2012

Lipid products from agriculture by-products and pyrolytic oil

Yi Liang
Iowa State University

Lipid products from agriculture by-products and pyrolytic oil

by

Yi Liang

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Program of Study Committee:
Lawrence A. Johnson, Major Professor
Ruth S. MacDonald
Tong Wang
Buddhi P. Lamsal
Shihwu Sung

Iowa State University
Ames, Iowa
2012

TABLE OF CONTENTS

ABSTRACT

This body of research focuses on two major areas related to microalgae-based fuel and chemical production. The first area is to produce algal lipid by utilizing fractionated pyrolytic bio-oil as feedstocks. The second area is the use of agriculture by-products as substrates for fermentative production of eicosapentaenoic acid (EPA).

The hypothesis of the first part of this work was that fractionated bio-oil can be used as feedstock for lipid-based fuel production by the microalga *Chlamydomonas reinhardtii*. The acetic acid-rich fraction of bio-oil derived from fast pyrolysis of softwood contains myriads of other compounds, some of which are toxic to *C. reinhardtii*. To enhance the fermentability of the acetic acid-rich bio-oil fraction by microalgae, activated carbon treatment was used to reduce the toxicity of this bio-oil fraction, while metabolic evolution was used to enhance the toxicity tolerance of the microalgae strain. Combining activated carbon treatment and using adapted algal strains through metabolic evolution resulted in significant improvement to algal growth performance on acetic acid-rich bio-oil fraction. A viable approach was discovered to produce fuels and chemicals from lignocellulosic biomass through the hybrid (fast pyrolysis-fermentation) process.

The hypothesis of the second part of this work was that agriculture by-products, including rendered animal proteins and thin stillage derived from corn ethanol production, can be used as nutritional sources for microbial growth and EPA (omega-3 fatty acid) synthesis. Rendered animal proteins were hydrolyzed into small peptides and free amino acids to facilitate nutrient absorption by the microalga *Schizochytrium limacinum* and the fungus *Pythium irregulare*. The utilities of using protein hydrolysates for growing

microorganisms depended on the hydrolysis method used and the type of microorganism. The enzymatic hydrolysates supported better cell growth performance than did alkali hydrolysates. *P. irregulare* displayed better overall growth performance on the experimental hydrolysates compared to *S. limacinum*. Under selected conditions for *P. irregulare* culture, cell growth, lipid synthesis, and omega-3 fatty acid production were similar to cultures using commercial yeast extract.

Thin stillage from dry-grind ethanol production contained various compounds that were ideal for fungal growth. Thin stillage concentration and temperature played important roles in fungal growth and EPA production. When 50% thin stillage was used in a stepwise temperature shift culture process, the cell density reached 23 g/L at day 9 with EPA yield and productivity of 243 mg/L and 27 mg/L·day, respectively. The fungal culture also generated a nutrient-depleted liquid by removing organic compounds from the raw thin stillage.

CHAPTER 1. GENERAL INTRODUCTION

PART I

1. INTRODUCTION TO HYBRID PROCESSING

The increase in energy consumption with limited availability of petroleum resources has aroused huge concern about the sustainability of our society and our way of life. Producing renewable fuels is one way to mitigate the potential for an energy crisis and, therefore, has attracted significant research efforts. Among all the work exploiting renewable energy, conversion of lignocellulosic biomass into fuels is of great interest.

Conventional approaches to degrade lignocellulosic biomass are through biochemical pathways (Saxena et al., 2009, Cardona et al., 2010). In this process, lignocellulosic biomass is first pretreated with acid to increase the accessible cellulose surface area and/or dissolve lignin, and then employing cellulase to depolymerize the cellulosic biomass. Oligosaccharides and monosaccharides derived from enzymatic hydrolysis of lignocellulosic biomass are utilized as feedstocks for fermentative production of fuels and chemicals. The sugar-based biochemical conversion platform has two major economical hurdles (i.e., high costs of pretreatment and hydrolytic enzymes), which make this process non-cost-effective (Cardona et al., 2010).

Another lignocellulosic biomass conversion approach, which has recently become promising, is the thermochemical conversion pathway (Goyal et al., 2008). Typically, there are three types of thermal treatments, i.e., gasification, pyrolysis, and hydrothermal liquefaction. The end-products of thermochemical conversion can be in liquid and/or gas forms, termed as bio-oil and synthesis gas (syngas), respectively. Bio-oil can be further

upgraded to a "drop-in fuel" via petroleum refinery systems, while syngas can be converted to liquid fuels through Fisher-Tropsch processes. The thermochemical conversion pathway is characterized as high temperature and high pressure, which has high capital cost and high energy input. Therefore, the application of this process has been limited due to economic considerations.

Hybrid processing, which employs both thermochemical and biochemical steps, was recently proposed as an alternative and promising approach for producing fuels and chemicals (Jarboe et al., 2011). In hybrid processing, thermochemical processing is first used to breakdown plant materials into significant amounts of fermentation substrates, followed by biological utilization of these substrates to produce desired fuels and chemicals. The advantages of this approach include: (i) thermochemical processing overcomes the recalcitrance of the lignocellulosic biomass; and (ii) biological conversion renders high product selectivity (Jarboe et al., 2011).

2. BIO-OIL PRODUCTION

2.1 Plant biomass composition

Plant biomass, as a temporary storage unit of sunlight-derived energy and atmospheric carbon, is one of the most abundant renewable energy resources on earth. Utilization of plant biomass for fuel and chemical production, therefore, is considered to be sustainable and benign to the environment (Mortensen et al., 2011). The chemical compositions and physical properties of plant biomass largely affect their applications in bio-

energy production (Goyal et al., 2008). The major components of plant biomass consist of cellulose, hemicellulose, and lignin.

Cellulose, comprising ~40-50 wt % of dry wood, provides strength to the structure of wood (Rowell, 1984). Cellulose is a linear polymer of β-1,4-D-glucopyranose units with an average molecular weight of around 100,000 (Goyal et al., 2008). Cellulose has crystalline structure, which makes it more resistant to thermal decomposition compared to hemicellulose (Mohan et al., 2006).

Hemicellulose is the second largest wood chemical constituent, usually accounting for 25-35 wt % of dry wood. Hemicellulose is a mixture of various polymerized monosaccharides, including glucose, mannose, xylose, galactose, arabinose, and methyl glucuronic and galacturonic acid. Hemicellulose possesses side-chain branches attached to the main polymeric chain and has smaller molecular weight than cellulose.

Lignin is the third major component of woody biomass, accounting for 23-33 wt % in softwoods and 16-25 wt % in hardwoods (Mohan et al., 2006). Lignin is a polyphenolic substance with highly branched structure. The primary monomers of lignin consist of hydroxyl- and methoxy-substituted phenylpropanes. The three general structures of phenylpropanes present in lignin are *p*-coumaryl, coniferyl, and sinapyl alcohols (Figure 1). These phenylpropane units undergo radical-radical coupling, polymerizing and cross-linking in lignin biosynthesis.

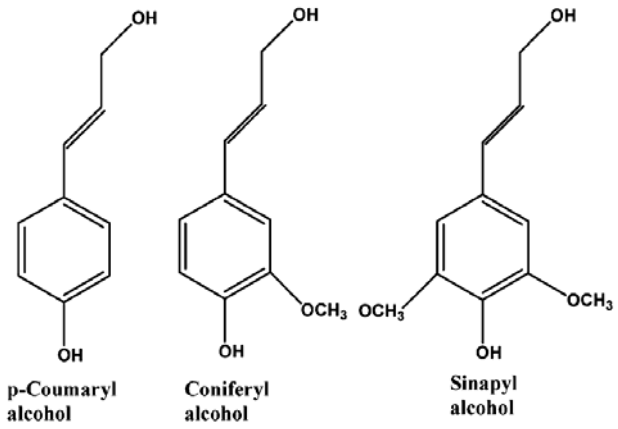


Figure 1. *p*-Coumaryl, coniferyl, and sinapyl structures

2.2 Fast pyrolysis

Pyrolysis is the thermal decomposition of biomass in the absence of air/oxygen with the production of three different types of products, liquid, gas, and char. Depending on the operating conditions used, pyrolysis can be characterized as slow or fast (Maschio et al., 1992). Fast pyrolysis employs faster heating rates and shorter residence times. It is usually conducted at moderate temperatures of around 500°C and very short reaction times of up to 2 s (Bridgwater, 2012a). The leading product of this process is liquid bio-oil. Rapid heating and quenching guarantees that the intermediate liquid products can condense before they are further degraded into gaseous products under high temperature. High reaction rates prevent the formation of char. In a typical fast pyrolysis process, the product distributions are 60-75 wt % of liquid bio-oil, 15-25 wt % of bio-char, and 10-20 wt % of non-condensable gases, respectively, depending on the feedstocks used (Mohan et al., 2006).

2.3 Characteristics of bio-oil

Bio-oil is a dark brown liquid with about 25 wt % of water. Fast pyrolysis derived bio-oil has a heating value of 17 MJ/kg (Bridgwater, 2012a), which is about one-half that of crude oil (Czernik and Bridgwater, 2004; Venderbosch and Prins, 2010). Unlike hydrocarbon liquids, bio-oil is highly oxygenated, which renders both opportunities and challenges for its utilization. Bio-oil is formed by simultaneous decomposition of cellulose, hemicellulose, and lignin with random bond cleavages. This leads to complexity in bio-oil chemical makeup. More than 300 different compounds have been identified in bio-oil and the specific product profile depends on the feedstock type and operational conditions used (Zhang et al., 2006). Compounds that have been discovered in bio-oil consist of acids, esters, alcohols, ketones, aldehydes, phenols, alkenes, furans, guaiacols, syringols, sugars, nitrogen compounds, and miscellaneous oxygenates (Diebold, 2000; Milne et al., 1997). Among the vast variety of compounds, hydroxyacetaldehyde (besides water) is present in the highest concentration of up to 10 wt %, followed by acetic and formic acids at the levels of ~5 and ~3 wt %, respectively.

The chemical mechanisms for forming the major compounds have been widely investigated. It is generally believed that cellulose initially undergoes a series of depolymerization and dehydration reactions and the corresponding products are primarily anhydrosugars, pyrans, and furans (Mettler et al., 2012). These C5-6 ring-contained compounds are not stable under pyrolytic conditions and serve as intermediates for secondary cracking, which is a possible chemical pathway for producing aliphatic oxygenated C2-4 organic compounds and light species/gases (Shen et al., 2011). Hemicellulose pyrolysis does

not yield significant amounts of anhydrosugars, instead, acetic acid is the predominant species generated from deacetylation of hemicellulose. Lignin pyrolysis yields aromatic compounds with guaiacyl-units or phenolic-units as well as other small molecules, for example, methanol, acetic acid, and acetone (Mohan et al., 2006).

2.4 Bio-oil fractionation

When bio-oil is used for fuel production, several problems arising from the chemical complexity of bio-oil have been noted (Bridgwater, 2012b). Firstly, bio-oil is rich in carboxylic acids, including primarily acetic and formic acids, which reduce bio-oil pH, and thus, promote corrosion of the reactor and storage vessels (Aubin and Roy, 1990). Secondly, bio-oil contains 15–35% water by weight, resulting in low heating value for the mixture (Bridgwater et al., 1999). The presence of highly oxygenated small molecules is another factor that contributes to the reduced bio-oil energy content (Pollard et al., 2011). In addition, the solids present in bio-oil may destabilize bio-oil during storage, and also cause clogging of the spray nozzles and other equipments (Mohan et al., 2006). To solve these problems, Pollard et al. (2011) developed a bio-oil recovery system, which separated bio-oil into five distinct stage fractions by selective condensation of classes of molecules according to their condensation points in the pyrolysis vapor stream. When employing this system, bio-oil can be fractionated into stage fractions with different physical and chemical characteristics.

3. FERMENTATION OF BIO-OIL DERIVED COMPOUNDS TO PRODUCE CHEMICALS AND FUELS

3.1 Fermentable substrates in bio-oil

While a number of studies have been carried out on catalytic upgrading bio-oil for fuel production (Mortensen et al., 2011), the use of bio-oil as a chemical reservoir for chemical production has been under-explored. Bio-oil, rich in sugars, carboxylic acids, and other fermentable substrates, is also a good feedstock for producing fuels and chemicals via biological conversion.

1,6-Anhydro-β-D-glucose, also known as levoglucosan, is a fermentable sugar derived from pyrolyzed cellulose. Under selected pyrolysis conditions, the levoglucosan content can reach ~30 wt % on a moisture-free basis (Scott et al., 1989), which leads to a great opportunity for utilizing this substrate for fermentation. Prosen et al. (1993) evaluated capabilities of a variety of fungi and yeasts to use levoglucosan-rich bio-oil for ethanol production. They found that some of the fungi and yeasts can use both activated charcoal treated liquid and acid hydrolysates of the bio-oil. However, ethanol yield was much higher when the acid hydrolysates were fed to microorganisms. This may be due to acid hydrolysis converting levoglucosan to glucose, which is a preferred substrate for ethanol production. Yu and Zhang (2003) investigated the fermentability of cellulosic pyrolysate containing high concentration of levoglucosan. The glucose derived from hydrolysis of the pyrolysate can be used for ethanol production by *Saccharomyces cerevisiae* and *Pichia* sp after lime and absorbent treatments, and the ethanol yield was 0.45 g/g glucose under optimum conditions. Recently, Lian et al. (2010) developed a new scheme to convert anhydrosugars (primarily

levoglucosan) in bio-oil into ethanol and lipids by yeasts. Solvent extraction was first employed to separate the sugars from the phenols, followed by hydrolyzing the anhydrosugars to produce glucose. A series of detoxification methods were then applied to produce a non-toxic glucose-rich aqueous phase, which could be fermented by yeasts into ethanol and lipid.

Acetic acid is another major component of woody biomass hydrolysates, which has long been considered to be undesirable due to its low energy density and corrosiveness when chemically converting bio-oil into fuels. Acetic acid, however, is an ideal fermentation substrate for various microorganisms, and therefore, can be used to produce different types of fuels and chemicals by biological methods (He et al., 2009; Nakamura et al., 2011; Chen and Johns, 1996). Lian et al. (2012) successfully used a detoxified pyrolytic aqueous phase, rich in acetic acid, for lipid synthesis via yeast fermentation, which proved the feasibility of using acetic acid in pyrolytic bio-oil as fermentation feedstock.

Other fermentable compounds found in bio-oil in appreciable amounts include glycoaldehyde and hydroxyacetone. Glycolaldehyde derived from pyrolytic bio-oil has been separated from bio-oil as feedstock for fermentation production of ethylene glycol (Vitasari et al., 2012). Hydroxyacetone, also known as acetol, is an important chemical intermediate in bio-oil and can be converted into propylene glycol by baker's yeast (Kometani et al., 1993).

3.2 Inhibitory compounds

Although bio-oil consists of various potential fermentation substrates, their utilization is hindered by the presence of other non-fermentable compounds toxic to the microorganisms.

The inhibitors identified in bio-oil range from furans, phenols, aldehydes, to organic acids (Jarboe et al., 2011). Prosen et al. (1993) found removing phenolics from bio-oil by activated carbon increased fermentability. Studies on utilization of cellulose hydrolysates also show that organic acids (formic acid, acetic acid, and levulinic acid, etc.), furfural, and hydroxymethyl furfural (HMF) inhibit yeast fermentation (Klinke et al., 2004; Olsson and Hahn-Hagerdal, 1996; Palmqvist and Hahn-Hagerdal, 2000; Palmqvist et al., 1999b; Larsson et al., 1999; Helle et al., 2003). Lian et al. (2010, 2012) further confirmed that the aforementioned compounds were toxic to yeast in ethanol production. These findings show not only that the non-fermentable compounds in bio-oil are toxic, but also fermentable substrates, such as acetic acid, can be inhibitory.

Because each type of compound can deliver toxic effects in different manners, understanding the mechanisms can be particularly helpful in addressing inhibition problems. Formic acid, as a by-product of lignocellulosic hydrolysis, negatively affects cell replication of *Debaryomyces hansenii* and *Rhodosporidium toruloides*. A possible explanation is that undissociated formic acid can diffuse across the plasma membrane, and thus influence cell metabolism (Zhao et al., 2012; Duarte et al., 2005; Huang et al., 2009).

Acetic acid, one of the most important fermentation inhibitors in lignocellulose hydrolyzate, possesses similar inhibition mechanism with formic acid. The severity of the toxicity effects of acetic acid is highly dependent on its concentration of undissociated form, which is determined by both concentration and pH. At low pH, acetic acid can diffuse into the cytosol, where pH is neutral and acetic acid dissociates. This reduces the intracellular pH

and subsequently affects energy production and impairs nutrient transport (Huang et al., 2009; Pienkos and Zhang, 2009).

Furfural, as an inhibitor to microorganism growth, reduces the specific growth rate, biomass yield, and ethanol productivity (Boyer et al., 1992; Navarro, 1994; Palmqvist et al., 1999a; Taherzadeh et al., 1999). Furfural can reversibly inactivate cell reproduction (Ding et al., 2011). In yeast fermentation, furfural is converted to less inhibitory furfuryl alcohol through aldehyde reductases. This process is controlled by multiple NADH-dependent genes (Taherzadeh et al., 1999; Liu et al., 2008).

Methanol, being an aliphatic alcohol, increases the cell membrane fluidity, and thus, adversely affects cell growth and metabolism (Venkataramanan et al., 2012).

The phenolic compounds from lignin decomposition have long been identified as growth inhibitors to microorganisms. The inhibition effects of phenolics have mainly been attributed to hydrophobicity, which makes them capable of being incorporated into biological membranes. Therefore, membrane deterioration and denaturation of membrane-associated enzymes are two major observations for phenolics toxicity (Zaldivar et al., 2000; Heipieper et al., 1994).

3.3 Mitigation of bio-oil toxicity

Although the abundance of biologically utilizable compounds in bio-oil makes it an appealing substrate for microbial growth, bio-oil also contains a myriad of other chemicals that are potentially inhibitory to microorganisms. To address the inhibition problem, two

strategies can be applied, including selectively removing the inhibitors and increasing the biocatalyst tolerance levels.

