leading to SRT >> HRT for continuous ethanol production with 38 g/L average cell density. Cell retention via membranes, hollow fiber membrane or depth filter perfusion system can hold all of the cell in the bioreactor while spending used media out. Settling of cell by gravity especially enhanced by flocculation or aggregation has already applied in commercialized ethanol plants. Among these cell retention systems, hollow fiber membrane perfusion system, with media flow tangentially to membrane, has the advantage to cycle big volume of media while the rate to spending used media is low, but faces the challenge of overgrowth of cells, and consequently membrane fouling.

Fermenters with perfusion culture can be applied in mixotrophic culture of microalgae for acetic acid rich pyrolytic bio-oil portion utilization, with capability of using high amount of pyrolytic acetic acid and high productivity of biochemical. This work is

aiming to test the toxicity of acetic acid rich bio-oil portion, apply perfusion culture system design to fermenters and optimize the continuous production of bioenergy and biochemical with Microalga *Chlamydomonas reinhardtii* (*C. reinhardtii*) as the employed strain.

2. Materials and methods

Microalgae culture and monitoring

The microalga *C. reinhardtii* ST 21 strain used in this work was obtained from Dr. Martin Spalding's laboratory at Iowa State University. Seed culture was established in in 250-mL Erlenmeyer flasks containing 50-mL tris-acetate-phosphate (TAP) medium under previously described procedure [1]. *C. reinhardtii* growth test was performed in flasks with 50 ml medium and 5 ml algal seed in each well. The flasks were shaken by an orbital shaker with a speed of 130 rpm. Ambient temperature was set at 25^{o}C. Continuous illumination at 110-120 μmol $s^{-1}m^{-2}$ was provided. The optical density of each sample at 730 nm (OD_{730}) were measured via spectrophotometer. Each test was performed in triplicates for statistical analysis.

SF5 preparation and storage

The fast pyrolysis and bio-oil stage fractionation were done at Central Iowa Research and Demonstration Farms by Dr Robert C. Brown's group. [2] The acetic-acid-rich SF5 was collected in 1-L Nalgene HDPE bottles. It was stored at 4^{o}C and would be shaken up before each use.

Alkaline treatment of SF5

SF5 solution was transferred to a 100-ml beaker. Different alkali species including NaOH, KOH and $Ca(OH)_2$ (in the form of dry power) were respectively added to the solution to reach pH 10. The solution temperature increased to around 80oC shortly after alkali addition

(within 10 minutes). The solution was stirred with a magnetic stir bar for about 1 hour until the temperature reduced and stabilized at room temperature. The treated SF5 solution was then centrifuged at 750 g for 5 minutes to remove the precipitants. The supernatants were transferred into fresh 50-ml tubes and stored at 4 oC prior to use.

Fermentation parameters

The fermentation was carried out in a bioreactor with 3L working volume. All of the media was autoclaved uner 121 ℃ for 2 hours. The growth parameters of algal cell were listed as below: Batch a) microalgae fermentation with pure TAP medium (4 g/L acetic acid); Batch b) microalgae fermentation with TAP medium (3.1 g/L acetic acid) + 1 wt% of treated SF5 (0.9 g/L acetic acid); Fed batch c) microalgae fermentation with pure TAP medium (3.1 g/L acetic acid) for 7 days and then added 1 wt% of treated SF5 (0.9 g/L acetic acid); Bleeding d) Bleeding rate = 0.1 ~ 0.7 day^{-1} (0.2 ~1.4 mL/min); Perfusion e) Perfusion rate = 0.7 day^{-1} (1.4 mL/min); Perfusion culture with cell bleeding f) Bleeding rate = 0.1 ~ 0.7 day^{-1} (0.2 ~1.4 mL/min) while Perfusion rate = 0.7 day^{-1} (1.4 mL/min). During the fermentation, the temperature was controlled at 20 ℃. Air was bubbled to the fermenter with a flow rate of. Continuous illumination at ~100 μmol $s^{-1}m^{-2}$ was provided. Samples were taken every half days for batch culture, and one sample per day was taken when dilution rate is not zero. The optical density of each sample at 730 nm (OD_{730}) were measured via spectrophotometer. Each test was performed in triplicates for statistical analysis.

3. Results and Discussion

Utilization of acetic acid of Chlamydomonas reinhardtii

Many microalgae strains were reported to have heterotrophic metabolism, or a ‘dark mechanism’, which is essentially the same as non-photosynthetic organisms. Daniela et al.