3.3.1 Selective removal of inhibitors

Activated carbon with its strong absorption capability is an efficient absorbent for various compounds, including formic acid, acetic acid, phenols, furfural and HMF (Lee et al., 2011; Dąbrowski et al., 2005). Prosen et al. (1993) used activated carbon to remove lignin and phenolics in a pyrolytic liquid and the resulting product could be fermented by yeasts for ethanol production. Lian et al. (2010, 2012) combined activated carbon absorption with a series of other treatments to detoxify bio-oil and produce either a sugar-rich or acetic acid-rich liquid for fermentation.

Distillation is another detoxification method, which separates compounds based on differences in their boiling points. In bio-oil detoxification, distillation has not been used as a stand-alone method due to the complex nature of bio-oil. Instead, distillation was used either in combination with activated carbon treatment or solvent extraction to generate less toxic fermentation substrates (Lian et al., 2012; Chan and Duff, 2010; Vitasari et al., 2012).

Solvent extraction separates compounds based on their relative solubilities in different liquids. Lian et al. (2010) used ethyl acetate to extract phenolics from bio-oil and the water phase was used for sugar-based fermentation. Chan and Duff (2010) employed a co-solvent system of tri-*n*-octylamine and 1-octanol to remove acetate from bio-oil. This system selectively eliminated 90% of acetic acid while retaining 100% of the glucose. Vitasari et al. (2012) used a combination of tri-*n*-octylamine and toluene to remove acetic

acid and formic acid from pyrolytic bio-oil, and used 1-octanol to recover the fermentation substrate glycolaldehyde.

Overliming is a widely used method for detoxifying cellulose hydrolysates (Kuhad et al., 2010; Huang et al., 2009; Okuda et al., 2008). Because some of the inhibitors in cellulose hydrolysates are also present in bio-oil, the same method was used to eliminate contaminants in pyrolytic bio-oil. Chan and Duff (2010) reported that ethanol yield was increased from 0.19 to 0.45 g ethanol/g glucose when the feedstock bio-oil hydrolysate was detoxified by using the overliming treatment. This improved fermentability was attributed to the removal of furans and phenolics.

Membrane technology is a promising method in bio-oil based fuel and chemical production (He et al., 2012). Membrane filtration has advantages of high detoxification efficiency, possible inhibitor recovery, simple operation, and ease of scale-up, and has been used in cellulosic ethanol production,

3.3.2 Increasing tolerance of biocatalysts by metabolic evolution

Bio-oil contains various types of toxic compounds that can be only removed by combination of different treatment methods, therefore, compared to multi-treatment detoxification process, it is more cost-effective to develop microorganisms that are tolerant of these inhibitory compounds. Metabolic evolution has been utilized to enhance strain tolerance when bio-oil-based substrates were fed to microorganisms (Nakagawa et al., 1984; Zhuang et al., 2001; Chan and Duff, 2010).

Metabolic evolution, also known as directed evolution, is frequently used to improve strain performance such as microorganism tolerance to inhibitory compounds (Miller et al., 2010; Miller et al., 2009a; Miller et al., 2009b). When using directed evolution to increase strain tolerance to the inhibitory compounds in bio-oil, the microorganism is grown in the presence of a slightly inhibitory amount of the toxic compound. At this concentration, cells are able to grow and divide, although at a lower rate than under normal conditions. During this time, genomic DNA can acquire mutations, either during DNA replication or by DNA damage. Although some of these mutations may negatively impact microbial fitness and some may have no effect, some mutations may confer increased tolerance to the toxic compound(s) or increased substrate use. If so, strains that acquire these mutations will grow faster than their non-mutated cohorts and eventually take over the entire population. By subdiluting the cultures on a regular basis and gradually increasing the concentration of the toxic compound(s), cells with beneficial mutations are able to grow faster and become the dominant strain in the culture. At the end of the process, single colonies are isolated and characterized for their tolerance to the inhibitory compound, and the most robust strain can be used for production.

4. ECONOMIC FEASIBILITY OF BIO-OIL FERMENTATION

A recent comparative analysis of the cost of biofuels revealed that fast pyrolysis is more promising than either enzymatic hydrolysis or gasification routes to advanced biofuels (Anex et al., 2010). In this study, transportation fuels in the near-term (5-7 years) were estimated to cost $2-3 per gallon gasoline equivalent (gge) for pyrolysis, $4-5 per gge for gasification, and $5-6 per gge for cellulosic ethanol via enzymatic hydrolysis. Therefore, bio-

oil derived from fast pyrolysis shows great potential for dedicated applications and for biofuel production.

So and Brown (1999) performed an economic analysis that found the cost of ethanol from this hybrid processing to be comparable to the cost of ethanol from acid hydrolysis or enzymatic hydrolysis of woody biomass. Sandvig et al. (2004) found fermentation of bio-oil-derived sugars to be economically attractive when integrated into the production of both bio-power and bio-based chemicals. These findings promise a great future for fast pyrolysis based hybrid processes and products.

PART II

1. OMEGA-3 POLYUNSATURATED FATTY ACIDS

Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (20:5 n-3, EPA) and docosahexaenoic acid (22:6 n-3, DHA) are a class of lipids with various biological functions. Both clinical and epidemiological studies show that consumption of PUFAs has preventive and therapeutic effects on a series of illnesses, such as rheumatoid arthritis, heart disease, cancers, schizophrenia, and Alzheimer's disease (Cohen and Ratledge, 2010). The health benefits delivered by PUFAs have led to significant efforts in exploring PUFA producers and developing commercial processes.

Fish oil is a PUFA rich source and has been commercially produced for decades. However, the peculiar taste and odor, and possible metal contamination of fish oil have limited its applications (Barclay et al., 1994). Microbial production of EPA and DHA, on the

other hand, avoids all the problems associated with fish-derived PUFAs, and therefore, has been a promising alternative source of high quality PUFAs (Kralovec et al., 2012).

A variety of microorganisms, including lower fungi, bacteria, and marine microalgae, are capable of producing PUFAs (Bajpai et al., 1991; Kendrick and Ratledge, 1992; Ratledge et al., 2001; Athalye et al., 2009; Johnson and Wen, 2009). Among all the microbial PUFA producers, bacteria are not suitable for commercial PUFA production because they cannot accumulate high levels of lipid, and the related fatty acid compositions are uncommon in other systems (Ratledge et al., 2001). On the contrary, oleaginous fungi and microalgae with high lipid production yields and high PUFA contents, are good candidates as PUFA sources. The marine microalga *Schizochytrium* sp (Barclay et al., 1994) and *Crypthecodinium cohnii* (Ratledge et al., 2001; de Swaaf et al., 2003) have been used for commercial DHA production. The highest productivity that has been reported is 138 $mg \cdot L^{-1} \cdot h^{-1}$ (Yaguchi et al., 1997). In comparison, EPA yield and productivity from microbial sources are very low, which hindered commercialization. Many studies have been conducted to identify EPA producers and to enhance production yield. Microbial species belonging to the genera of *Mortierella*, *Pythium* and *Saprolegnia* are capable of producing appreciable amount of EPA (Cohen and Ratledge, 2010) and genetic modification has also been introduced to improve strain performance (Cohen et al., 1992). However, microbial production of EPA is still in the infancy stage. Further increase in EPA level to the economic viable point requires in-depth understanding of factors that influence EPA production.

2. RENDERED ANIMAL PROTEINS

In meat processing, the low-value tissues (bone, offal, feather, and blood) from animal carcasses are typically rendered into dry meals that can be used as protein components in animal feeds or pet foods (Cramer et al., 2007; Wang et al., 2008). These meals include meat and bone meal (MBM), feather meal (FM), and blood meal (BM). Protein qualities of rendered animal meals have been evaluated and these meals are generally nutritionally adequate protein resources in animal diets (Wang et al., 2008; Hendriks et al., 2002).

The traditional markets for these products are maturing and, in some cases, threatening to shrink due to the concern of bovine spongiform encephalopathy caused by specific risk materials (SRMs) contained in rendered protein products (FDA). As a result, efforts are necessary to develop non-feed applications for rendered animal proteins. For example, Garcia et al. (2011) investigated the hydrolysis of MBM, FM, and BM to increase the solubility of the raw protein and prepare small peptides with potential nutritional value for growth of microorganisms. They used enzyme-hydrolyzed MBM as a medium ingredient for fermentation of biopolymer-producing *E. coli* (Solaiman, 2011). The non-nutritional characteristics of the protein hydrolysates, such as hygroscopicity, chromicity, autoclave stability, viscosity, and foaming, were also reported. Rendered protein hydrolysates can be used as a low-cost substitute for commercial peptone (Garcia et al., 2010). These results demonstrate an opportunity of exploring animal proteins as a low-cost nutrition source for fermentation.

PART III

1. THIN STILLAGE DERIVED FROM DRY-GRIND ETHANOL PRODUCTION

Fuel ethanol production from corn in the United States has expanded rapidly over the past decade. According to the Renewable Fuels Association, the annual corn fuel ethanol production reached 13 billion gallons in 2010 (http://www.ethanolrfa.org/pages/statistics). Currently, the majority of corn-derived fuel ethanol is produced using a dry-grind process (Figure 2). In this process, the residual ethanol-free slurry (whole stillage) after ethanol is distilled from fermented beer is separated into a solids fraction (wet distillers grains) and a liquid fraction (thin stillage) (Liu, 2011). While part of the thin stillage is recycled to slurry the corn meal at the beginning of the dry-grind process, the majority of thin stillage is sent to an evaporator to be condensed into a syrup-like paste containing about 35% solids. The condensed syrup is usually combined with the wet distiller's grains and dried into dried distillers grains with solubles (DDGS), which are primarily used as cattle feed.

Evaporating the thin stillage is an energy-intensive process and a major cost to the ethanol plant. DDGS, as animal feed, has only moderate value for ethanol producers. Therefore, developing an efficient way to use thin stillage and increase product value will enhance the economics of dry-grind ethanol production.

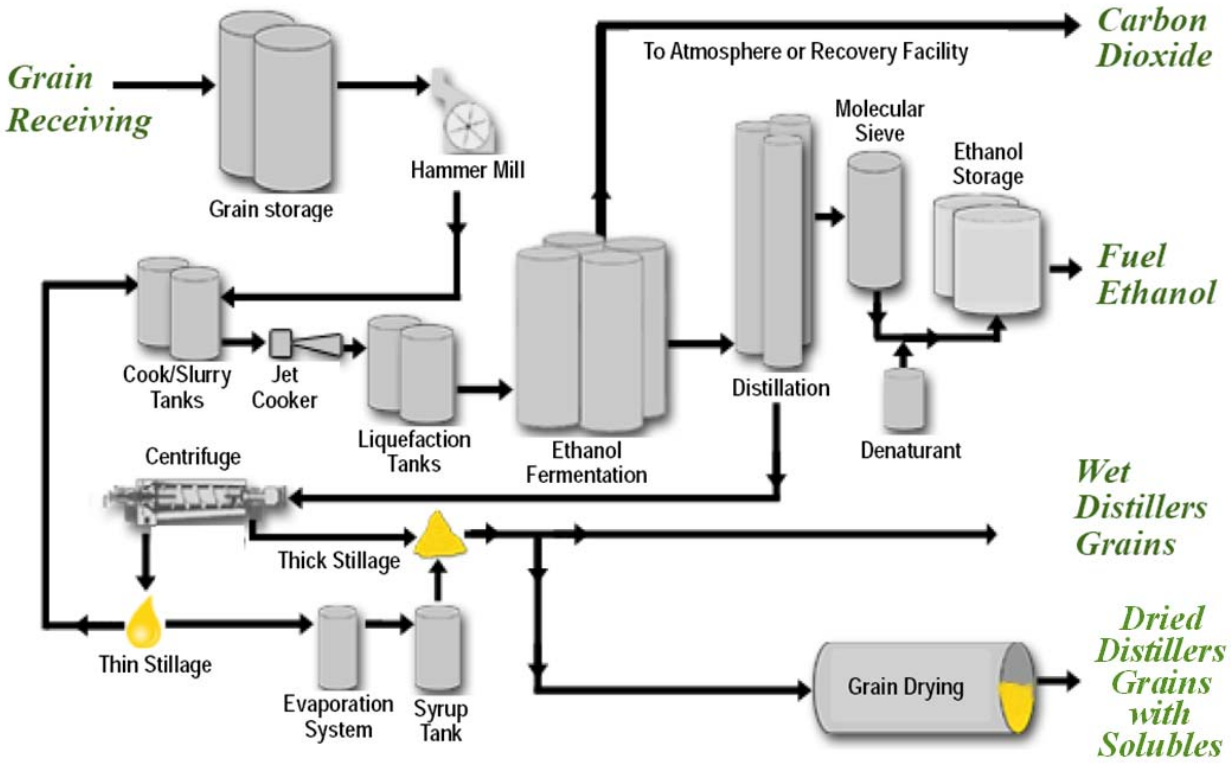


Figure 2. Diagram of the dry-grind process for ethanol production (*http://ciitn.missouri.edu/cgi-bin/pub_view_project_ind.cgi?g_num=6&c_id=2007008*)

2. USE OF THIN STILLAGE AS FEEDSTOCK FOR FERMENTATION PRODUCTION OF OMEGA-3 PUFA

Thin stillage contains various unfermented components of the grains (e.g., fiber, oil, and protein) and yeast cells (Kim et al., 2008). These materials are excellent nutrients for microorganisms (Dowd et al., 1993; Kim et al., 2008). For example, thin stillage was used as a carbon source for growing the fungus *Ganoderma lucidum* to produce polysaccharides (Hsieh et al., 2005). van Leeuwen et al. (2010) used thin stillage to grow a variety of fungi, which can be used as animal feeds, human food, and sources of nutraceuticals. In particular,

they recently reported using thin stillage for growing the oleaginous fungus *Mucor circinelloides* in an air-lift bioreactor, resulting high mass concentration with high oil content (Mitra et al., 2012). Ahn et al. (2011) also used thin stillage to grow the bacterium *Clostridium pasteurianum* for butanol production. Using anaerobic digestion for treating thin stillage to improve water quality and energy efficiency in dry-grind ethanol production was also investigated (Alkan-Ozkaynak and Karthikeyan, 2011). Using thin stillage to produce eicosapentaenoic acid (EPA, C20:5, n-3) by fungal fermentation provides another outlet for this currently underutilized material.

Research has been conducted using less-expensive agricultural by-products for growing the fungus *Pythium irregulare*, which is a good EPA producer. These by-products include crude soybean oil and soy protein isolate (Cheng et al., 1999), and crude glycerol from a biodiesel plant (Athalye et al., 2009). These materials, however, have only one function for microbial growth. For example, crude glycerol serves as a carbon source while rendered animal proteins serve as a nitrogen source for the microorganism; there is still need to supplement other nutrients in the growth medium, which may increase the medium cost. In contrast, thin stillage can be used as the sole nutrient source for the fungal growth due to its complete nutrient composition for microorganisms. The objective of this part of work was to test the feasibility of using thin stillage as a nutrient source for EPA production by *Pythium irregulare*.

REFERENCES

Ahn, J.H., Sang, B.I., Um, Y., 2011. Butanol production from thin stillage using *Clostridium pasteurianum*. Bioresour. Technol. 102, 4934-4937.

Alkan-Ozkaynak, A., and Karthikeyan, K.G., 2011. Anaerobic digestion of thin stillage for energy recovery and water reuse in corn-ethanol plants. Bioresour. Technol. 102, 9891-9896.

Anex, R.P., Aden, A., Kazi, F.K., Fortman, J., Swanson, R.M., Wright, M.M., Satrio, J.A., Brown, R.C., Daugaard, D.E., Platon, A., Kothandaraman, G., Hsu, D.D., Dutta, A., 2010. Techno-economic comparison of biomass-to-transportation fuels via pyrolysis, gasification, and biochemical pathways. Fuel 89, S29-S35.

Athalye, S.K., Garcia, R.A., Wen, Z.Y., 2009. Use of biodiesel-derived crude glycerol for producing eicosapentaenoic acid (EPA) by the fungus *Pythium irregulare*. J. Agric. Food Chem. 57, 2739-2744.

Aubin, H., Roy, C., 1990. Study on the corrosiveness of wood pyrolysis oils. Fuel Sci. Technol. Int. 8, 77-86.

Bajpai, O., Bajpai, P.K., Ward, O.P., 1991. Production of docosahexaenoic acid by *Thraustochytrium aureum*. App. Microbiol. Biotechnol. 35, 706-710.

Barclay, W. R., Meager, K. M., Abril, J. R. 1994. Heterotrophic production of long chain omega-3 fatty acids utilizing algae and algae-like microorganisms. J. Appl. Phycol. 6, 123-129.

Boyer, L.J., Vega, J.L., Klasson, K.T., Clausen, E.C., Gaddy, J.L., 1992. The effects of furfural on ethanol production by *Saccharomyces cerevisiae* in batch culture. Biomass Bioenergy 3, 41-48.

Bridgwater, A.V., 2012a. Review of fast pyrolysis of biomass and product upgrading. Biomass Bioenergy 38, 68-94.

Bridgwater, A.V., 2012b. Upgrading biomass fast pyrolysis liquids. Environ. Prog. Sustainable Energy 31, 261-268.

Bridgwater, A.V., Meier, D., Radlein, D., 1999. An overview of fast pyrolysis of biomass. Org. Geochem. 30, 1479-1493.

Cardona, C.A., Quintero, J.A., Paz, I.C., 2010. Production of bioethanol from sugarcane bagasse: Status and perspectives. Bioresour. Technol. 101, 4754-4766.

Chan, J.K.S., Duff, S.J.B., 2010. Methods for mitigation of bio-oil extract toxicity. Bioresour. Technol. 101, 3755-3759.

Chen, F., Johns, M.R., 1996. Heterotrophic growth of *Chlamydomonas reinhardtii* on acetate in chemostat culture. Proc. Biochem. 31, 601-604.

Cheng, M.H., Walker, T.H. Hulbert, G.J., Raman, D.R., 1999. Fungal production of eicosapentaenoic and arachidonic acids from industrial waste streams and crude soybean oil. Bioresour. Technol. 67, 101-110.

Cohen, Z., Didi, S. and Heimer, Y.M., 1992. Overproduction of gamma-linolenic and eicosapentaenoic acids by algae. Plant Physiol. 98, 569-572.

Cohen, Z. and Ratledge, C., 2010. Single cell oils: microbial and algal oils, Urbana, IL, AOCS Press.

Cramer, K.R., Greenwood, M.W., Moritz, J.S., Beyer, R.S., Parsons, C.M., 2007. Protein quality of various raw and rendered by-product meals commonly incorporated into companion animal diets. J Animal Sci. 85, 3285-3293.

Czernik, S., Bridgwater, A.V., 2004. Overview of applications of biomass fast pyrolysis oil. Energy Fuel 18, 590-598.

Dąbrowski, A., Podkościelny, P., Hubicki, Z., Barczak, M. 2005. Adsorption of phenolic compounds by activated carbon - A critical review. Chemosphere 58, 1049-1070.

de Swaaf, M.E., Pronk, J.T., Sijtsma, L., 2003. Fed-batch cultivation of the docosahexaenoic-acid-producing marine alga *Crypthecodinium cohnii* on ethanol. Appl. Microbiol. Biotechnol., 61, 40-43.

Diebold, J.P., 2000. A Review of the chemical and physical mechanism of the storage stability of fast pyrolysis bio-oils. NREL report no. NREL/SP-570-27613, National Renewable Energy Laboratory, CO, USA.

Ding, M.Z., Wang, X., Yang, Y., Yuan, Y.J., 2011. Metabolomic Study of interactive effects of phenol, furfural, and acetic acid on *Saccharomyces cerevisiae.* Omics-A J. Integr. Biol., 15, 647-653.

Dowd, M.K., Reilly, P.J., Trahanovsky, W.S., 1993. Low molecular weight organic composition of ethanol stillage from corn. Cereal Chem. 70, 204-209.

Duarte, L.C., Carvalheiro, F., Neves, I., Girio, F.M., 2005. Effects of aliphatic acids, furfural, and phenolic compounds on *Debaryomyces hansenii* CCMI 941. Appl. Biochem. Biotechnol., 121, 413-425.

FDA. Substances Prohibited From Use in Animal Food or Feed. Federal Register. http://edocket.access.gpo.gov/2008/pdf/08-1180.pdf 2008**,** 73.

Garcia, R.A., Piazza, G.J., Wen, Z., Pyle, D.J., Solaiman, D.K.Y., 2010. The non-nutritional performance characteristics of peptones made from rendered protein. J. Ind. Microb. Biotechnol. 37, 95-102.

Garcia, R.A., Pyle, D.J., Piazza, G.J., Wen, Z., 2011. Hydrolysis of animal protein meals for improved utility in non-feed applications. Appl. Eng. Agric. 27, 269-275.

Goyal, H.B., Seal, D., Saxena, R.C., 2008. Bio-fuels from thermochemical conversion of renewable resources: A review. Renew. Sust. Energ. Rev. 12, 504-517.

He, Q., Lokken, P.M., Chen, S., Zhou, J.Z., 2009. Characterization of the impact of acetate and lactate on ethanolic fermentation by *Thermoanaerobacter ethanolicus*. Bioresour. Technol. 100, 5955-5965.

He, Y., Bagley, D.M., Leung, K.T., Liss, S.N., Liao, B.Q., 2012. Recent advances in membrane technologies for biorefining and bioenergy production. Biotechnol. Adv. 30, 817-858.

Heipieper, H.J., Weber, F.J., Sikkema, J., Kewelon, H., Debont, J.A.M., 1994. Mechanisms of resistance of whole cells to toxic organic solvents. Trends Biotechnol. 12, 409-415.

Helle, S., Cameron, D., Lam, J., White, B., Duff, S., 2003. Effect of inhibitory compounds found in biomass hydrolysates on growth and xylose fermentation by a genetically engineered strain of *S. cerevisiae*. Enzyme Microb. Technol. 33, 786-792.

Hendriks, W.H., Butts, C.A., Thomas, D.V., James, K.A.C., Morel, P.C.A., Verstegen, M.W.A., 2002. Nutritional quality and variation of meat and bone meal. Asian-Austral J. Animal Sci. 15, 1507-1516.

Huang, C., Zong, M.H., Wu, H., Liu, Q.P., 2009. Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*. Bioresour. Technol. 100, 4535-4538.

Jarboe, L.R., Wen, Z., Choi, D., Brown, R.C., 2011. Hybrid thermochemical processing: fermentation of pyrolysis-derived bio-oil. Appl. Microbiol. Biotechnol. 91, 1519-1523.

Johnson, M.B., Wen, Z.Y., 2009. Production of biodiesel fuel from the microalga *Schizochytrium limacinum* by direct transesterification of algal biomass. Energy Fuel 23, 5179-5183.

Kendrick, A. and Ratledge, C., 1992. Lipids of selected molds grown for production of n-3 and n-6 polyunsaturated fatty acids. Lipids 27, 15-20.

Kim, Y., Mosier, N.S., Hendrickson, R., Ezeji, T., Blaschek, H., Bien, B., Cotta, M., Dale, B., Ladisch, M.R., 2008. Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage. Bioresour. Technol. 99, 5165-5176.

Klinke, H.B., Thomsen, A.B., Ahring, B.K., 2004. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. Applied Microbiol. Biotechnol. 66, 10-26.

Kometani, T., Yoshii, H., Takeuchi, Y., Matsuno, R., 1993. Large-scale preparation of (R)-1,2-propanediol through baker yeast mediated bioreduction. J. Ferment. Bioeng. 76, 414-415.

Kralovec, J. A., Zhang, S. C., Zhang, W., Barrow, C. J., 2012. A review of the progress in enzymatic concentration and microencapsulation of omega-3 rich oil from fish and microbial sources, Food Chem. 131, 639-644.

Kuhad, R.C., Gupta, R., Khasa, Y.P., Singh, A., 2010. Bioethanol production from *Lantana camara* (red sage): Pretreatment, saccharification and fermentation. Bioresour. Technol. 101, 8348-8354.

Larsson, S., Palmqvist, E., Hahn-hagerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebrant, N.Q., 1999. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme Microb. Technol. 24, 151-159.

Lee, J.M., Venditti, R.A., Jameel, H., Kenealy, W.R., 2011. Detoxification of woody hydrolyzates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum*. Biomass Bioenergy 35, 626-636.

Lian, J., Chen, S., Zhou, S., Wang, Z., O'Fallon, J., Li, C.Z., Garcia-Perez, M., 2010. Separation, hydrolysis and fermentation of pyrolytic sugars to produce ethanol and lipids. Bioresour. Technol. 101, 9688-9699.

Lian, J., Garcia-Perez, M., Coates, R., Wu, H., Chen, S., 2012. Yeast fermentation of carboxylic acids obtained from pyrolytic aqueous phases for lipid production. Bioresour. Technol. 118, 177-86.

Liu, Z.L., Moon, J., Andersh, B.J., Slininger, P.J., Weber, S., 2008. Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 81, 743-753.

Liu, K.S., 2011. Chemical composition of distillers grains, a review. J. Agric. Food Chem. 59, 1508-1526.

Maschio, G., Koufopanos, C., Lucchesi, A., 1992. Pyrolysis, a promising route for biomass utilization. Bioresour. Technol. 42, 219-231.

Mettler, M.S., Vlachos, D.G., Dauenhauer, P.J., 2012. Top ten fundamental challenges of biomass pyrolysis for biofuels. Energy Environ. Sci. 5, 7797-7809.

Miller, E.N., Jarboe, L.R., Turner, P.C., Pharkya, P., Yomano, L.P., York, S.W., Nunn, D., Shanmugam, K.T., Ingram, L.O., 2009a. Furfural inhibits growth by limiting sulfur assimilation in ethanologenic *Escherichia coli* Strain LY180. Appl. Environ. Microbiol. 75, 6132-6141.

Miller, E.N., Jarboe, L.R., Yomano, L.P., York, S.W., Shanmugam, K.T., Ingram, L.O., 2009b. Silencing of NADPH-dependent oxidoreductase genes (yqhD and dkgA) in furfural-resistant ethanologenic *Escherichia coli*. Appl. Environ. Microbiol. 75, 4315-4323.

Miller, E.N., Turner, P.C., Jarboe, L.R., Ingram, L.O., 2010. Genetic changes that increase 5-hydroxymethyl furfural resistance in ethanol-producing *Escherichia coli* LY180. Biotechnol. Lett. 32, 661-667.

Milne, T.A., Elam, L.C., Evans, R.C., 1997. Hydrogen from biomass—State of the art and research challenches. NREL report no. IEA/H2/TR-02/001, NREL, Golden, CO, USA.

Mohan, D., Pittman, C.U., Steele, P.H., 2006. Pyrolysis of wood/biomass for bio-oil: A critical review. Energy Fuel. 20, 848-889.

Mitra, D, Rasmussen, M.L., Chand, P., Chintareddy, V.R., Yao, L., Grewell, D., Verkade, J.G., Wang, T., van Leeuwen, J., 2012. Value-added oil and animal feed production from corn-ethanol stillage using the oleaginous fungus *Mucor circinelloides*. Bioresour. Technol. 107, 368-375.

Mortensen, P.M., Grunwaldt, J.D., Jensen, P.A., Knudsen, K.G., Jensen, A.D., 2011. A review of catalytic upgrading of bio-oil to engine fuels. Appl. Catal. A: Gen. 407, 1-19.

Nakagawa, M., Sakai, Y., Yasui, T., 1984. Itaconic acid fermentation of levoglucosan. J. Ferment. Technol. 62, 201-203.

Nakamura, Y., Miyafuji, H., Kawamoto, H., Saka, S., 2011. Acetic acid fermentability with *Clostridium thermoaceticum* and *Clostridium thermocellum* of standard compounds found in beech wood as produced in hot-compressed water. J. Wood Sci. 57, 331-337.

Navarro, A.R., 1994. Effects of furfural on ethanol fermentation by Saccharomyces cerevisiae: mathematical models. Curr. Microbiol, 29, 87-90.

Okuda, N., Soneura, M., Ninomiya, K., Katakura, Y., Shioya, S., 2008. Biological detoxification of waste house wood hydrolysate using *Ureibacillus thermosphaericus* for bioethanol production. J. Biosci. Bioeng. 106, 128-133.

Olsson, L., Hahn-hagerdal, B., 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. Enzyme Microb. Technol. 18, 312-331.

Palmqvist, E., Almeida, J.S., Hahn-hagerdal, B., 1999a. Influence of furfural on anaerobic glycolytic kinetics of *Saccharomyces cerevisiae* in batch culture. Biotechnol. Bioeng. 62, 447-454.

Palmqvist, E., Grage, H., Meinander, N.Q., Hahn-hagerdal, B., 1999b. Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol. Bioeng. 63, 46-55.

Palmqvist, E., Hahn-hagerdal, B., 2000. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresour. Technol. 74, 17-24.

Pienkos, P.T., Zhang, M., 2009. Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. Cellulose 16, 743-762.

Pollard, A.S., Rover, M.R., Brown, R.C., 2011. Characterization of bio-oil recovered as stage fractions with unique chemical and physical properties. J. Anal. Appl. Pyrol. 93, 129-138.

Prosen, E.M., Radlein, D., Piskorz, J., Scott, D.S., Legge, R.L., 1993. Microbial utilization of levoglucosan in wood pyrolysate as a carbon and energy source. Biotechnol. Bioeng. 42, 538-541.

Pyle, D.J., Garcia, R.A., Wen, Z., 2008. Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: Effects of impurities on DHA production and algal biomass composition. J. Agric. Food Chem. 56, 3933-3939.

Ratledge, C., Kanagachandran, K., Anderson, A.J., Grantham, D.J., Stephenson, J.C., 2001. Production of docosahexaenoic acid by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source. Lipids 36, 1241-1246.

Rowell, R.M., 1984. The chemistry of solid wood, American Chemical Society.

Sandvig, E., Walling, G., Daugaard, D.E., Pletka, R.J., Radlein, D., Johnson, W., Brown, R.C., 2004. The prospects for integrating fast pyrolysis into biomass power systems. Int. J. Power Energy Syst. 24, 228-238.

Saxena, R.C., Adhikari, D.K., Goyal, H.B., 2009. Biomass-based energy fuel through biochemical routes: A review. Renew. Sust. Energy Rev. 13, 156-167.

Scott, D.S., Czernik, S., Piskorz, J., Radlein, D., 1989. Sugars from biomass cellulose by a thermal conversion process. In: Klass DL (ed) Energy from biomass and wastes, Institute of Gas Technology, Chicago, IL, 1349-1360.

Shen, D., Xiao, R., Gu, S., Luo, K., 2011. The pyrolytic behavior of cellulose in lignocellulosic biomass: A review. Rsc Adv. 1, 1641-1660.

So, K.S., Brown, R.C., 1999. Economic analysis of selected lignocellulose-to-ethanol conversion technologies. Appl. Biochem. Biotechnol. 77-9, 633-640.

Solaiman, D.K.Y., Garcia, R.A., Ashby, R.D., Piazza, G.J., Steinbuchel, A., 2011 Rendered-protein hydrolysates for microbial synthesis of cyanophycin biopolymer. New Biotechnol. 28, 552-558.

Taherzadeh, M.J., Gustafsson, L., Niklasson, C., Liden, G., 1999. Conversion of furfural in aerobic and anaerobic batch fermentation of glucose by Saccharomyces cerevisiae. J. Biosci. Bioeng. 87, 169-174.

van Leeuwen, J., Khanal, S.K., Pometto, A.L., Rasmussen, M.L., Mitra, D., 2010. Fungi cultivation on alcohol fermentation stillage for useful products and energy savings. US patent application publication. Pub. No. US 2010/0196994 A1.

Vechtlifshitz, S.E., Almas, K.A., Zomer, E., 1990. Microbial growth on peptones from fish industrial wastes. Lett. Appl. Microbiol. 10, 183-186.

Venderbosch, R.H., Prins, W., 2010. Fast pyrolysis technology development. Biofuels, Bioprod. Biorefin. 4, 178-208.

Venkataramanan, K.P., Boatman, J.J., Kurniawan, Y., Taconi, K.A., Bothun, G.D., Scholz, C., 2012. Impact of impurities in biodiesel-derived crude glycerol on the fermentation by *Clostridium pasteurianum* ATCC 6013. Appl. Microbiol. Biotechnol. 93, 1325-1335.

Vitasari, C.R., Meindersma, G.W., De Haan, A.B., 2012. Laboratory scale conceptual process development for the isolation of renewable glycolaldehyde from pyrolysis oil to produce fermentation feedstock. Green Chem. 14, 321-325.

Wang, Y., Li, K., Han, H., Zheng, Z.X., Bureau, D.P., 2008. Potential of using a blend of rendered animal protein ingredients to replace fish meal in practical diets for malabar grouper (Epinephelus malabricus). Aquaculture 281, 113-117.

Yaguchi, T., Tanaka, S., Yokochi, T., Nakahara, T., Higashihara, T., 1997. Production of high yields of docosahexaenoic acid by *Schizochytrium* sp. strain SR21. J. Amer. Oil Chem. Soc. 74, 1431-1434.

Yu, Z.S., Zhang, H.X., 2003. Pretreatments of cellulose pyrolysate for ethanol production by *Saccharomyces cerevisiae*, *Pichia* sp YZ-1 and *Zymomonas mobilis*. Biomass Bioenergy 24, 257-262.

Zaldivar, J., Martinez, A., Ingram, L.O., 2000. Effect of alcohol compounds found in hemicellulose hydrolysate on the growth and fermentation of ethanologenic *Escherichia coli*. Biotechnol. Bioeng. 68, 524-530.

Zhang, Q., Liu, Y., Wang, M., Liu, Q., Hu, J., Yin, Y., 2006. Highly efficient C—C bond-forming reactions of an α-cyanoketene dithioacetal with aldehydes and ketones. Synthesis 18, 3009-3014.

Zhao, X., Peng, F., Du, W., Liu, C., Liu, D., 2012. Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast Rhodosporidium toruloides and preparation of biodiesel by enzymatic transesterification of the lipid. Bioproc. Biosyst. Eng. 35, 993-1004.

Zhuang, X.L., Zhang, H.X., Yang, J.Z., Qi, H.Y., 2001. Preparation of levoglucosan by pyrolysis of cellulose and its citric acid fermentation. Bioresour. Technol. 79, 63-66.

CHAPTER 2 UTILIZATION OF ACETIC ACID RICH PYROLYTIC BIO-OIL BY MICROALGA CHLAMYDOMONAS REINHARDTII: REDUCING BIO-OIL TOXICITY AND ENHANCING ALGAL TOXICITY TOLERANCE

Submitted to Bioresource Technology

Yi Liang, Xuefei Zhao, Zhanyou Chi, Marjorie Rover, Patrick Johnston, Robert Brown, Laura Jarboe, and Zhiyou Wen

ABSTRACT

Bio-oil derived from fast pyrolysis of lignocellulosic biomass contains various substrates that can be fermented by microorganisms to produce various fuels and chemicals. The aim of the present work was to utilize the acetic acid-rich fraction of bio-oil for growth and lipid production in the microalga *Chlamydomonas reinhardtii*. The acetic acid-rich fraction of bio-oil derived from fast pyrolysis of softwood contains around 26% (w/w) of acetic acid, formic acid, methanol, furfural, acetol, and various phenolics as identified compounds, and 13% (w/w) of unidentified compounds. Among those identified compounds, phenolics were most inhibitory to algal growth, followed by acetol. To enhance the fermentability of the acetic acid-rich bio-oil fraction by microalgae, activated carbon treatment was used to reduce the toxicity of this bio-oil fraction, while metabolic evolution was used to enhance the toxicity tolerance of the microalgae strain. Combining the activated carbon treatment and using adapted algal strain through metabolic evolution resulted in significant improvement of algal growth performance on acetic acid-rich bio-oil fraction; the algae were capable of growing in medium with 100% of acetic acid being replaced by bio-oil fraction. The biomass derived from different treatments exhibited similar fatty acid profiles,

with slightly decreasing total fatty acid content. The fast pyrolysis-fermentation process is a viable approach for producing fuels and chemicals from lignocellulosic biomass.