(2015) reviewed a bunch of microalgae species capable of utilize organic carbon sources to achieve high productivity of biomass and value-added products. The benefits of heterotrophic growth are less lighting and O_2 requirement, high cell density and productivity, easy-operation bioreactors and low costs. As to *C. reinhardtii*, the algal species is potential for bio-lipids and protein production through heterotrophic growth with acetate as the carbon source. In addition, the species is amenable for genetic manipulations [15,16,17] for new strain with higher productivity or resistance to toxic effect from substrate or product.

As a result, *C. reinhardtii* is suitable as a model strain in heterotrophic growth test for bioenergy and biochemical production. In this work, due to the providing of light to cells, the cultivation is composed both by heterotrophic growth and autotrophic photosynthesis. The cells during mixotrophic cultivation can utilize both oxygen from common air and CO_2 generated during heterotrophic growth. Based on previously reported flask culture, the total fatty acid concentration of *C. reinhardtii* can achieve 20 wt% of biomass when cultured with pure TAP media and only 10 wt% of biomass when more than 1wt% of pyrolytic bio-oil acetic acid rich portion was added. The cultivation of *C. reinhardtii* in different types of bioreactors were listed at Table 6-1.

The cultivation of *C. reinhardtii* was established in a batch bioreactor with acetate as the carbon substrate as control. After a 3 day lag phase, the strain started growing exponentially with a specific growth rate of 0.70 day^{-1}. After 8 day culture, the cell density become stable and the growth rate decreased to zero. The maximum cell density is achieved at day 9, which is 4.13 g/L. All of the cells were harvested at day 10 and the maximum cell productivity was 1.38 g/L/day. The maximum productivity of fatty acid may achieve 0.28 g/L/day.

Then *C. reinhardtii* was used to test mixotrophic growth with pyrolytic acetate as carbon resource. It is reported that SF5 contains lots of toxic compounds, such as weak carboxylic acids, aldehydes, ketones and phenols, which can inhibit microalgae cell growth through broking cell membrane integrity and adversely affecting cell breath and so on. Based on previous multiwall plate culture and flask culture result, *C. reinhardtii* cannot tolerate more than 0.1 wt% of untreated SF5. In batch culture with 1 wt% of untreated SF5, inoculated cells become dead at day 2 (data not shown).

After alkaline treatment, the toxicity of SF5 decreased significantly. When *C. reinhardtii* was cultured with 1 wt% of overlimed SF5, cell growth was found. 5 day were cost for this strain to get adapted to the environment and start rapid growth with a specific growth rate of 0.41 day^{-1}. After 12 day culture, the cell density become stable and the growth rate decreased to zero. The maximum cell density is achieved at day 12, which is 2.23 g/L. All of the cells were harvested at day 13 and the cell maximum productivity was 0.20 g/L/day. The maximum productivity of fatty acid may achieve 0.02 g/L/day. Compared to control batch fermentation, the growth performance and product formation was significantly limited by 1 wt% addition of SF5, even already detoxified by overliming. Thus some fermentation strategies for further increasing of utilization of SF5 and productivity of biomass and product are needed.

Fed-batch

Fed batch cultivation provides an effective technique for high cell density fermentation. It is reported to be commercialized in pharmaceutical and protein production. By controlling the rate of the addition of carbon and energy source, osmotic or toxic effects from substrate can be eliminated. In addition, with higher cell density, the substrate

inhibitory effect per cell would be lower compared to batch cultivation. Fed batch strategy improves the productivity of product formed strictly associated with cell growth.

As to bioreactor where product accumulation is negatively correlated to the cell density, or product accumulation and cell density would be affected differently by the culture condition, fed batch cultivation can also be effective to improving the productivity. Lipid usually accumulated at the cost of cell density. It is reported that high lipid accumulation is corresponding to low cell density and vice versa in *C. reinhaditii*. Astaxanthin can be produced from heterotrophic growth of microalga, Haematococcus pluvialis with acetate; however, high light density is needed for astaxanthin accumulation but inhibitory to immature cells growth. Multiple fed batch stage in sequential mode may solve these problems.

In this work, *C. reinhaditii* was cultured in two stages. At the first stage, this strain was inoculated into the 3 L bioreactor, and cultured with pure TAP media like a batch culture for one week, when the cell density is about 3.21 g/L. And then 30 mL of overlimed SF5 was added to the fermenter and the second culture stage started. After another 3 day culture, the cell growth is almost stopped. The maximum cell density is achieved at day 10, which is 5.45 g/L. The maximum cell productivity was 1.64 g/L/day. The total productivity of fatty acid may achieve 0.16 g/L/day.