KEYWORDS: Bio-oil, Acetic acid, *Chlamydomonas reinhardtii*, Activated carbon, Metabolic evolution, Lipid production

1. INTRODUCTION

Significant research has been conducted in producing biofuels from lignocellulosic biomass. Most of these efforts have been based on the biological platform, by which lignocellulosic biomass is converted into reduced sugars through pretreatments and enzymatic hydrolysis, followed by yeast or bacterial fermentation of the sugars into biofuels. This conversion pathway faces several challenges such as high pretreatment and enzyme costs, lack of robust microbes capable of fermenting mixed sugars (hexose and pentose), and under-utilization of lignin compounds in the biomass.

Thermochemical-based process (such as fast pyrolysis) is another approach to convert lignocellulosic biomass into fuels and chemicals. Fast pyrolysis is the thermal decomposition of biomass in the absence of oxygen to produce an energy-rich liquid (bio-oil), a flammable gas mixture (syngas), and a carbon- and nutrient-rich solid (biochar) (Brown et al., 2011). It is usually conducted at temperatures of around 500°C and very short reaction times (Bridgwater, 2012a). Raw bio-oil, a major product of fast pyrolysis, can be upgraded into hydrocarbon, which in turn can be refined into drop-in fuels by using existing petroleum refining technology and infrastructure (Mortensen et al., 2011). It can also provide pyrolytic

substrates which can be used in various fermentation processes (Jarboe et al., 2011; Layton et al., 2011).

However, using raw bio-oil for fuel production also faces several challenges (Bridgwater, 2012b). First, bio-oil has 15 – 35% (w/w) water, which leads to low heating value of the product (Bridgwater et al., 1999). Second, bio-oil is rich in carboxylic acids, of which acetic acid produced via lignin depolymerization is the most plentiful. The carboxylic acids are undesirable due to their high corrosiveness and low heating value (Aubin and Roy, 1990). Third, although pyrolytic substrates in bio-oil can be used for fermentation processes, various contaminants contained in bio-oil strongly inhibit microorganisms (Jarboe et al., 2011). Due to the extreme chemical complexity of bio-oil, those inhibitory compounds are difficult to be identified and thus, there is a lack of a rational approach to remove those compounds.

To address the above problems, Pollard et al. (2011) developed a unique system to fractionate bio-oil into five distinct stage fractions (SF) with different physical and chemical characteristics, and each fraction can be used for different purposes. For example, the stage fraction #1 (SF1), which contains the majority of levoglucosan in crude bio-oil, can be used as a fermentation substrate (Jarboe et al., 2011) while stage fraction #5 (SF5), which contains the majority of water and acetic acid, can be excluded from bio-oil refinery for fuel production but used for other applications .

Carboxylic acids from bio-oil have been used to produce road deicers, which was superior to calcium magnesium acetate deicers (Oehr and Barrass, 1992). Recently, Lian et al. (2012), reported using the acetic acid-rich bio-oil phase for yeast fermentation and identified the major hurdle of this process to be the strong inhibition from using this fraction. Although

various pretreatment methods have been employed to reduce inhibition (Jarboe et al., 2011; Lian et al., 2010, 2012), these methods are complicated and expensive. The aim of the present study was to develop simple methods to address this toxicity problem, i.e, reducing the toxicity of bio-oil through a simple active carbon adsorption and/or enhancing the toxicity tolerance of the microorganisms through metabolic evolution of the organism. We used the microalgae *Chlamydomonas reinhardtii* as the model organism for testing our approaches, as this specific species has been widely reported on due to its capability to use acetic acid for heterotrophic growth (Chen and Johns, 1994, 1996). In particular, the genome sequence of *C. reinhardtii* can be modified to enhance lipid production (Work et al., 2010). The lipid contents of the genetically modified species can reach 25-30%, which gives a great opportunity for utilizing acetic acid- derived from bio-oil for lipid-based biofuel production.

2. MATERIALS AND METHODS

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

The raw bio-oil and acetic acid-rich stage fraction #5 (SF5) were prepared by using the pyrolysis fractionation system previously described (Pollard et al., 2011). The feedstock used for pyrolysis was mixed softwood. The SF5 was collected and stored at 4^{o}C in 1-L Nalgene HDPE bottles. Prior to use, the samples were mixed by hand shaking.

2.2. Microorganism, medium and culture conditions

The microalga *Chlamydomonas reinhardtii* 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. To prepare a seed culture, the cells on the agar slant were transferred to 250-

mL Erlenmeyer flasks containing 50 mL Tris-Acetate-Phosphate (TAP) medium with 1 ml/L acetic acid (Hoober, 1989). To test algal growth on bio-oil, the acetic acid-rich SF5 was added to the medium at different concentrations to replace pure acetic acid. The pH of the medium was adjusted to 7 prior to autoclaving at 121^{o}C for 15 min. The flasks were placed in an orbital shaker (200 rpm) at 25^{o}C. Although cells can grow in complete dark areas, providing light to the culture promotes cell growth, therefore, continuous illumination at 110-120 μmol $s^{-1}m^{-2}$ was used. Algal growth was monitored by measuring the optical density at 730 nm and then converted to cell dry weight concentration by using a calibration curve.

2.3. Treatment of SF5 by activated carbon

SF5 was diluted 20-fold with distilled water, adjusted to different pH levels as per experimental set up. The diluted samples were mixed with activated carbon powder (Strem Chemicals, Inc., Newburyport, MA) in 50-mL centrifuge tubes which were placed onto a Wrist Action® Shaker for a thorough mixing. The activated carbon-treated solution was centrifuged and the supernatant was filtered through a 0.45-μm membrane filter.

2.4. Metabolic evolution of *C. reinhardtii* strain

Metabolic evolution of *C. reinhardtii* was performed by maintaining the total acetic acid concentration constant (1 mL/L) in TAP medium while increasing stepwise the proportion of SF5 and decreasing pure acetic acid addition. The culture was initiated with 5% of total acetic acid derived from SF5 and then sub-diluted 10-fold until normal growth performance was observed. An increment (5%) of the bio-oil-derived acetic acid was subsequently employed, and sub-culturing was repeated. By gradually increasing SF5

concentrations and sub-culturing the cells, strains with enhanced tolerance were acquired and high levels of acetic acid replacement were reached.

2.5. Analysis

Water content of SF5 was determined by using a Karl Fischer MKS-500 moisture titrator (Kyoto Electronics Manufacturing Co., Ltd., Japan). The titrant was Hydranal Composite 5 K (Sigma) and the solvent was Hydranal Working Medium K (Sigma). Prior to analysis, the instrument was calibrated by using a water standard purchased from Brinkmann Instrument Company (Delran, NJ).

The content of water-insoluble compounds in SF5 was determined by diluting the bio-oil fraction with water (80°C) at 80:1 ratio and placed in a 50 mL centrifuge tube. The solution was homogenized by using a vortex mixer, treated by ultrasound for 30 min, and then thoroughly mixed for 60 min. The treated solution was filtered through Whatman Grade 42# filter paper. The centrifuge tube and the filter paper were dried at 50°C for 24 h to determine the solid residual in the centrifuge tube and the solids retained by filter paper.

Gas chromatography/mass spectroscopy (GC/MS) analysis was used to identify the chemical compositions of SF5 following procedures previously described (Pollard et al., 2011). The GC was equipped with flame ionization detector (GC-FID) and used to quantify these compounds. All samples were diluted 20-fold with deionized water and filtered through a 0.45-µm membrane filter prior to injection. 1 µL sample (split ratio of 25:1) was injected into a Varian 450-GC (Palo Alto, CA) with Zebron® ZB-WAXplus column (30 m × 0.25 mm × 0.25 µm) (Torrance, CA). The injection temperature was 250°C. The column temperature was kept at 35°C for 6 min, subsequently increased to 60°C at 5°C/min and kept

at this temperature for 2 min. The temperature was further raised to 120°C at 8°C/min, maintained for 2 min, and then increased at the same rate to 230°C. The column then was maintained at 230°C for 2 min for purging. The carrier gas was helium at a constant 1.0 ml/min flow rate. The calibration curves of acetol, methanol, and furfural were linear in the concentration ranges studied.

Formic acid and acetic acid in the SF5 were analyzed by a Dionex ion chromatography ICS 5000 system with a Dionex IonPac® ICE-AS1 column (4 × 250 mm). The column temperature was set at 19°C and the eluent (1.0 mM heptafluorobutyric acid) flow rate was 0.120 mL/min. The concentrations were determined by using standard curves.

Total phenolic compounds contained in SF5 were measured by using Folin-Ciocalteau method with gallic acid being used as calibration standard (Singleton et al., 1999). All values of phenolics concentration were expressed as gallic acid equivalent, and toxicity was based on gallic acid equivalents as well.

Algal biomass was analyzed for fatty acid compositions. The fatty acid methyl esters (FAME) preparation and quantification were described previously (Liang et al., 2011).

2.6. Statistic analysis

Each experiment was conducted in triplicates and means and standard deviations were determined. Cell growths at different bio-oil-derived compound addition levels were analyzed by one-way analysis of variance (ANOVA) by using JMP software (SAS Inc., Cary, NC). The data to be analyzed were combined into a group. For each group, ANOVA was applied to evaluate the difference between mean values. The probability value of <5% ($P<0.05$) was defined as statistically significant. For those compounds significantly inhibiting

cell growth ($P<0.05$), a pair-wise comparison was conducted to determine the inhibitory levels for each compound. To compare the active carbon removal efficiency, ANOVA test was also applied.

3. RESULTS AND DISCUSSIONS

3.1. Characterization of SF5 and the feasibility of *C. reinhardtii* growth on raw SF5

Table 1 shows the major components of SF5 derived from mixed softwood. It contains 59.25 % water, resulting in the low energy content and unsuitability for upgrading into fuel. Water-insoluble compounds account for < 1% of the stream. A variety of compounds were identified in SF5, including acetic acid, formic acid, methanol, acetol, phenolic compounds, and furfural. Due to the extreme complexity of the bio-oil generated from biomass pyrolysis process, there are ~13% compounds that could not be identified by the analytical methods used.

Table 1 shows that the high acetic acid content of SF5 may render this fraction of bio-oil a suitable substrate for fermentation, because acetic acid is a good carbon source for various microorganisms (Ratledge et al., 2001; Barbosa et al., 2001; Fei et al., 2011). We used the microalga *Chlamydomonas reinhardtii* as the model strain to test the fermentability of SF5. This algal species is capable of using acetic acid as a substrate for heterotrophic high-cell-density cultivation (Chen, 1996). In addition, studies have shown that this species can be genetically modified to enhance the lipid content (Li et al., 2010; Work et al., 2010), which provides a great opportunity for producing lipid-based fuel.

Figure 1 shows the cell growth of *C. reinhardtii* on TAP medium containing pure acetic acid (control), and 5 and 10% of acetic acid replacement by SF5, respectively. When 5%

acetic acid was replaced by SF5, algae growth was partially inhibited within the first two days. Starting from day 3, the cell growth caught up the control culture. When SF5 addition was increased to 10% acetic acid replacement, the inhibition became significant and algal cells were not able to recover in the entire growth period. The above results indicate the inhibitory effect of SF5 on algal growth, particularly at high concentrations. To better understand the mechanisms of this toxicity, the effect of each individual major compound in bio-oil on algal growth was tested.

3.2. Effects of bio-oil derived compounds on growth of *C. reinhardtii*

To determine inhibitory effects of the compounds contained in SF5, an appropriate concentration range needed to be determined for each compound. Our previous work showed that *C. reinhardtii* grows in medium containing up to 4 ml/L of acetic acid without inhibition (data not shown). Therefore, 4 ml/L acetic acid was used to determine the maximum volume of bio-oil fraction to be added. The concentration of the other compounds at this maximum bio-oil addition level was set as the highest end of the concentration range for those compounds.

Figure 2 shows the algal growth in medium added different compounds. As shown in Figures 2A-2B, adding formic acid and methanol resulted in similar growth as the control within the concentration ranges studied. ANOVA test indicted that the inhibitory effects of these two compounds were insignificant ($P>0.05$). These results differed from previous reports. For example, formic acid as a byproduct of lignocellulosic hydrolysates has shown negative effects on the replication of *Debaryomyces hansenii* and *Rhodosporidium toruloides*, because undissociated formic acid can diffuse across the plasma membrane, and

consequently influence the metabolisms of the cells (Zhao et al., 2012; Duarte et al., 2005). Methanol increases cell membrane fluidity, and thus, adversely affects cell growth and metabolism (Venkataramanan et al., 2012). In the present work, the absence of inhibitory effects observed for these two compounds may be due to the lower concentration range used as previous studies showing the inhibitory effect of these compounds used much higher concentrations. For example, the inhibition of formic acid on the growth of the oleaginous yeast *Rhodosporidium toruloides* was only found when its concentration reached 2 g/L(Zhao et al., 2012); methanol did not affect the growth of *Clostridium pasteurianum* even at a concentration of 5.0 g/L (Venkataramanan et al., 2012).

Figures 2C- 2E show that the cell growth in medium containing furfural, acetol and phenolics decreased with increasing concentrations of these compounds. A pair-wise comparison showed significant (P<0.05) inhibition of these compounds on *C. reinhardtii* growth. Within the concentration range studied, however, the inhibitory effects of furfural and acetol were not as strong as phenolic compounds. Up to 0.6 g/L furfural and 3.2 g/L acetol, the biomass was reduced by ~30%. Furfural has also been considered to be a strong growth inhibitor for microorganisms (Zhao et al., 2012), while the effect of acetol on cell growth is highly species specific (Lian et al., 2012). For instance, acetol inhibited the replication of the yeast *R. glutinis*, however, it supported the growth of another yeast species, *C. curvatus*, when used as the sole carbon source. The phenolic compounds resulting from lignin decomposition have long been identified as growth inhibitors to microorganisms. The inhibitory effects are mainly attributed to the hydrophobicity of the phenolics, which makes them capable of incorporating into biological membranes, and thus, deteriorate cell membranes and denature membrane-associated enzymes (Zaldivar et al., 2000; Heipieper et

al., 1994). Figure 2E shows that phenolics at >0.5 g/L significantly inhibited the growth of *C. reinhardtii.*

3.3. Activated carbon treatment for detoxification of SF5

The previous results showed the inhibition of SF5 when used as algal culture substrate. Therefore, an appropriate treatment to remove the inhibitory compounds in bio-oil is needed. In the present work, activated carbon was used to reduce toxicity of bio-oil fraction SF5.

Table 2 shows the effect of contact time for activated carbon treatment. The ANOVA test showed that activated carbon did not significantly (P<0.05) adsorb acetic acid, formic acid, and methanol; but, did adsorb furfural, acetol, and phenolic compounds during the 2.5-h period of contact time. For each compound, treatment time from 0.5 to 2.5 h did not result in significant (P< 0.05) different removal efficiency (ANOVA). Therefore, 0.5 h was sufficient to reach the adsorption equilibrium for activated carbon treatment.

Further investigations on the effects of activated carbon loading and pH on the removal of different compounds were conducted. As shown in Figure 3A, the adsorption of acetic acid was highly dependent on pH and no adsorption was observed at pH 6 and 8. At pH 2 and 4, however, increased loading of activated carbon significantly reduced residual acetic acid in the aqueous phase. Adsorption of acetic acid (pKa=4.75) only took place with undissociated molecules. This same adsorption trend applied to formic acid (pKa=3.75) as well (Figure 3B). Figure 3C shows that methanol was not absorbed by activated carbon at any of the treatment conditions. While Figure 3D shows that acetol was partially removed by activated carbon and removal efficiency of this compound was not significantly different

(P>0.05) among all test conditions. Activated carbon removed furfural very efficiently (Figure 3E). Regardless of pH, an activated carbon loading of over 0.7 g/mL SF5 completely removed furfural. The removal of phenolic compounds was also appreciable although not as efficient as for furfural (Figure 3F). The removal efficiency of phenolics was a function of activated carbon loading, independent of pH. The purpose of activated carbon treatment was to retain acetic acid while reducing other inhibitory compounds. The optimal condition was therefore chosen as pH 6 with active carbon loading of 1.0 g/mL SF5.

3.4. Metabolic evolution of *C. reinhardtii* for developing inhibitors-tolerant strains

The previous results show activated carbon treatment was an effective method for removing inhibitory compounds (e.g., acetol and phenolics) from SF5. However, the SF5 sample also contained ~13% unidentified compounds, some of which might also inhibit the cell growth. Due to the extreme complexity of the bio-oil composition, it was challenging to identify and quantify unknown compounds and, thus, to develop a pretreatment method to remove all those compounds. In addition, the methods reported for bio-oil detoxification, such as distillation and solvent extraction, are expensive to be implemented at commercial scale (Lian et al., 2010, 2012; Chan and Duff, 2010; Vitasari et al., 2012). Therefore, another approach for improving the bio-oil utilization is to develop robust strains that can tolerate bio-oil toxicity. In the present study, we used metabolic evolution approach to achieve this goal.

As shown in Figure 4, *C. reinhardtii* was first sub-cultured in medium with 5% acetic acid being replaced by SF5. Algal cells were able to grow and divide at similar performance as that of the control. After sub-culturing at this SF5 level (SF5 replacing 5% acetic acid) for

three generations, the algal cells were transferred to medium containing 10% acetic acid being replaced by SF5. Sub-culturing was repeated at the SF5 level for 3-4 generations before increasing to the next level of acetic acid replacement by SF5. Over 170 days of operation, the algal cells successfully adapted to 50% replacement of acetic acid by SF5 (Figure 4). Compared to the feasibility test of *C. reinhardtii* growth on raw SF5 stream (Figure 1), in which the algal cells barely survived in 10% acetic acid replacement medium, metabolic evolution was an effective approach to enhance the cells' tolerance to bio-oil toxicity (Figure 4).