Compared to the batch culture with 1 wt% of overlimed SF5, it is clear that both high cell density and high productivity was achieved via fed batch cultivation. However, there are still several drawbacks for this type of bioreactors. Firstly, with the accumulation of cells and products, inhibitory effect may emerge due to high concentration of metabolites and product. Secondly, the scaling up of fed batch cultivation will occupy large amount of area. Both of

these two problems were reported to be solved via perfusion technology. Last but not the least, no productivity can be obtained during fed batch cultivation. Continuous culture following the fed batch cultivation when high cell density is achieved may provide a chance to optimize the cell productivity.

Continuous culture for improved productivity

Continuous cultivation can achieve optimized productivity but low cell density. Microalgal biomass production in large-scale generanlly is in continuous culture via raceway ponds (Terry and Raymond, 1985; Molina Grima, 1999) and tubular photobioreactors (Molina Grima et al., 1999, Tredici, 1999 and Sánchez Mirón et al., 1999). Biomass increase via photosynthesis during daylight and as much as 25% of biomass might be lost during the night via respiration. Another advantage of continuous culture is that reliable measurement of bioenergy and biochemical productivity can be achieved during steady state.

In this work, a range of dilution rates were applied to mixotrophic cultivation. The growth and acetic acid concentration change was shown on Figure 6-3. With dilution rate is 0, the steady state cell density is about 5.70 g/L. With the increase of dilution rate, the steady state cell density decreased. Cell washing out happened when dilution rate is 0.35 day-1. The optimized cell productivity, 0.43 g/L/day was achieved when dilution rate 0.15 day-1. Compared to the two stage fed batch cultivation, the cell density where optimized cell productivity is achieved is lower in continuous culture. Optimized cell productivity in continuous cell culture is less than the cell productivity in two state fed batch cultivation due to the high toxicity per cell from SF5.

Perfusion culture for improved density

Perfusion strategy can be applied in bioreactors for high cell productivity and eliminated metabolite inhibition. Perfusion culture with hollow fiber membrane retention cell is reported to be applied to yeast fermentation for bioethanol production, microalgae mixothrophic culture for enhanced astaxanthin and fatty acid production. However, perfusion culture with hollow fiber membrane retention of cells for high cell density cultures meets the major challenge: membrane fouling and decreased light density. Reported methods to clean membrane are pressure pulsing and backwashing, which are applied in commercial monoclonal antibody production by animal cells and commercial wastewater treatment plants, respectively.

In this work, after 25 day culture, the cell density can be stable and achieve ~30 g/L, which is as 10 times high as batch culture. However, all of the cells were hold and transfer back to the bioreactor, no cell productivity during perfusion cultivation. If harvested at day 26, the theoretical productivity is ~1.14 g/day/L. Since perfusion strategy is helpful to increase cell density and eliminate metabolites inhibition, adding it to continuous cultivation and the optimized cell productivity might be further improved.

Combination of Bleeding culture and Perfusion culture

In this investigation, perfusion rate was set as 0.7 day^{-1}, the bleeding rate varies from 0.1 to 0.5 day-1 (Figure 6-5). With perfusion strategy, all of the cells were kept inside of the hollow fiber membrane and transferred back the bioreactor. The cell retention brings several benefits to continuous cultivation. Firstly, washing out cells from the bioreactor is hard. When bleeding rate is 0.5 day^{-1}, the cell density is still as high as 2.77 g/L. Secondly, the advantage from two stage adding of pure TAP media and SF5 at cell density is kept, which means inhibition form SF5 on cell basis is eliminated. Thirdly, the optimized cell

productivity and fatty acid productivity is increased to 2.05 g/L/day, which is as 10 times as that in batch cultivation. Last but not the least, the yield on acetic acid basis improved a lot when perfusion strategy is added to continuous culture (Figure 6-7).

6.4 Conclusions

This study demonstrated different types of cultivation in microalgal mixotrophic growth for utilization of fast pyrolysis bio-oil. Two stage fed batch cultivation is helpful to increase cell density rapidly and eliminate the substrate inhibition on cell basis. Continuous cultivation provide the chance to optimize cell productivity but cell density needs to be improved. Perfusion strategy with cell retention by hollow fiber membrane leads to as 10 times high cell density as batch culture but no cell productivity. The combination of all these cultivation strategies show as 10 times high cell productivity as batch culture. In summary, this result shows an effective cultivation method to improve the fermentative production of biofuels and biobased chemicals through pyrolytic substrates.

6.5 Acknowledgement

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Table 6-1 Growth performance of algal cell in different types of bioreactors

Batch a) microalgae fermentation with pure TAP medium (4 g/L acetic acid); Batch b) microalgae fermentation with TAP medium (3.1 g/L acetic acid) + 1 wt% of treated SF5 (0.9 g/L acetic acid); Batch c) microalgae fermentation with pure TAP medium (3.1 g/L acetic acid) for 7 days and then added 1 wt% of treated SF5 (0.9 g/L acetic acid); Bleeding) Bleeding rate = 0.1 ~ 0.7 day^{-1} (0.2 ~1.4 mL/min); Perfusion) Perfusion rate = 0.7 day^{-1} (1.4 mL/min); Perfusion culture with cell bleeding) Bleeding rate = 0.1 ~ 0.7 day^{-1} (0.2 ~1.4 mL/min) while Perfusion rate = 0.7 day^{-1} (1.4 mL/min)

Fermentation	Max Cell Density (g/L)	Productivity (g/L/day)	Specific Growth Rate (day^{-1})	Yield ($g_{cell}/g_{acetate}$)
Batch a	4.24±0.07	0.48±0.01	0.70	
Batch b	2.23±0.00	0.19±0.00*	0.41	0.40±0.08
Fed-Batch	5.49±0.07	0.55±0.01	----	
Bleeding (continuous)	3.00±0.07	0.43±0.01		0.61±0.03
Perfusion	32.12±0.06	1.14±0.01(26)		
Perfusion culture with cell bleeding	10.08±0.09	2.05±0.04		0.75±0.02

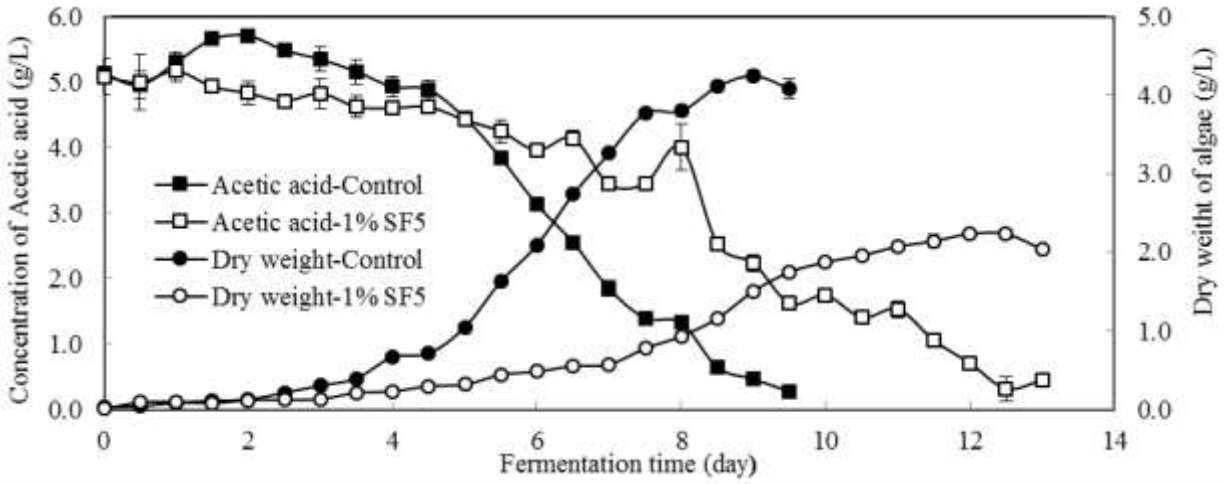

Figure 6-1 Growth and substrate utilization of algal cell in batch culture.
A) microalgae fermentation with pure TAP medium (4 g/L acetic acid);
B) microalgae fermentation with TAP medium (3.1 g/L acetic acid) + 1 wt% of treated SF5 (0.9 g/L acetic acid)