Metabolic evolution (also called directed evolution) is frequently used to improve strain performance by increasing the biocatalyst tolerance to inhibitory compounds such as furfural (Miller et al., 2009a, 2009b, 2010) and isobutanol (Atsumi et al., 2010). This method has been used with *C. reinhardtii* culture to build a model system for pro-active herbicide resistance. Under selective pressures, the level of herbicide tolerance of *C. reinhardtii* was greatly increased after 10 generations of growth (Reboud et al., 2007). The mechanism of metabolic evolution is that when the cells are subjected to non-lethal toxic environment, they tend to adapt to it by acquiring mutations in DNA, either during DNA replication or by DNA damage. Although some of these mutations may negatively impact algal fitness and some may have no effect, some mutations often increase tolerance to toxic compound(s). Strains that acquire these mutations grow faster than their non-mutated cohorts and eventually take over the entire population (Nealson et al., 2003). By sub-culturing cells on a regular basis and gradually increasing the concentration of toxic compound(s), cells with beneficial mutations are able to grow faster and become the dominant strains in the culture (Nealson et al., 2003).

3.5. Fermentability of *C. reinhardtii* on acetic acid-rich SF5

Different algal cultures were performed to evaluate activated carbon treatment and metabolic evolution as an approach to improve fermentability of SF5. The fermentation conditions include: (i) wild type strain in standard TAP medium (wild type -TAP); (ii) wild type strain in medium with 10% acetic acid replaced with activated-carbon-treated SF5 (wild type -10% -AC); (iii) adapted strain in medium with 50% acetic acid replaced with raw SF5 (adapted - 50% -Raw); (iv) adapted strain in medium with 50% acetic acid replaced with activated-carbon-treated SF5 (adapted-50%-AC); and (v) adapted strain in medium with 100% acetic acid replaced with activated-carbon-treated SF5 (adapted-100%-AC).

Although with longer lag phase, the wild type strain successfully grew in activated-carbon-treated SF5 at 10% acetic acid replacement (wild-10%-AC, Figure 5). Compared with the growth performance of the same strain at 10% replacement using non-treated SF5 (Figure 1), activated carbon positively affected bio-oil detoxification. However, when acetic acid replacement was increased to 20% activated-carbon-treated SF5, cell growth was significantly inhibited (data not shown), indicating the limitation of using activated carbon alone for treatment. This may be due to the presence of some unidentified toxic compounds in activated-carbon-treated SF5, even at trace amounts.

Figure 5 also shows that the adapted strains through metabolic evolution were capable of growing in medium with 50% acetic acid substituted by raw SF5 (adapted-50%-Raw). When using adapted strains for growing on activated-carbon-treated SF5 (adapted-50%-AC), algal growth was further improved as indicated by the shortened lag phase. The adapted

strains could even grow in medium with 100% acetic acid being replaced by activated-carbon-treated SF5 (adapted-100%-AC).

The fatty acid content and composition of the algal biomass were analyzed to evaluate the potential of producing lipid-based biofuel from pyrolytic bio-oil. As shown in Table 3, the contents of major fatty acids derived from different strains and bio-oil sources were similar, indicating that SF5 did not change the fatty acid composition, and the wild type and metabolically evolved strains possessed similar fatty acid synthesis pathways. The control culture had higher total fatty acid (TFA) content than those grown on bio-oil streams, particularly with activated-carbon-treated culture. The reason may be that activated carbon removed some growth inhibitors as well as lipid synthesis promoters in bio-oil. Indeed, Lian et al (2012) reported that the acetate consumption yeast *L. starkeyi* can use acetol for lipid production, and the addition of external acetol, *L. starkeyi* resulted in higher level of lipids.

4. CONCLUSIONS

The present study proves the feasibility of using acetic acid-rich bio-oil fractions for microalgae cultivation with the potential of producing lipid-based biofuel. Because bio-oil consists of myriads of compounds, which exhibit strong inhibition to algal growth, complete separation or purification of bio-oil is not easy to achieve. Activated carbon adsorption and development of toxicity-tolerant strains are economically viable tools, which make biological utilization of bio-oil possible. The present work opens up a great opportunity for fermentative production of fuels and chemicals by using bio-oil derived substrates.

REFERENCES

Atsumi, S., Wu, T.Y., Machado, I.M.P., Huang, W.C., Chen, P.Y., Pellegrini, M., Liao, J.C., 2010. Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*. Mol. Syst. Biol. 6, (449), 1-11.

Aubin, H., Roy, C., 1990. Study on the corrosiveness of wood pyrolysis oils. Fuel Sci. Technol. Int. 8, 77-86.

Barbosa, M.J., Rocha, J.M.S., Tramper, J., Wijffels, R.H., 2001. Acetate as a carbon source for hydrogen production by photosynthetic bacteria. J. Biotechnol. 85, 25-33.

Bridgwater, A.V., 2012a. Review of fast pyrolysis of biomass and product upgrading. Biomass Bioenergy 38, 68-94.

Bridgwater, A.V., 2012b. Upgrading Biomass Fast Pyrolysis Liquids. Environ. Prog. Sustainable Energy 31, 261-268.

Bridgwater, A.V., Meier, D., Radlein, D., 1999. An overview of fast pyrolysis of biomass. Org. Geochem. 30, 1479-1493.

Brown, T.R., Wright, M.M., Brown, R.C., 2011. Estimating profitability of two biochar production scenarios: Slow pyrolysis vs fast pyrolysis. Biofuel Bioprod. Biorefin. 5, 54-68.

Chan, J.K.S., Duff, S.J.B., 2010. Methods for mitigation of bio-oil extract toxicity. Bioresour. Technol. 101, 3755-3759.

Chen, F., 1996. High cell density culture of microalgae in heterotrophic growth. Trends Biotechnol. 14, 421-426.

Chen, F., Johns, M.R., 1994. Substrate inhibition of *Chlamydomonas reinhardtii* by acetate in heterotrophic culture. Proc. Biochem. 29, 245-252.

Chen, F., Johns, M.R., 1996. Heterotrophic growth of *Chlamydomonas reinhardtii* on acetate in chemostat culture. Proc. Biochem. 31, 601-604.

Duarte, L.C., Carvalheiro, F., Neves, I., Girio, F.M., 2005. Effects of aliphatic acids, furfural, and phenolic compounds on *Debaryomyces hansenii* CCMI 941. Appl. Biochem. Biotechnol. 121, 413-425.

Fei, Q., Chang, H.N., Shang, L., Choi, J.D.R., Kim, N., Kang, J., 2011. The effect of volatile fatty acids as a sole carbon source on lipid accumulation by *Cryptococcus albidus* for biodiesel production. Bioresour. Technol. 102, 2695-2701.

Heipieper, H.J., Weber, F.J., Sikkema, J., Keweloh, H., Debont, J.A.M., 1994. Mechanisms of resistance of whole cells to toxic organic solvents. Trends Biotechnol. 12, 409-415.

Hoober, J.K., 1989. The *Chlamydomonas* sourcebook: a comprehensive guide to biology and laboratory use, Harris, E.H. (Ed), Academic Press, San Diego, CA, 246, 1503-1504.

Jarboe, L.R., Wen, Z., Choi, D., Brown, R.C., 2011. Hybrid thermochemical processing: fermentation of pyrolysis-derived bio-oil. Appl. Microbiol. Biotechnol. 91, 1519-1523.

Layton, D.S., Ajjarapu, A., Choi, D.W., Jarboe, L.R., 2011. Engineering ethanologenic Escherichia coli for levoglucosan utilization. Bioresour. Technol. 102, 8318-8322.

Lee, J.M., Venditti, R.A., Jameel, H., Kenealy, W.R., 2011. Detoxification of woody hydrolyzates with activated carbon for bioconversion to ethanol by the thermophilic anaerobic bacterium *Thermoanaerobacterium saccharolyticum*. Biomass Bioenergy 35, 626-636.

Li, Y.T., Han, D.X., Hu, G.R., Sommerfeld, M., Hu, Q.A., 2010. Inhibition of starch synthesis results in overproduction of lipids in *Chlamydomonas reinhardtii*. Biotechnol. Bioeng. 107, 258-268.

Lian, J., Chen, S., Zhou, S., Wang, Z., O'Fallon, J., Li, C.Z., Garcia-Perez, M., 2010. Separation, hydrolysis and fermentation of pyrolytic sugars to produce ethanol and lipids. Bioresour. Technol. 101, 9688-9699.

Lian, J., Garcia-Perez, M., Coates, R., Wu, H., Chen, S., 2012. Yeast fermentation of carboxylic acids obtained from pyrolytic aqueous phases for lipid production. Bioresour. Technol. 118, 177-86.

Liang, Y., Garcia, R.A., Piazza, G.J., Wen, Z., 2011. Nonfeed application of rendered animal proteins for microbial production of eicosapentaenoic acid by the fungus *Pythium irregulare*. J. Agri. Food Chem. 59, 11990-11996.

Miller, E.N., Jarboe, L.R., Turner, P.C., Pharkya, P., Yomano, L.P., York, S.W., Nunn, D., Shanmugam, K.T., Ingram, L.O., 2009a. Furfural inhibits growth by limiting sulfur assimilation in ethanologenic *Escherichia coli* Strain LY180. Appl. Environ. Microbiol. 75, 6132-6141.

Miller, E.N., Jarboe, L.R., Yomano, L.P., York, S.W., Shanmugam, K.T., Ingram, L.O., 2009b. Silencing of NADPH-dependent oxidoreductase genes (yqhD and dkgA) in furfural-resistant ethanologenic *Escherichia coli*. Appl. Environ. Microbiol. 75, 4315-4323.

Miller, E.N., Turner, P.C., Jarboe, L.R., Ingram, L.O., 2010. Genetic changes that increase 5-hydroxymethyl furfural resistance in ethanol-producing *Escherichia coli* LY180. Biotechnol. Lett. 32, 661-667.

Mortensen, P.M., Grunwaldt, J.D., Jensen, P.A., Knudsen, K.G., Jensen, A.D., 2011. A review of catalytic upgrading of bio-oil to engine fuels. Appl. Catal. A: Gen. 407, 1-19.

Nealson, K.H., Rye, R., 2003. Evolution of metabolism. Treatise on Geochemistry, William, H. (Ed) Oxford, Pergamon, Vol. 8.

Oehr, K.H., Barrass, G., 1992. Biomass derived alkaline carboxylate road deicers. Resour. Conserv. Recy. 7, 155-160.

Pollard, A.S., Rover, M.R., Brown, R.C., 2011. Characterization of bio-oil recovered as stage fractions with unique chemical and physical properties. J. Anal. Appl. Pyrol. 93, 129-138.

Ratledge, C., Kanagachandran, K., Anderson, A.J., Grantham, D.J., Stephenson, J.C., 2001. Production of docosahexaenoic acid by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source. Lipids 36, 1241-1246.

Reboud, X., Majerus, N., Gasquez, J., Powles, S., 2007. *Chlamydomonas reinhardtii* as a model system for pro-active herbicide resistance evolution research. Biol. J. Linn Soc. 91, 257-266.

Singleton, V.L., Orthofer, R., Lamuela-Raventos, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol. 299, 152-178.

Venkataramanan, K.P., Boatman, J.J., Kurntawan, Y., Taconi, K.A., Bothun, G.D., Scholz, C., 2012. Impact of impurities in biodiesel-derived crude glycerol on the fermentation by *Clostridium pasteurianum* ATCC 6013. Appl. Microbiol. Biotechnol. 93, 1325-1335.

Vitasari, C.R., Meindersma, G.W., De Haan, A.B., 2012. Laboratory scale conceptual process development for the isolation of renewable glycolaldehyde from pyrolysis oil to produce fermentation feedstock. Green Chem. 14, 321-325.

Work, V.H., Radakovits, R., Jinkerson, R.E., Meuser, J.E., Elliott, L.G., vineyard, D.J., Laurens, L.M.L., Dismukes, G.C., Posewitz, M.C., 2010. Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. Eukaryotic Cell 9, 1251-1261.

Zaldivar, J., Martinez, A., Ingram, L.O., 2000. Effect of alcohol compounds found in hemicellulose hydrolysate on the growth and fermentation of ethanologenic *Escherichia coli*. Biotechnol. Bioeng. 68, 524-530.

Zhao, X., Peng, F., Du, W., Liu, C., Liu, D., 2012. Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast *Rhodosporidium toruloides* and preparation of biodiesel by enzymatic transesterification of the lipid. Bioproc. Biosyst. Eng. 35, 993-1004.

Table 1. Chemical characterization of SF5

Chemical compounds	% (w/w)
Water	59.25±2.44
Water insoluble	0.75±0.08
Acetic acid	11.08±0.15
Formic acid	0.82±0.03
Methanol	1.63±0.33
Furfural	1.47±0.15
Acetol	9.27±0.71
Total phenolics	2.16±0.22
Unknown compounds	13.64±2.58
Mass closure	100

Data are means of triplicates ± standard deviations.

Table 2. The concentrations (% w/w) of each individual compound in SF5 after treatment with active carbon for different durations [a]

Contact time (h)	Acetic acid	Formic acid	Methanol	Furfural	Acetol	Phenolics
0.5	10.52±0.80	0.81±0.02	1.54±0.21	0.35±0.16	5.63±1.62	1.94±0.18
1	10.78±0.33	0.86±0.05	1.55±0.21	0.54±0.11	6.51±1.07	1.89±0.24
1.5	11.12±0.12	0.79±0.04	1.46±0.37	0.43±0.03	5.45±0.41	1.92±0.07
2	10.94±0.32	0.78±0.02	1.50±0.20	0.43±0.18	5.98±0.66	1.86±0.11
2.5	10.62±0.51	0.80±0.04	1.57±0.23	0.40±0.08	6.33±0.87	1.90±0.15
w/o AC[b]	11.08±0.15	0.82±0.03	1.63±0.33	1.47±0.15	9.27±0.71	2.16±0.22

[a] Experiments were conducted at pH 7 and 25°C with activated carbon loading of 0.2 g /mL of diluted bio-oil solution (20-fold). Data are means of triplicates ± standard deviations.
[b] No activated carbon was used.

Table 3. Fatty acid composition (% TFA) and total fatty acid (TFA) content (% DW) of *C. reinhardtii* (wild-type and adapted cells through metabolic evolution) produced at different culture conditions [a]

Fatty acid (%TFA)	Wild type -TAP	Wild type-10% -AC	Adapted-50% -Raw	Adapted-50% -AC	Adapted-100% -AC
16:0	27.68±0.93	28.06±1.76	31.99±1.20	28.06±0.39	24.53±0.43
16:1	1.85±0.05	1.61±0.15	2.59±0.24	1.55±0.71	1.92±0.21
18:0	3.11±0.65	3.22±0.31	N/D	3.03±0.32	1.35±0.29
18:1	15.42±0.98	15.03±1.76	16.57±1.39	23.55±1.55	28.25±3.24
18:2	17.33±1.25	16.10±1.15	13.19±0.66	18.52±0.43	12.32±0.84
20:0	5.78±0.33	7.21±0.69	5.36±0.42	7.55±0.45	4.63±1.00
18:3	28.88±1.31	28.75±2.37	26.45±1.57	30.84±1.81	27.01±2.14
20:2	N/D	N/D	3.86±0.84	N/D	N/D
TFA (%DW)	18.35±0.14	13.04±0.23	16.6±0.47	15.50±0.25	14.35±0.26

[a] Data are means of triplicates ± standard deviations.

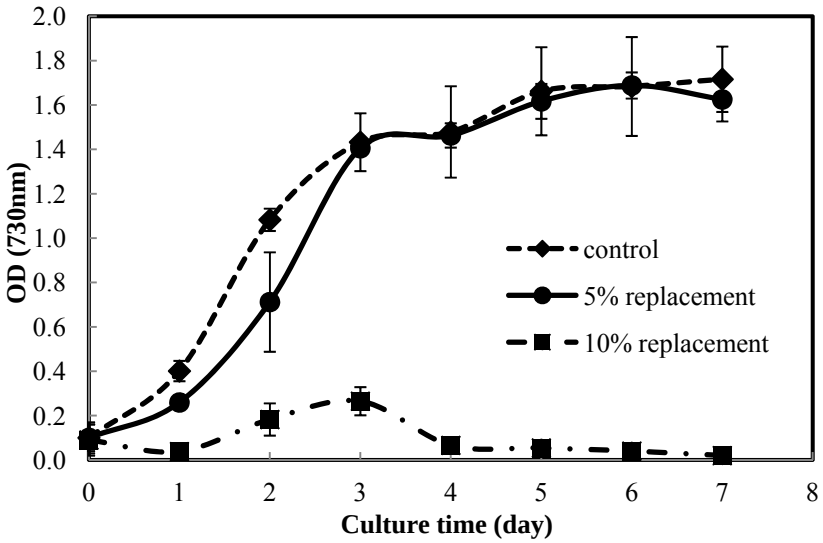


Figure 1. Growth of *C. reinhardtii* strain in TAP medium with 1 ml/L pure acetic acid (control); 5% acetic acid in TAP replaced with SF5; and 10% acetic acid in TAP replaced with SF5. Data are means of triplicates and error bars show standard deviations.