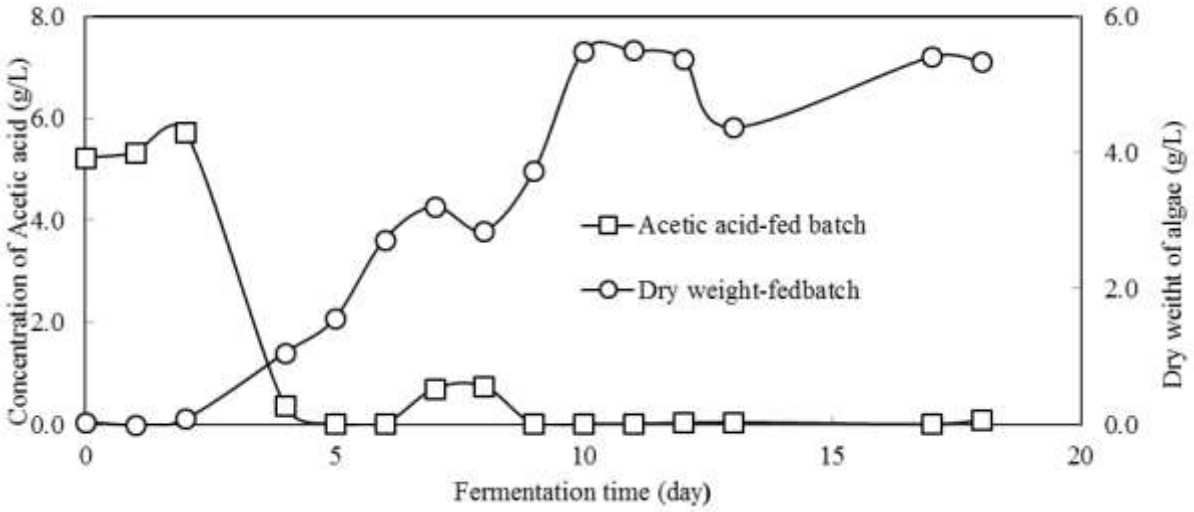

Figure 6-2 Growth and substrate utilization of algal cell in fed batch culture.
Microalgae fermentation with pure TAP medium (3.1 g/L acetic acid) for 7 days and then added 1 wt% of treated SF5 (0.9 g/L acetic acid)

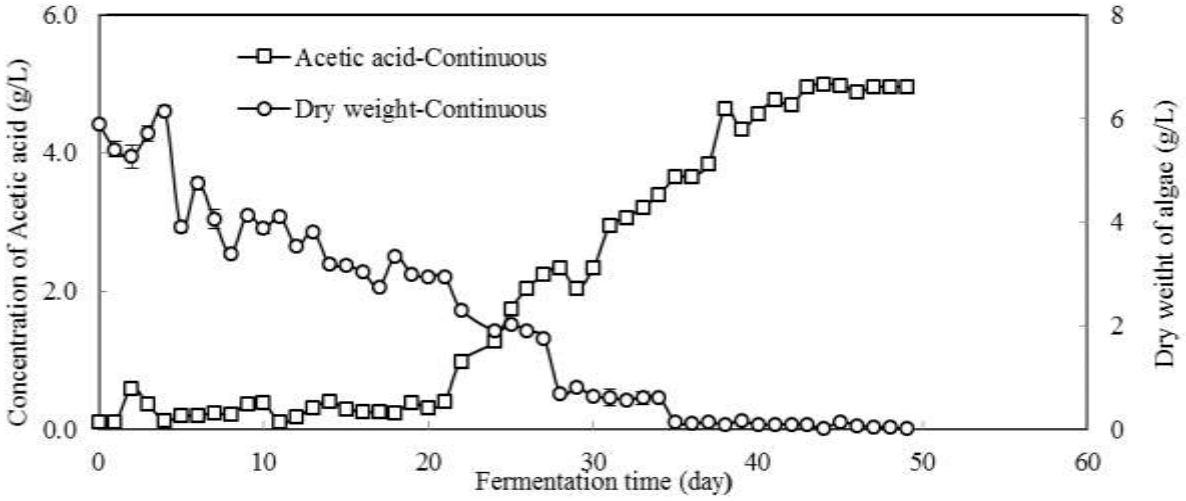

Figure 6-3 Growth and substrate utilization of algal cell in continuous (bleeding) culture
Bleeding rate = 0.1 ~ 0.7 day^{-1} (0.2 ~1.4 mL/min)

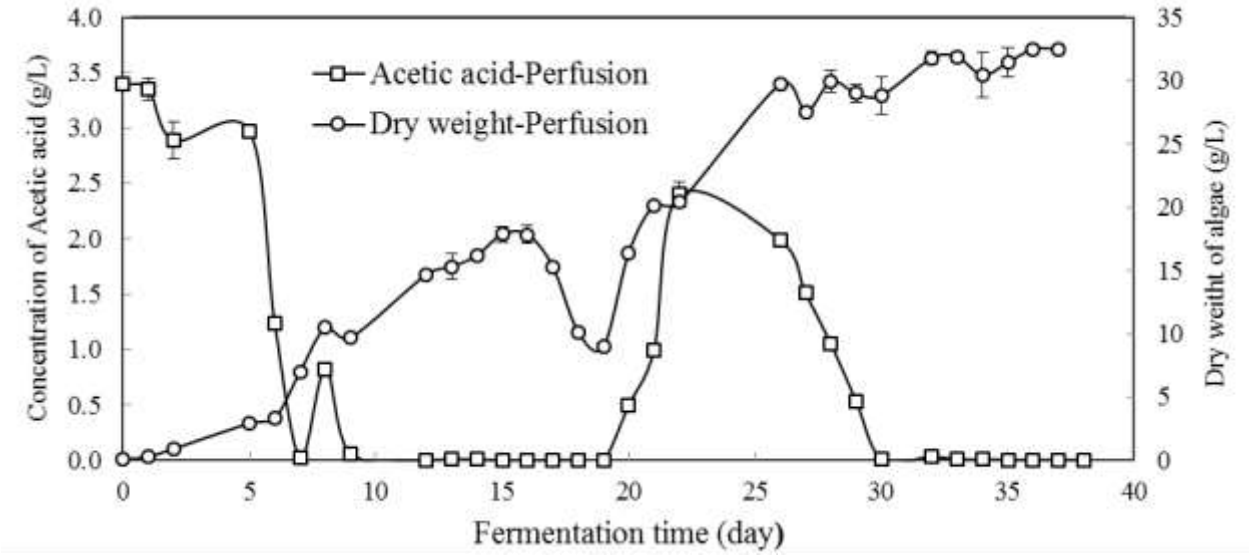

Figure 6-4 Growth and substrate utilization of algal cell in perfusion culture

With Perfusion rate = 0.7 day^{-1} (1.4 mL/min)

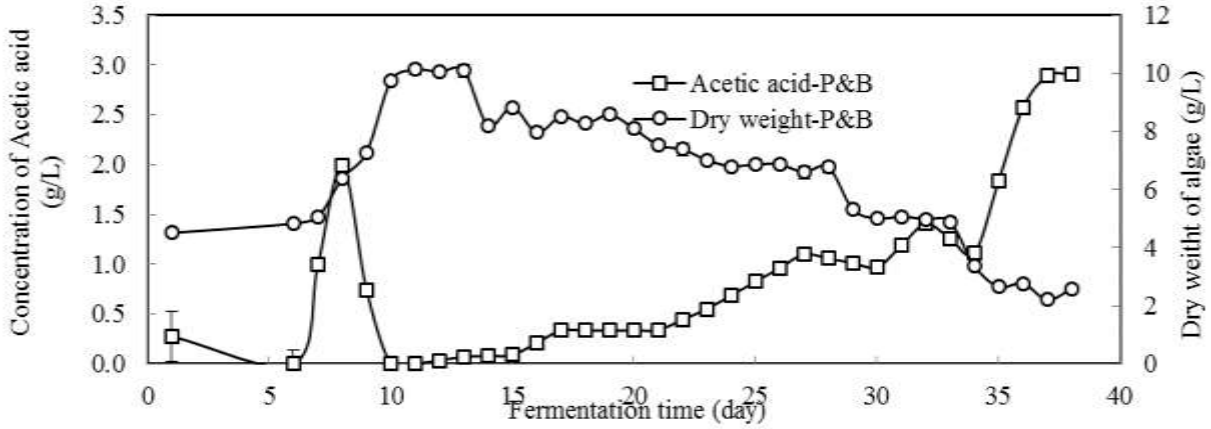

Figure 6-5 Optimization of algal fermentation in continuous reactor with cell perfusion

Bleeding rate = 0.1 ~ 0.7 day^{-1} (0.2 ~1.4 mL/min) while Perfusion rate = 0.7 day^{-1} (1.4 mL/min)