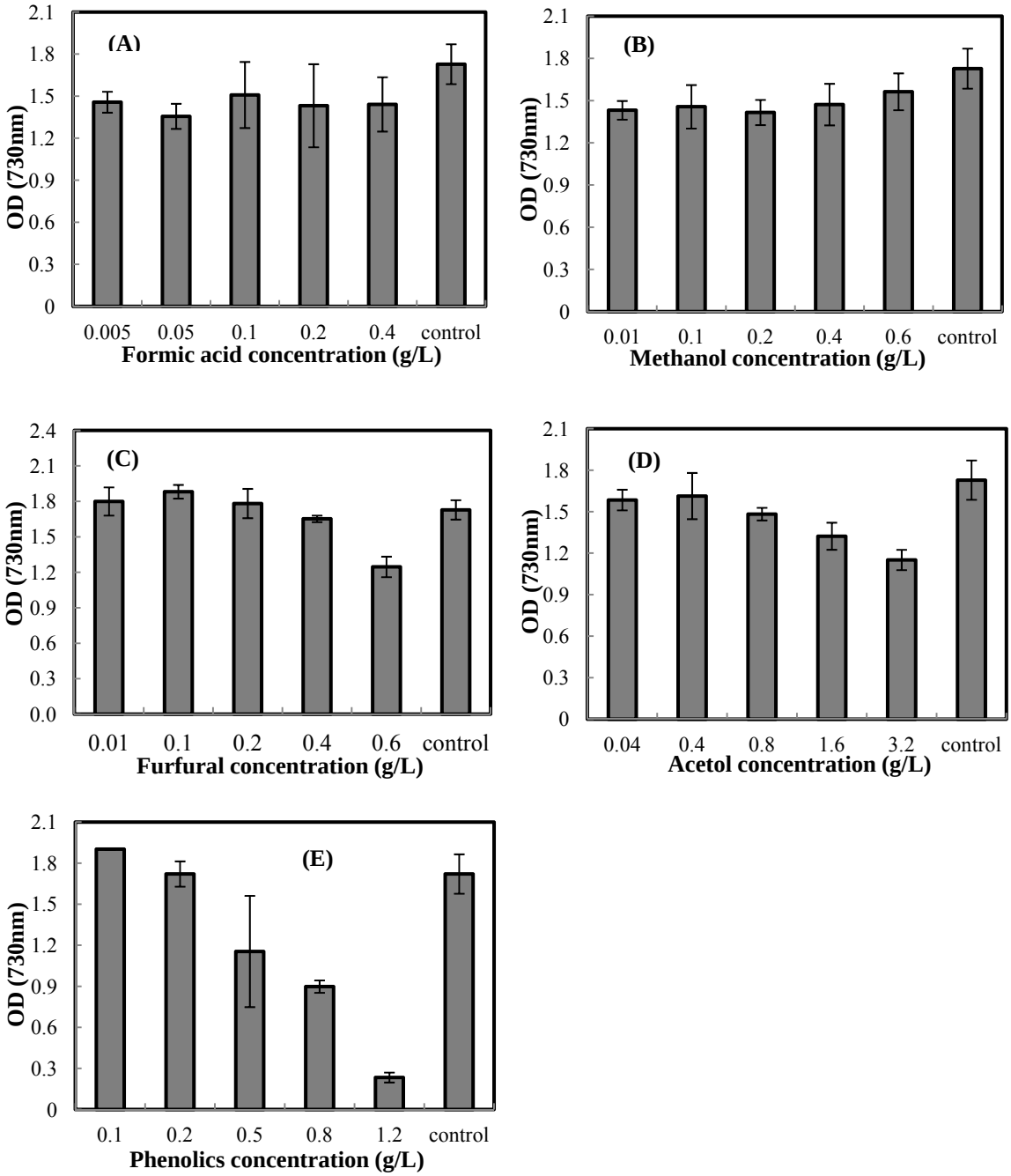


Figure 2. Effects of formic acid (A), methanol (B), furfural (C), acetol (D), and phenolic compounds (E) on the growth of *C. reinhardtii* at different concentrations. OD values were measured at day 5. Data are means of triplicates and error bars show standard deviations.

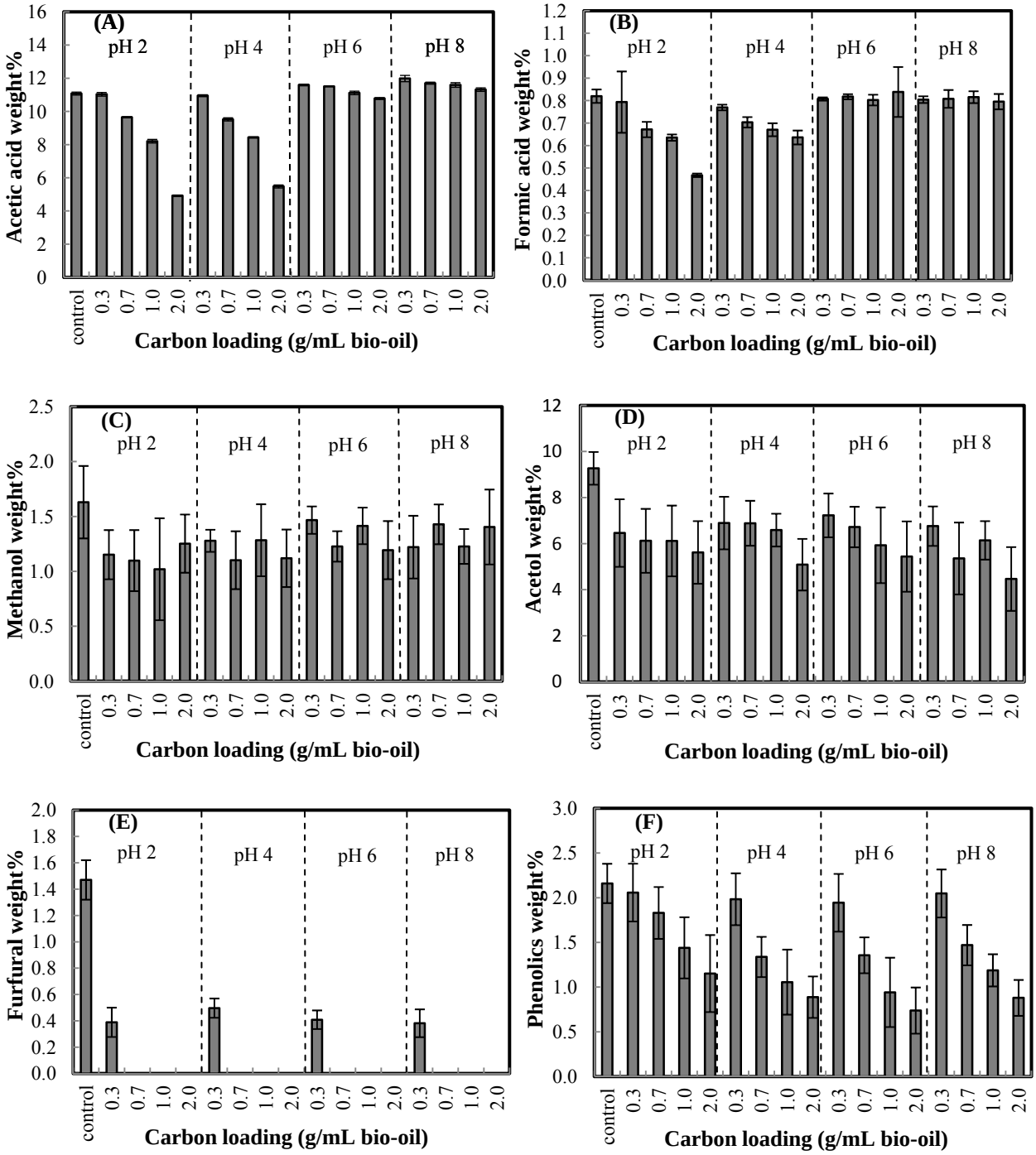


Figure 3. The percentages of (A) acetic acid, (B) formic acid, (C) methanol, (D) acetol, (E) furfural, and (F) phenolics remained in SF5 after activated carbon treatment at different carbon loading and pH. Data are means of triplicates and error bars indicate standard deviations. Treatment time was 0.5 h.

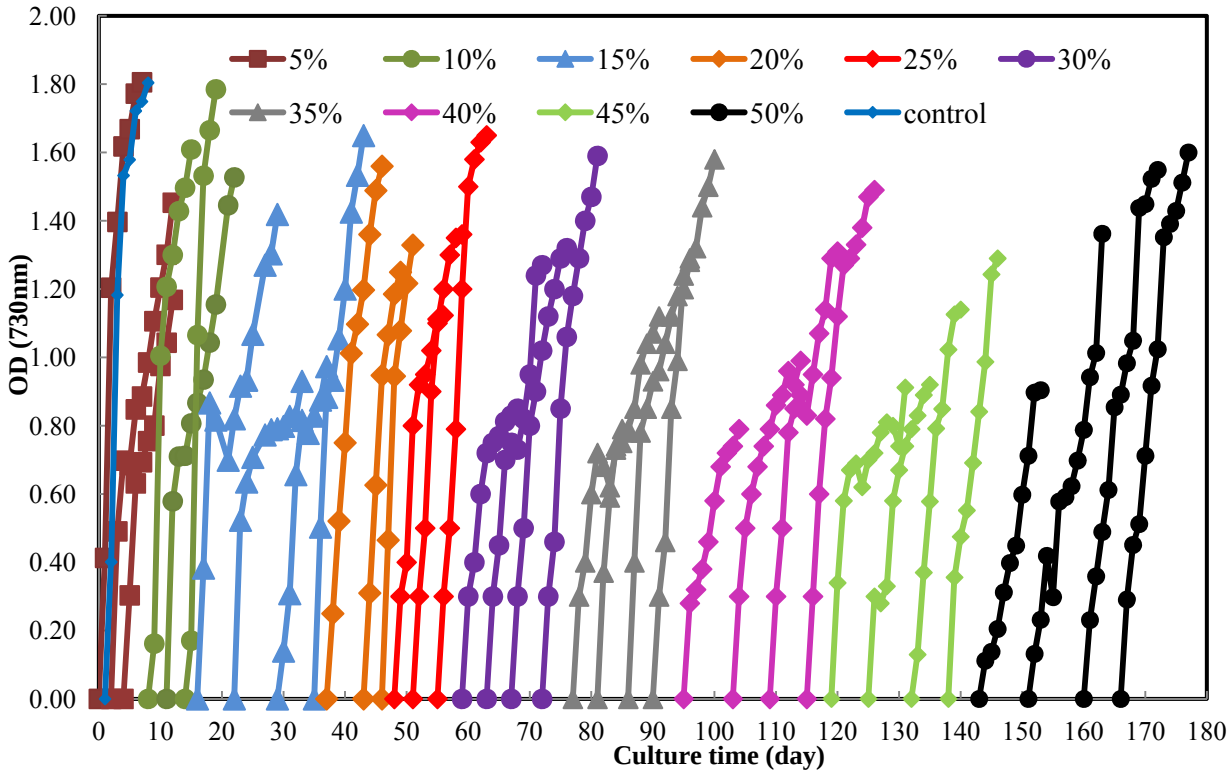


Figure 4. Growth of *C. reinhardtii* with increasing stepwise acetic acid replacement percentage by SF5.

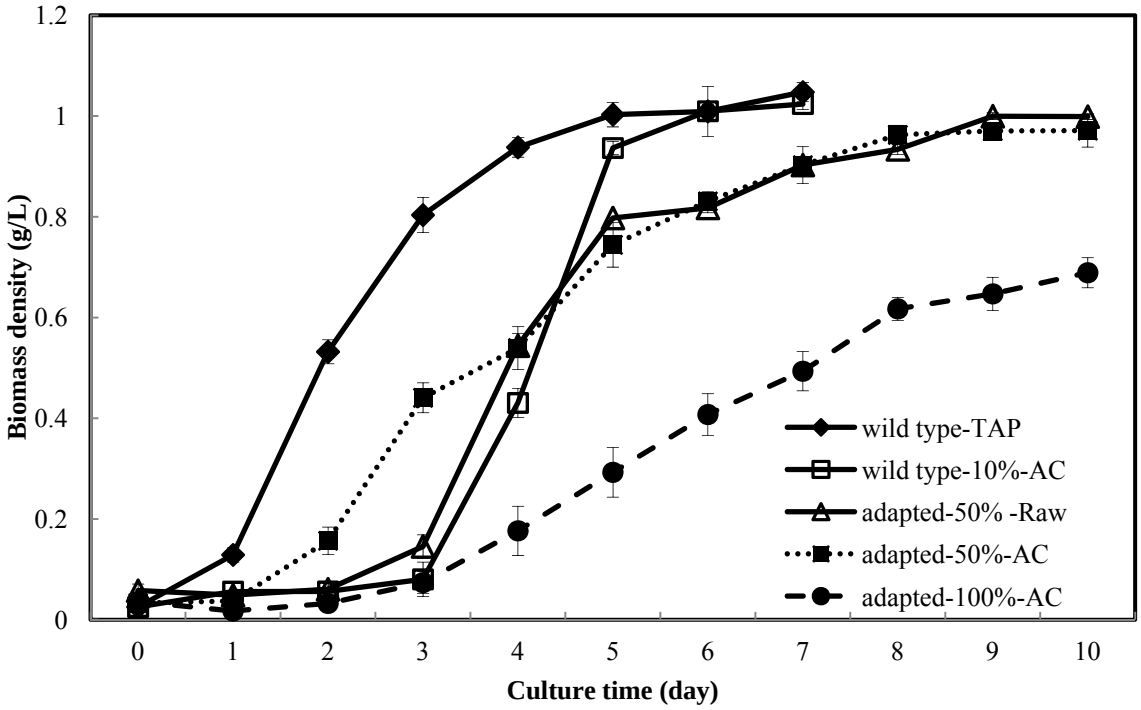


Figure 5. Growth of *C. reinhardtii* (both wild type and the metabolic evolution adapted strain) at different culture conditions. Data are means of triplicates and error bars indicate standard deviations.

CHAPTER 3 NON-FEED APPLICATION OF RENDERED ANIMAL PROTEINS FOR MICROBIAL PRODUCTION OF EICOSAPENTAENOIC ACID BY THE FUNGUS *PYTHIUM IRREGULARE*

Published in Journal of Agriculture and Food Chemistry

Yi Liang, Rafael A. Garcia, George J. Piazza, Zhiyou Wen

ABSTRACT

Rendered animal proteins are well suited for animal nutrition applications, but the market is maturing, and there is a need to develop new uses for these products. The objective of the present study was to explore the potential of using animal proteins as a nutrient source for microbial production of omega-3 polyunsaturated fatty acids by the microalga *Schizochytrium limacinum* and the fungus *Pythium irregulare*. In order to be absorbed by the microorganisms, the proteins needed to be hydrolyzed into small peptides and free amino acids. The utility of the protein hydrolysates for microorganisms depended on the hydrolysis method used and the type of microorganism. The enzymatic hydrolysates supported better cell growth than did the alkali hydrolysates. *P. irregulare* displayed better overall growth performance on the experimental hydrolysates compared to *S. limacinum*. When *P. irregulare* was grown in medium containing 10 g/L enzymatic hydrolysate derived from meat and bone meal or feather meal, cell growth, lipid synthesis, and omega-3 fatty acid production were similar to the culture using commercial yeast extract. The fungal biomass derived from the animal proteins contained 26-29% lipid, 32-34% protein, 34-39% carbohydrate, and < 2% ash. It was possible to hydrolyze rendered animal protein and feed

industrial microorganisms which can produce omega-3 fatty acids for making omega-3 fortified foods or feeds.

KEYWORDS: Rendered protein; yeast extract; *Pythium irregulare*; lipid; omega-3 fatty acid; meat and bone meal; feather meal; blood meal

1. INTRODUCTION

In meat processing, the low-value tissues (bone, offal, feather, and blood) from animal carcasses are typically rendered into dry meals that can be used as protein components in animal feeds or pet foods. These meals include meat and bone meal (MBM), feather meal (FM), and blood meal (BM). The traditional markets for these products are maturing and, in some cases, shrinking due to the concern of bovine spongiform encephalopathy caused by specific risk materials (SRMs) contained in the rendered protein products (FDA, 2008). As a result, development of a new outlet for large quantities of rendered animal proteins is needed.

Research has been conducted to increase the non-feed usage of the rendered animal proteins. For example, Garcia et al. (2011) investigated the hydrolysis of MBM, FM and BM to increase the solubility of the raw protein and prepare small peptides with potential nutritional value for microorganism growth. They used enzyme hydrolyzed MBM as a medium ingredient for fermentation of biopolymer producing *E. coli* (Solaiman et al., 2011). The non-nutritional characteristics of the protein hydrolysates, such as hygroscopicity, chromicity, autoclave stability, viscosity, and foaming, were also reported (Garcia et al.,

2010). These results demonstrate the opportunity to explore animal proteins as a low-cost nutrition source for microorganism fermentation. This is particularly attractive for industrial fermentation focusing on commodity and moderate-value products, because this type of fermentation is characterized by being larger volume and more sensitive to the cost of growth medium ingredients (Van Hoek et al., 2003).

Omega-3 polyunsaturated fatty acids, including eicosapentaenoic acid (EPA, C20:5, n-3) and docosahexaenoic acid (DHA, C22:6, n-3) possess many health promoting properties, such as prevention of human cardiovascular disease, cancer, schizophrenia, and Alzheimer's disease (Simopoulos, 1999) and thus, can be used for making various omega-3 fortified food products. In the aquaculture industry, omega-3 fatty acids are essential nutrients for feeding marine fish. Currently, fish oil is still the major commercial source of omega-3 fatty acids although it has various limitations such as odor/taste problems, heavy metal contamination, and limited supply. Microbial fermentative production of omega-3 fatty acids for moderate-value products, such as fish and animal feeds, is still not economical due to high production cost. Indeed, commercial production of omega-3 fatty acid through microbial fermentation is only available in products with high profit margins (e.g., infant formula).

In previous studies, we developed algal and fungal fermentation processes to produce omega-3 fatty acids (Pyle et al., 2008; Athalye et al., 2009). The microalga *Schizochytrium limacinum* (DHA producer) and the fungus *Pythium irregulare* (EPA producer) can use crude glycerol from the biodiesel industry as a carbon source, so the growth medium cost can be significantly reduced. However, these two microorganisms also need a large amount of peptone and yeast extract as sources of nitrogen, vitamins, and trace elements for growth; the inclusion of commercial peptone and yeast extract represents another significant portion of

the medium cost (Pyle et al., 2008; Athalye et al., 2009; Vechtlifshitz et al., 1990). This cost could be reduced if yeast extract and peptone were replaced by less expensive rendered protein meals. The objective of the present work was to test the feasibility of using these low-cost animal protein meals as substitutes for commercial peptone and yeast extract in the fermentation of *S. limacinum* and *P. irregulare.*

2. MATERIALS AND METHODS

2.1 Raw animal protein materials and hydrolysis reagent

Ruminant MBM and flash-dried cattle BM were obtained from Darling International (Irving, TX); FM was obtained from Carolina By-Products (Winchester, VA). The hydrolytic agents included Bell Mine high calcium hydrated lime (Tannin Corp., Peabody, MA), Versazyme (BioResource International, Morrisville, NC), Alcalase 2.4L and Flavourzyme (Novozymes, Bagsvaerd, Denmark). Commercial Bacto Yeast Extract and Bacto Proteose Peptone were obtained from DIFCO laboratories (Detroit, MI) and Becton, Dickinson and Company (Sparks, MD), respectively.