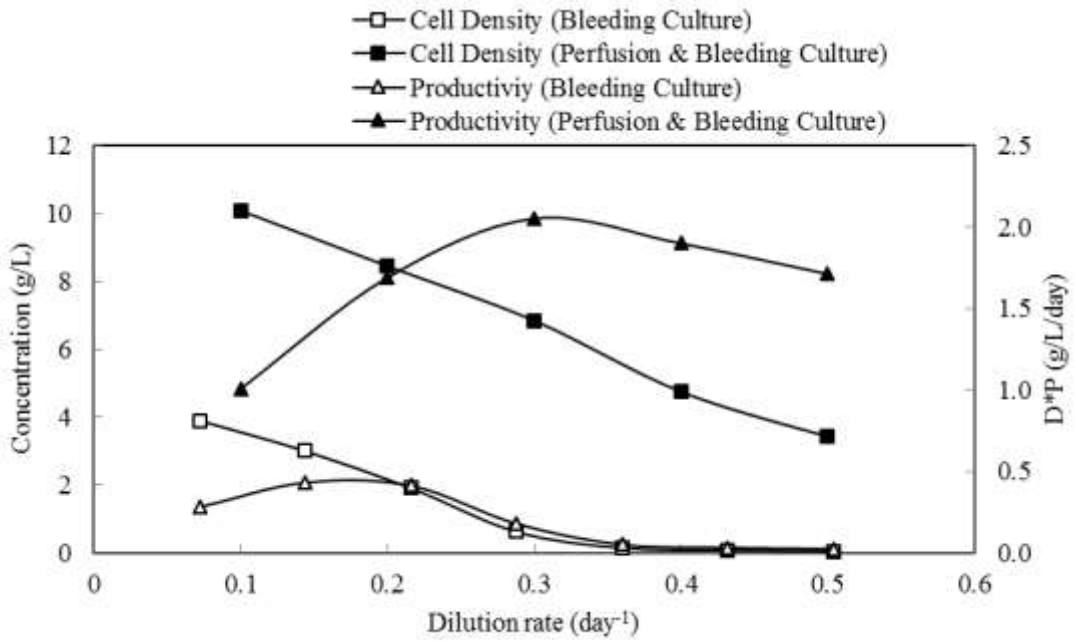

Figure 6-6 Optimization of algal fermentation in continuous culture

With Perfusion rate = 0 for bleeding culture only
With Perfusion rate = 0.7 day^{-1} (1.4 mL/min) for bleeding culture with cell perfusion

Bleeding rate = 0.1 ~ 0.7 day^{-1} (0.2 ~1.4 mL/min) /Continuous feeding rate = 0.1 ~ 0.7 day^{-1} (0.2 ~1.4 mL/min)

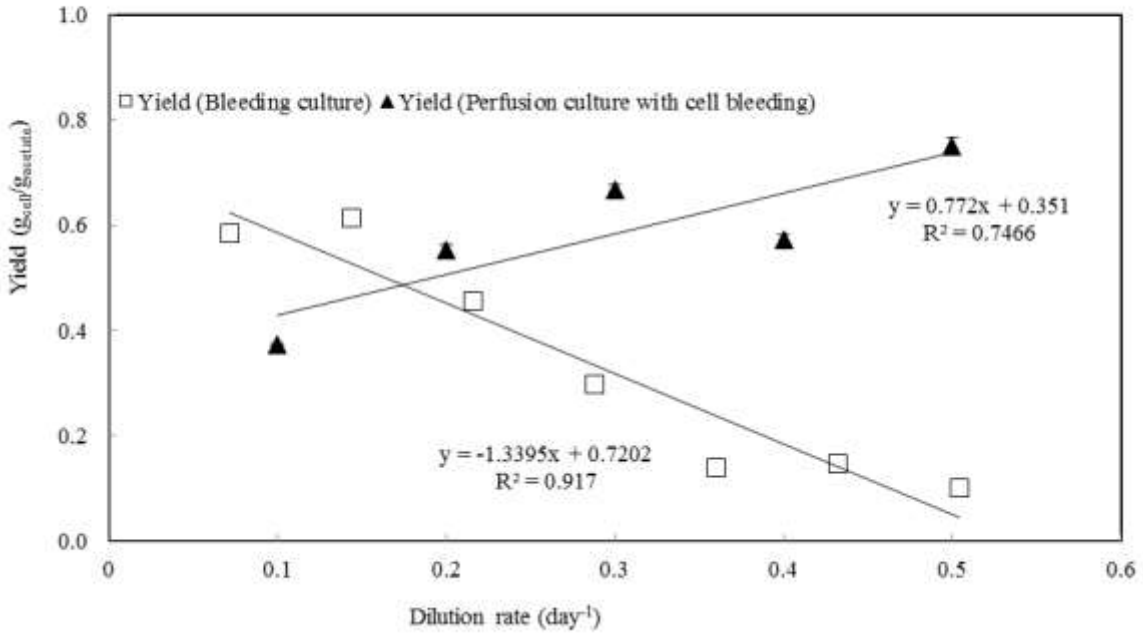

Figure 6-7 Yield change for bleeding culture and perfusion & bleeding culture

CHAPTER 7 GENERAL CONCLUSIONS

7.1 General conclusions

The present body of work focused on lipid rich algal biomass production from lignocellulosic biomass. Based on the concept of hybrid processing, fermentative substrate (acetic acid) is produced through thermochemical decomposition of lignocellulose, and biochemical utilization of this substrate is used to produce liquid rich biomass of the microalga *Chlamydomonas reinhardtii*.