2.2 Protein hydrolysate preparation

The preparation of animal protein hydrolysate was previously described (Garcia et al., 2011). In brief, the protein meals were first defatted by using hexane extraction, followed by alkaline or enzymatic hydrolysis. The durations of alkaline hydrolysis were 4, 8 and 16 h (Table 1). For enzymatic treatment, three different enzymes were involved, including alcalase, versazyme and flavourzyme. Alcalase is an endo-protease of the serine type having broad substrate specificity; versazyme is a proteolytic enzyme used for the conversion of

animal by-products into protein sources for use in manufacturing animal feed; and flavourzyme is an aminopeptidase. The hydrolysis conditions are shown in Table 2. The hydrolysis reactions were terminated following protocols previously described (Garcia et al., 2011), then the residual solid material was removed from the liquid and the remaining hydrolysate was dehydrated using a Büchi B-191 Mini Spray Drier (Flawil, Switzerland).

2.3 **Microorganisms, medium and culture conditions**

The microalga *S. limacinum* SR-21 (ATCC MYA-1381) and the fungus *P. irregulare* (ATCC 10951) were used. The two species were maintained in culture medium as recommended by ATCC. The medium composition for *S. limacinum* was 5 g/L glucose, 1.0 g/L yeast extract, and 1.0 g/L peptone dissolved in artificial seawater. The artificial seawater consisted of 18 g/L NaCl, 2.44 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L KCl, 1.0 g/L $NaNO_3$, 0.3 g/L $CaCl_2 \cdot 2H_2O$, 0.05 g/L KH_2PO_4, 1.0 g/L Tris buffer (Sigma Co.), 0.027 g/L NH_4Cl, 15.0×10^{-8} g/L vitamin B_{12}, 3 mL/L chelated iron solution, and 10 mL/L trace element solution including boron, cobalt, manganese, zinc, and molybdenum (Starr and Zeikus, 1993). The medium for *P. irregulare* consisted of 20 g/L glucose and 5 g/L yeast extract. The pH for both of the two subculture media was adjusted to 7.5-8.0 before autoclaving at 121°C for 15 min. The cells were grown in 250-mL Erlenmeyer flasks each containing 50 mL of medium and incubated at 25°C in an orbital shaker set at 180 rpm. In the investigation of cell growth on animal protein hydrolysates, yeast extract, and peptone in the subculture media were replaced with protein hydrolysates prepared from either alkaline or enzymatic hydrolysis, while all the other micronutrients were the same as those in the subculture media.

2.4 Analyses

Cell dry weight and glucose concentration. The cell dry weight of *S. limacinum* and *P. irregulare* was determined using a previously reported procedure (Pyle et al., 2008; Athalye et al., 2009). Glucose concentrations were determined by using the dinitrosalicylic acid (DNS) method (Ghose, 1987).

Proximate analysis. The freeze-dried algal/fungal biomass was subjected to proximate analysis. The lipids were extracted and quantified according to the Folch method (Folch et al., 1957). The crude protein content was estimated by measuring the total Kjeldahl nitrogen (TKN) and multiplying by the conversion factor of 6.25. The carbohydrate was estimated by subtracting lipid and protein contents.

Fatty acid analysis. The procedure for fatty acid methyl esters (FAME) preparation was the same as previously reported (Pyle et al., 2008). The fatty acid profile was analyzed by a Varian GC-450 gas chromatography (Palo Alto, CA) equipped with a flame ionization detector and a SGE SolGel-Wax capillary column (30 m × 0.25 mm × 0.25 μm). The fatty acids were identified by comparing the retention times with those of standard fatty acids (Nu-Chek Prep Inc., Elysian, MN) and quantified by comparing their peak areas with those of the internal standard (C17:0) (Chi et al., 2007).

2.5 Statistical analyses

Each experiment was performed in triplicates and means and standard deviations were determined. Cell growth and fatty acid production at different experiment conditions were analyzed by one-way analysis of variance (ANOVA) by using JMP software (SAS Inc., Cary, NC). The data analyzed were combined into groups. For each group, ANOVA was

applied to evaluate the difference between mean values. The probability value of <10% (P< 0.1) was defined as statistically significant.

3. RESULTS

3.1 Characteristics of animal protein hydrolysates

The hydrolysates of MBM, FM, and BM prepared under various hydrolysis conditions were characterized for changes in their physical and chemical properties relating to their utility as media ingredients. Properties including the solubility of organic matter, molar mass distribution, amino acid composition, and the proximate analysis have been reported previously (Garcia et al., 2011). In brief, both alkali and enzyme hydrolyses increased the solubility of organic matter and reduced the molar mass of the rendered proteins. However, the average masses of the peptides in the hydrolysates were larger than that of the commercial yeast extract. Both alkali- and enzyme-hydrolysates were comprised of largely peptides and amino acids extracted from the rendered protein materials (Garcia et al., 2011). Alkali-hydrolysis destroyed some amino acids and created cross-linked amino acids, while enzyme hydrolysis affected the amino acid composition much less (Garcia et al., 2011). Little fat or ash from rendered protein ends up in the hydrolysate, but both alkali and enzyme hydrolysates agents significantly contribute to the ash content (Garcia et al., 2011).

3.2 Feasibilities of utilizing animal protein hydrolysates by *S. limacinum* and *P. irregulare*

Different protein hydrolysates were tested for their capabilities of supporting the growth of *S. limacinum* and *P. irregulare*. When *S. limacinum* was incubated in medium

containing alkali-hydrolysates, no cell growth was observed; when grown in enzyme-hydrolysate supplemented medium, slight cell growth was observed, but the cell biomass accumulated after 5-6 days was still appreciably less than that from yeast extract culture (Table 3). The growth of *P. irregulare* on protein hydrolysate was much better than that of *S. limacinum*, with enzyme hydrolysate resulting in better growth than alkali hydrolysate (Table 3). Both alkali- and enzyme-hydrolysates were not suitable substitutes for yeast extract in the culture of *S. limacinum.* Consequently, *P. irregulare* was selected for subsequent studies.

Figure 1 shows the cell growth, total fatty acid content, and EPA content of *P. irregulare* growing in different types of hydrolysates. Overall, cell dry weights obtained from enzyme hydrolysates were higher than those from alkali hydrolysates. Among various enzyme hydrolysates, MBM- and FM-derived hydrolysates resulted in better growth performance than the BM-hydrolysates (Figure 1A). Although cell growth performance from different protein hydrolysates varied widely, the total fatty acid content of the biomass was more consistent, similiar or even higher than for the control (Figure 1B). The EPA content of the protein hydrolysate derived biomass, however, was relatively low compared to the control. The trends in EPA content were similar to that of cell growth (Figure 1C).

3.3 Effects of protein hydrolysate concentration and C/N ratio

The feasibility study showed that protein hydrolysate, particularly the MBM and FM enzyme hydrolysates, support good growth and fatty acid accumulation in *P. irregulare*. To further increase cell growth and EPA production in *P. irregulare*, the effects of protein hydrolysate concentrations and C/N ratio were investigated by using the MV and FV hydrolysates prepared from the hydrolysis of MBM and FM.

As shown in Table 4, cell dry weight increased with MV from 1-10 g/L and leveled off from 10-15 g/L. Cell dry weight from the 10-15g/L MV culture was approximately 20% higher than that of the control. The biomass contained C14:0, C16:0, C16:1, C18:1 and C18:2 as the major fatty acids, and the proportions of those fatty acids were relatively stable with changing C/N ratio. The total fatty acid (TFA) content was also maintained at a relatively constant level except at 1 g/L MV concentration. Compared to the control, *P. irregulare* grown in MV had higher TFA content (mg/g DW) but lower EPA proportion (%TFA) and EPA content (Table 4). Eventually, MV at high concentration (10-15 g/L) resulted in EPA yield similar to that of the control culture (Table 4). Table 5 shows that the trends of cell growth, fatty acid profile, and EPA production of *P. irregulare* growing in FV were similar to those of the MV-based culture.

3.4 Growth kinetics of P. irregulare on MV- and FV-containing medium

The previous results indicated that 10 g/L MV and FV with 30 g/L glucose led to the best cell growth and EPA yield. The kinetics of cell growth, nutrient consumption and TFA/EPA production in *P. irregulare* were then investigated with this medium composition. The culture with 30 g/L glucose and 10 g/L yeast extract was also included as control. As shown in Figures 2A and 2B, the trends of biomass concentration and glucose consumption of the protein hydrolysate culture were similar to those of the control. The trends in TFA contents were similar for all three cultures, with the protein hydrolysates consistently having a higher TFA content than the control (Figure 2C). The EPA composition (%TFA), however, showed the opposite trend to that of the TFA content (Figure 2D). Figure 2E shows that the EPA content ranged from 5-10 mg/g DW, with a slight increase at the end of the culture

period. The EPA yield, however, monotonically increased with culture time (Figure 2F) indicating a later harvest time is preferred due to greater algae concentrations. The kinetic parameters of the three cultures are summarized in Table 6. The protein hydrolysate-based culture resulted in a higher specific growth rate, cell dry weight, and biomass productivity, but lower EPA content compared to those of the control. The EPA yield and productivity of the protein hydrolysate culture, however, were similar to those of the control (Table 6).

3.5 Characteristics of fungal biomass

The fungal biomass obtained from MV- and FV-medium as well as the control medium (yeast extract) were further characterized for their potential nutrition value. Table 7 shows the proximate analysis of the biomass. The lipid contents of biomass grown on MV- and FV-media were higher than that of the yeast extract culture, while the protein content showed an opposite trend. The carbohydrate contents of the three cultures were similar. The fatty acid profiles of the biomass are described in Table 4 (MV, MBM hydrolysate) and Table 5 (FV, FM hydrolysate). The two protein sources resulted in a similar fatty acid composition. Although the EPA portion in TFA was low, the TFA contents of the MV- and FV-derived biomass were much higher than that of yeast extract-derived biomass.

4. DISCUSSION

Microorganisms, which do not secrete proteolytic enzymes, typically cannot utilize large peptides and proteins in their growth medium. Transportation across the cell membrane into the cytoplasm largely depends on molecules being soluble and of low molar mass. Rendered proteins are poor candidates for meeting a microorganism's amino acid

requirements, because these proteins tend to be large (Garcia and Phillips, 2009) and poorly soluble (Garcia et al., 2006). On the contrary, amino acids in yeast extract, a common commercial complex nutrient source for growth media, are small, soluble, and easy for most microorganisms to utilize. Therefore, hydrolyzing proteins into smaller peptides and free amino acids should facilitate their utilization by microorganisms.

Protein hydrolysis is usually achieved by using enzyme, alkali, or acid catalysis. In the present study, the enzyme hydrolysates resulted in better growth performance of *S. limacinum* and *P. irregulare* than did alkali hydrolysates (Table 3). Some amino acids were destroyed during alkali hydrolysis. For example, compared with the original raw protein materials, the content of arginine, serine, and threonine in the alkali hydrolysates decreased while enzyme hydrolysis caused little change in those amino acids (Garcia et al, 2011). Alkali treatment of proteins also caused the formation of unusual cross-linked amino acids, such as lysinoalanine and lanthionine, which may not be utilized by microorganisms (Garcia et al, 2011; Friedman, 1999), while cross-linking of amino acids in enzyme hydrolysis was very minor (Garcia et al, 2011). The high ash contents in alkali hydrolysates may also result in poor cell growth. The ash content of the alkali hydrolysate of MBM, FM and BM ranged from 14.8 to 27.2%, which is much higher than that of enzyme hydrolysate (8.5 to 15.1%). The majority of the ash originated from reagents used in the hydrolysis, i.e., calcium salt, which may inhibit the growth of *S. limacinum* and *P. irregulare* (Garcia et al., 2010, 2011). Finally, we speculated that the enzyme hydrolyzed materials were richer in growth promoting factors, e.g. B vitamins, which are not stable to the high pH of alkali hydrolysis.

In addition to the hydrolysis method, the protein source also influences growth performance. MBM- and FM-hydrolysate resulted in better cell growth than BM-hydrolysate,

even if the three proteins were all hydrolyzed by the same enzyme (Figure 1). Such a difference in the cell growth may be caused by any of a number of factors, including differences in amino acid composition (Garcia et al, 2011), differences in contents of growth promoting factors, and the presence of inhibitory substances.

The effects of protein hydrolysates on cell growth were also species specific. Compared with *P. irregulare*, *S. limacinum* was more sensitive to the protein hydrolysate. *S. limacinum* has more complex nutritional requirements which are not fully met by the animal protein hydrolysates. On the contrary, the growth medium for *P. irregulare* was much simpler, with glucose and yeast extract being the only ingredients. Yeast extract is generally prepared by the autolysis of yeast cells under well-controlled conditions in order to preserve naturally occurring B vitamins. The primary function of yeast extract in growth media is to provide organic nitrogen as well as various vitamins and trace elements. The growth performance of *P. irregulare* (Figure 1A) indicated that the animal protein hydrolysates provided the similar function as yeast extract for the growth of *P. irregulare*. However, the TFA and EPA synthesis of *P. irregulare* cells growing on protein hydrolysate medium were different from the yeast extract culture (Figures 1B and 1C).

Tables 4 and 5 indicate that the concentration of protein hydrolysate and the C/N ratio of the culture medium significantly affected cell growth and fatty acid compositions of *P. irregulare*; a C/N ratio above approximately 9 changed growth limitations from carbon to nitrogen. Nitrogen starvation usually increases in lipid content due to metabolism switching from protein synthesis to lipid and carbohydrate synthesis (Otero et al., 1997; Sukenik, 1991). However, the results obtained from this work (Tables 4 and 5) did not show the same trend

for *P. irregulare*, indicating the effects of nitrogen starvation on lipid synthesis is species-specific.

In a previous study, we characterized the biomass of *P. irregulare* when using biodiesel-derived crude glycerol and yeast extract as culture medium ingredients (Athalye et al., 2009). Compared with biomass obtained from glycerol and yeast extract, the biomass obtained from glucose and animal protein hydrolysates contained more lipid but less protein. The composition of the *P. irregulare* biomass highly depended on the nutrients used in the medium. Amino acid composition can influence the lipid accumulation of microorganisms (Evans and Ratledge, 1984). Therefore, the amino acid profile of the protein hydrolysates may favor lipid production.

Our results indicate that rendered animal proteins can be an effective nutrient source for growing microorganisms, provided that the proteins are appropriately hydrolyzed into smaller molecules. Specifically, the enzyme hydrolysates were able to replace yeast extract as the nitrogen source to support growth and EPA production in *P. irregulare*. The growth performance, EPA production and proximate composition of *P. irregulare* when using enzyme protein hydrolysate were comparable to the culture using commercial yeast extract as nutrient supply. The present work provides a new alternative to the rendering industry as an outlet for the large amounts of animal proteins. More fermentation tests using different industrial microorganisms are needed to determine the breadth of applicability for protein hydrolysates. The hydrolysis process will also need to be optimized to prevent the loss of growth-promoting factors and improve cost effectiveness.

REFERENCES

Athalye, S.K., Garcia, R.A., Wen, Z.Y., 2009. Use of biodiesel-derived crude glycerol for producing eicosapentaenoic acid (EPA) by the fungus *Pythium irregulare*. J. Agric. Food Chem. 57, 2739-2744.

Chi, Z., Pyle, D., Wen, Z., Frear, C., Chen, S., 2007. A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. Proc. Biochem. 42, 1537-1545.

Evans, C., Ratledge, C., 1984. Effect of nitrogen source on lipid accumulation in oleaginous yeasts. J. Gen. Microbiol. 130, 1693-1704.

FDA. 2008. Substances Prohibited From Use in Animal Food or Feed. Federal Register. http://edocket.access.gpo.gov/2008/pdf/08-1180.pdf .

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Bio. Chem. 226, 497-509.

Friedman, M., 1999. Chemistry, biochemistry, nutrition, and microbiology of lysinoalanine, lanthionine, and histidinoalanine in food and other proteins. J. Agric. Food Chem. 47, 1295-1319.

Garcia, R.A., Rosentrater, K.A., Flores, R.A., 2006. Characteristics of North American meat and bone meal relevant to the development of non-feed applications. Appl. Eng. Agric. 22, 729-736.

Garcia, R.A., Phillips, J.G., 2009. Physical distribution and characteristics of meat and bone meal protein. J. Sci. Food Agri. 89, 329-336.

Garcia, R.A., Piazza, G.J., Wen, Z., Pyle, D.J., Solaiman, D.K.Y., 2010. The non-nutritional performance characteristics of peptones made from rendered protein. J. Ind. Micro. Biotechnol. 37, 95-102.

Garcia, R.A., Pyle, D.J., Piazza, G.J., Wen, Z., 2011. Hydrolysis of animal protein meals for improved utility in non-feed applications. Appl. Eng. Agric. 27, 269-275.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure. Appl. Chem. 59, 257-268.

Otero, A., Garcia, D., Fabregas, J., 1997. Factors controlling eicosapentaenoic acid production in semicontinuous cultures of marine microalgae. J. Appl. Phycol. 9, 465-469.

Pyle, D.J., Garcia, R.A., Wen, Z., 2008. Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition. J. Agric. Food Chem. 56, 3933-3939.

Simopoulos, A.P., 1999. Essential fatty acids in health and chronic disease. Am. J. Clin. Nutr. 70, 560S-569S.

Starr, R.C., Zeikus, J.A., 1993. UTEX - The Culture Collection of Algae at the University of Texas at Austin 1993 List of Cultures. J. Phycol. 29, 1-106.

Solaiman, D.K.Y., Garcia, R.A., Ashby, R.D., Piazza, G.J., Steinbuchel, A., 2011 Rendered-protein hydrolysates for microbial synthesis of cyanophycin biopolymer. New Biotechnol., 28, 552-558.

Sukenik, A., 1991. Ecophysiological consideration in the optimization of eicosapentaenoic acid production by *Nannochloropsis* - sp (Eustigmatophyceae). Biores. Technol. 35, 263-269.

Van Hoek, P., Aristidou, A., Hahn, J.J., Patist, A., 2003. Fermentation goes large-scale. Chem. Eng. Prog. 99, 37S-42S.