Alkali treatment, or NaOH neutralization particularly to pH 10, is a feasible and effective method for detoxification of the acetic acid-rich fraction of bio-oil for improved growth of the microalga *C. reinhardtii.* Detoxification was mainly due to removal of HMF, furfural, phenolics, and acetol from the bio-oil fraction. As a result of this detoxification, it was possible to grow the metabolically evolved strain *C. reinhardtii* YL01 on media in which the acetic acid was completely derived from the bio-oil fraction. This result suggests a new pathway for fermentative production of biofuels and biobased chemicals through pyrolytic substrates.

Compared to NaOH neutralization, overliming treatment can further reduce the toxicity and improve fermentability of SF5. When SF5 was treated by $Ca(OH)_2$, its fermentability was improved to the greatest level and algal cells could grow in medium containing up to 5.5 wt% SF5, where all the acetic acid in the medium was replaced by SF5. The detoxification effect by $Ca(OH)_2$ was due to the removal of various compounds including furans, phenols, ketones, aldehydes, ethers, esters, and alcohols. The synergistic effects of alkaline pH, high temperature, and presence of Ca^{2+} contribute to the high effectiveness of detoxification by $Ca(OH)_2$.

Among different oxidative treatment, ozone oxidation is the most feasible oxidative method for detoxification of fast pyrolysis bio-oil. With 3 hour ozone treatment under pH 10, the fermentability of Stage Fractionation 5 of bio-oil was increased from 0.05 wt% to 4.0 wt%. Based on the GC-MS identification, the inhibition of SF5 is mainly from ketones, aldehydes and phenolics. Adjusting pH from 2 to 10 can significantly remove these inhibitors and improve the fermentability SF5. Ozone treatment under basic pH mainly works on further oxidation of phenolics compound to ameliorate the maximum cell density and algal productivity. In summary, this result shows an effective oxidative method to improve the fermentative production of biofuels and biobased chemicals through pyrolytic substrates.

The growth performance and ability to uptake of acetic acid as carbon source by algal cells might be inhibited due to the damage of cell membrane. Overliming treatment of SF5, direct evolution and addition of organic protectants are possible methods, which can significantly protect cell membrane integrity and improve the cells tolerance of SF5 to 40 g/L, 4 g/L and 2 g/L, respectively. The mechanism behind each methods are quite different and need further investigation.

Cultivation of *C. reinhardtii* in different types of bioreactors for utilization of fast pyrolysis bio-oil are tested. Two-stage fed batch cultivation is helpful to increase cell density rapidly and eliminate the substrate inhibition on cell basis. Continuous cultivation provide the chance to optimize cell productivity but cell density needs to be improved. Perfusion strategy with cell retention by hollow fiber membrane leads to as 10 times high cell density as batch culture but no cell productivity. The combination of all these cultivation strategies show as 10 times high cell productivity as batch culture. In summary, this result shows an

effective cultivation method to improve the fermentative production of biofuels and biobased chemicals through pyrolytic substrates.

7.2 Future work

Additional work is recommended for further study about hybrid processing of lignocellulosic biomass for producing liqud-rich algae biomass.

i) Combination of physical and chemical methods can be applied in order to detoxify acetic acid-rich fraction of pyrolytic bio-oil (SF5). For example, the inhibitors in SF5 can be removed via a series of treatments, composed of overliming, ozone oxidation and then activated carbon adsorption, for better fermentability.

ii) Additional insight on the inhibitory effect on cells is needed to understand the inhibition mechanism and thus find appropriate methods to protect cells from the damage. Cell membrane integrity disruption is supposed to be one of the reasons why cells are damaged when exposed to certain levels of SF5. Other inhibition pathway, such as ROS increase, intracellular pH increase and osmic pressure increase, may also explain the adverse effect of SF5.

iii) Reverse engineering, providing tool kits to check the genes responsible for enhance tolerance to pyrolytic substrate is necessary. These genes can be isolated and applied to other microorganism to improve their tolerance to pyrolytic substrate as well as other types of toxic media.

iv) Based on metabolic engineering tool kits, produce high value products from pyrolytic substrate via bioreactors equipped with perfusion strategy. With improved utilization efficiency, increased yield and higher revenue, the commercialization of hybrid processing can be realized.