Vechtlifshitz, S.E., Almas, K.A., Zomer, E., 1990. Microbial growth on peptones from fish industrial-wastes. Lett. Appl. Micro. 10, 183-186.

Table 1. Conditions used for alkali hydrolysis and hydrolysate identifications (ID) [a]

Animal proteins	Time (h)	Hydrolysate ID
Meat and bone meal (MBM)	4	MA4
	8	MA8
	16	MA16
Feather meal (FM)	4	FA4
	8	FA8
	16	FA16
Blood meal (BM)	4	BA4
	8	BA8
	16	BA16

[a] Hydrolysis was conducted in saturated calcium hydroxide solution at 85°C (Garcia et al., 2011).

Table 2. Conditions used for enzymatic hydrolysis and hydrolysate identifications (ID) [a]

Animal protein	Enzyme treatment	pH	Time (h)	Hydrolysate ID
Meat and bone meal (MBM)	Alcalase + Flavourzyme; 0.4 AU/ g substrate,50 LAPU/g substrate	8.5/7.0	4 each	M ALC FLA
	Alcalase; 0.4 AU/ g substrate	8.5	4	M ALC
	Versazyme; 8 mg/g substrate	7.5	8	MV
Feather meal (FM)	Alcalase + Flavourzyme; 0.4 AU/ g substrate,50 LAPU/g substrate	8.5/7.0	4 each	F ALC FLA
	Alcalase; 0.4 AU/ g substrate	8.5	4	F ALC
	Versazyme; 8 mg/g substrate	7.5	8	FV
Blood meal (BM)	Alcalase + Flavourzyme; 0.4 AU/ g substrate,50 LAPU/g substrate	8.5/7.0	4 each	B ALC FLA
	Alcalase; 0.4 AU/ g substrate	8.5	4	B ALC
	Versazyme; 8 mg/g substrate	7.5	8	BV

[a] (Garcia et al., 2011).

Table 3. Algal growth performance on different protein hydrolysates [a]

Hydrolysate	*S. limacinum*	*P. irregulare*
Alkali-based hydrolysate	-	+ + +
Enzyme-based hydrolysate	+	+ + + + + [b]

[a.] The cell growth performance was defined as: - no growth; + poor (cell has some growth but the cell density was < 10% of the control); + + + good (cell density was more than 10% but < 80% of the control); + + + ++: excellent (cell density was at least 80% of the control). The control was the cell culture using commercial yeast extract and/or peptone.

[b.] Blood meal resulted in a rating of "good" (+++) growth performance

Table 4. Effects of MV (MBM hydrolysate) concentration and C/N ratio on growth and fatty acid composition of *P. irregulare*

Parameter	Unit	Media composition, C/N ratio, cell growth and fatty acid composition						Control [b]
Glucose	g/L	30	30	30	30	30	30	30
MBM hydrolysate	g/L	1	2	3	5	10	15	10 (yeast extract)
C/N ratio [a]	-	85	43	28	17	9	6	10-12 [c]
Cell dry weight	g/L	3.34±0.42	5.50±0.41	7.97±0.53	9.99±0.12	13.09±0.16	12.85±1.38	10.73±1.40
Fatty acid composition								
C 14:0	%TFA	10.95±0.48	11.59±1.80	11.06±0.78	10.98±0.78	11.29±2.53	11.15±0.59	10.20±0.41
C 16:0	%TFA	28.18±0.83	29.19±2.51	29.38±0.24	28.24±0.36	28.39±4.73	28.72±1.86	28.62±1.92
C 16:1	%TFA	20.73±0.53	20.27±1.82	21.24±0.75	20.87±0.62	18.41±3.16	16.67±1.13	7.91±1.15
C 18:0	%TFA	1.01±0.09	1.08±0.21	1.12±0.30	0.91±0.04	1.14±0.17	1.16±0.09	2.92±0.27
C 18:1	%TFA	24.33±1.15	24.51±0.94	24.22±0.98	24.66±1.17	24.64±1.90	23.31±2.28	16.43±0.73
C 18:2 (n-6)	%TFA	9.65±0.12	9.21±1.52	9.03±0.71	9.56±0.72	9.15±1.39	10.68±1.66	18.07±0.38
C 18:3 (n-3)	%TFA	0.55±0.03	0.57±0.11	0.50±0.03	0.55±0.01	0.64±0.09	0.75±0.08	1.63±0.07
C 20:4 (n-6)	%TFA	2.07±0.47	1.61±0.43	1.65±0.16	2.02±0.22	3.04±0.47	3.66±0.93	6.77±1.09
C 20:5 (n-3)	%TFA	2.53±0.38	1.96±0.82	1.78±0.11	2.23±0.33	3.10±0.23	3.89±1.15	7.44±1.08
TFA content	mg/g DW	166.03±16.76	191.96±2.36	207.40±23.80	241.97±18.17	226.50±14.48	197.34±30.85	113.48±13.71
EPA content	mg/g DW	4.19±0.62	3.77±1.56	4.10±0.92	5.69±0.50	6.18±0.59	7.62±2.36	8.44±1.14
EPA yield	mg/L	13.89±1.75	20.46±7.64	32.36±5.36	56.80±4.81	80.99±8.31	95.79±18.40	90.56±4.16

[a] N content based on total Kjeldahl nitrogen measured for MV. [b] Yeast extract used [c] N content of commercial yeast extract ranged from 10.0-12.5%. Data are means of triplicates ± standard deviations

Table 5. Effects of FV (FM hydrolysate) concentration and C/N ratio on growth and fatty acid composition of *P. irregulare*

Parameter	Unit	Media composition, C/N ratio, cell growth and fatty acid composition						Control [b]
glucose	g/L	30	30	30	30	30	30	30
FM hydrolysates	g/L	1	2	3	5	10	15	10 (yeast extract)
C/N ratio [a]	-	99	50	25	20	10	7	10-12 [c]
cell dry weight	g/L	4.30±0.47	7.21±0.49	8.53±0.38	9.39±1.19	12.51±0.42	12.30±1.11	10.73±1.40
Fatty acid composition								
C 14:0	%TFA	11.96±0.22	12.52±0.45	12.19±1.15	12.47±0.11	13.21±2.25	12.68±1.79	10.20±0.41
C 16:0	%TFA	27.12±0.38	28.49±1.04	27.16±0.78	27.64±0.68	29.25±4.29	27.48±0.50	28.62±1.92
C 16:1	%TFA	23.06±0.34	23.23±1.53	23.02±1.62	21.47±0.05	17.17±2.34	18.59±0.89	7.91±1.15
C 18:0	%TFA	0.84±0.04	0.85±0.14	0.85±0.03	1.12±0.12	1.31±0.42	0.89±0.18	2.92±0.27
C 18:1	%TFA	23.51±0.59	23.34±1.17	24.86±2.26	23.73±0.67	22.55±3.20	23.74±2.97	16.43±0.73
C 18:2 (n-6)	%TFA	8.45±0.18	7.37±0.34	7.56±0.36	8.54±0.61	10.10±1.73	10.40±0.46	18.07±0.38
C 18:3 (n-3)	%TFA	0.50±0.03	0.45±0.01	0.48±0.05	0.71±0.17	0.60±0.17	0.58±0.02	1.63±0.07
C 20:4 (n-6)	%TFA	1.82±0.13	1.55±0.05	1.69±0.22	2.37±0.48	3.06±0.64	2.86±0.11	6.77±1.09
C 20:5 (n-3)	%TFA	2.74±0.17	2.20±0.05	2.19±0.34	1.97±0.15	2.74±0.26	2.78±0.09	7.44±1.08
TFA content	mg/g DW	198.87±13.78	207.16±19.70	242.78±11.58	266.74±23.05	268.28±9.59	239.63±6.94	113.48±13.71
EPA content	mg/g DW	5.46±0.61	4.57±0.55	5.31±0.84	5.24±0.29	6.37±0.58	6.66±0.38	8.44±1.14
EPA yield	mg/L	23.27±0.71	33.08±5.73	45.41±9.03	49.00±4.65	79.88±9.95	81.77±6.69	90.56±4.16

[a] N content based on total Kjeldahl nitrogen measured for FV. [b] Yeast extract. [c] N content of commercial yeast extract ranged from 10.0-12.5%. Data are means of triplicates ± standard deviation.

Table 6. Cell growth and EPA production parameters of *P. irregulare* on media containing 30 g/L glucose and 10 g/L different protein hydrolysates

		Protein hydrolysate [a]		
Parameter	Unit	Yeast extract	MV	FV
Specific growth rate, μ	day^{-1}	0.48±0.07	0.55±0.03	0.55±0.01
Max cell dry wt	g/L	10.73±1.40	12.40±0.33	12.83±0.55
Biomass productivity	g/L·day	1.53±0.20	1.77±0.05	2.14±0.09
Growth yield	g/g	0.42±0.04	0.43±0.02	0.45±0.03
EPA content	mg/g DW	8.44±1.14	6.85±0.71	7.05±1.39
EPA yield	mg/L	90.56±4.16	85.02±11.06	89.98±14.18
EPA productivity	mg/L·day	12.93±0.59	12.15±0.10	15.00±0.29

[a.] Refer to Table 2 for hydrolysate identifications
Data are means of triplicates ± standard deviations

Table 7. Proximate analysis (% dry biomass) of freeze-dried fungal biomass grown on different protein hydrolysates

Component	Protein hydrolysate [a]		
	Yeast extract	MV	FV
Lipid	18.27±1.61	26.24±1.44	29.58±1.67
Crude protein	43.69±9.31	32.88±1.38	34.13±0.31
Carbohydrate	34.67±1.49	39.52±0.55	34.71±1.53
Ash	3.36±0.30	1.37±0.06	1.58±0.13

[a.] Refer to Table 2 for hydrolysate identifications.
Data are means of triplicates ± standard deviations

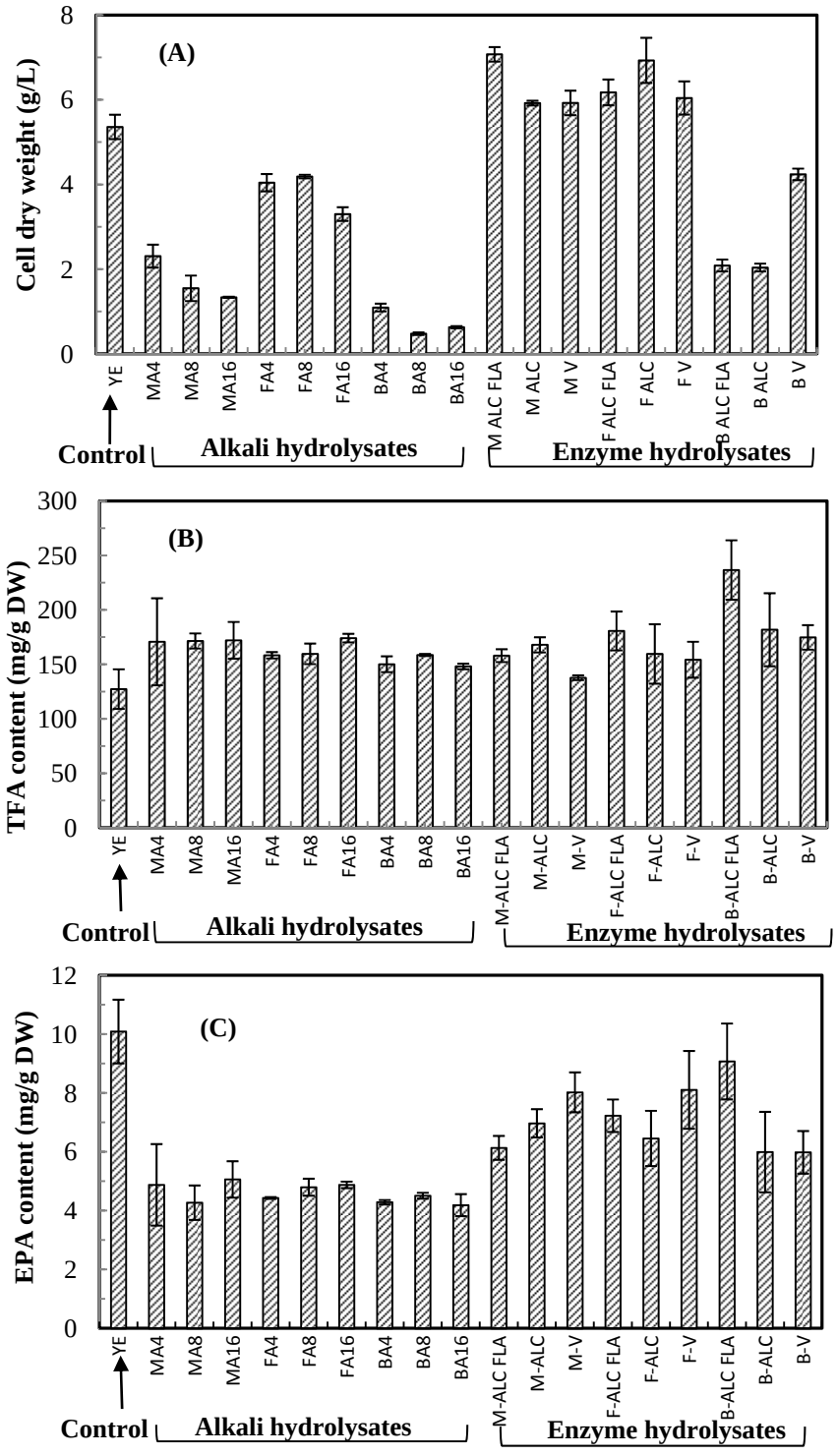


Figure 1. Cell growth (**A**), total fatty acid content (**B**), and EPA content (**C**) of *P. irregulare* grown in Control (YE, yeast extract), alkali-hydrolysates, and enzyme-hydrolysates containing medium (Refer to Tables 1 & 2 for hydrolysate identifications). Data are means of triplicates and error bars show standard deviations.

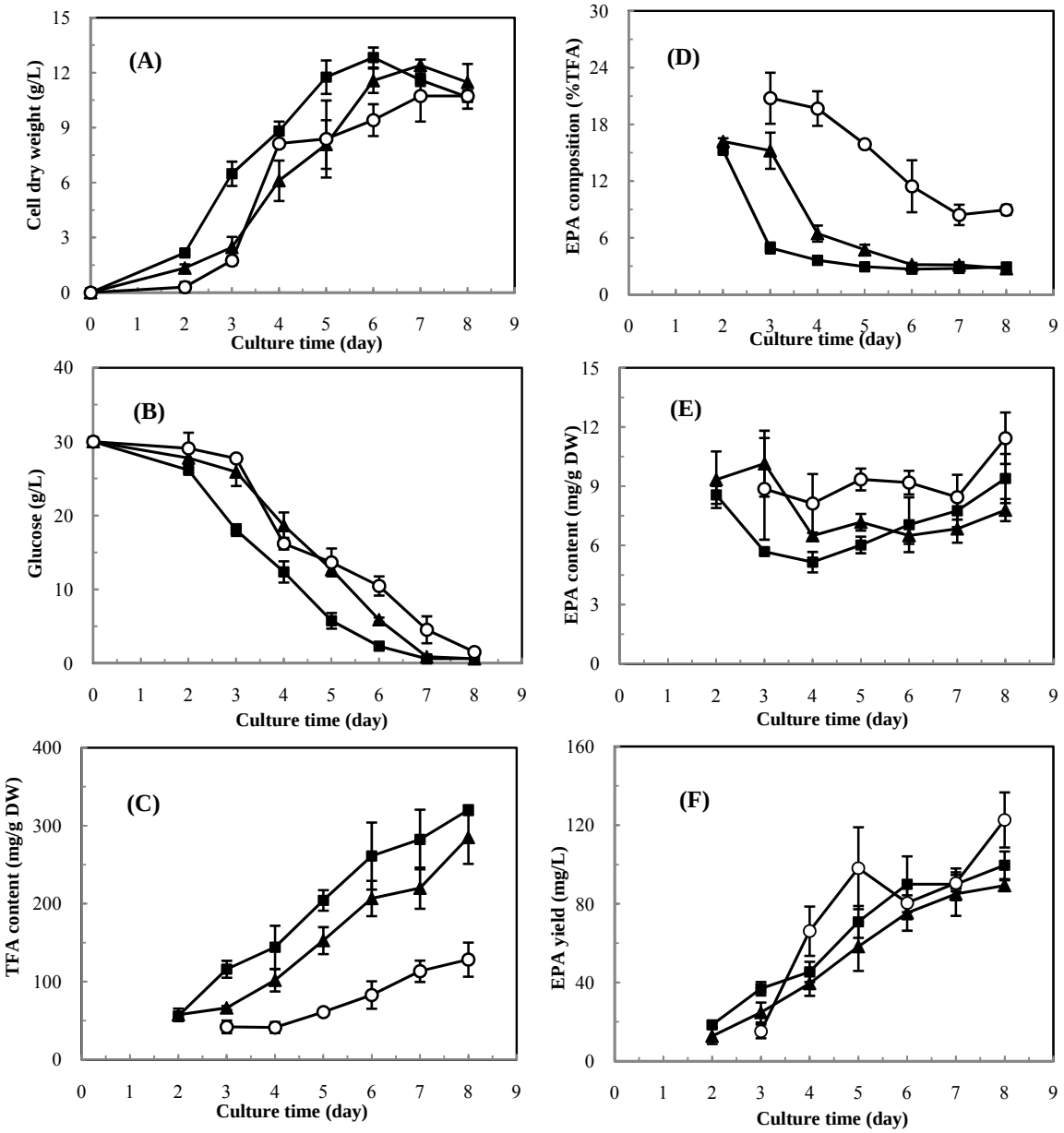


Figure 2. Time course of cell dry weight (**A**), residual glucose concentration (**B**), TFA content (**C**), EPA composition (**D**), EPA content (**E**), and EPA yield (**F**) of *P. irregulare* grown in medium supplemented with MV (MBM hydrolysate) (▲); FV (FM hydrolysate) (■) and yeast extract (○) as nutrients (refer to Table 2 for hydrolysate IDs). Data are means of triplicates, and error bars show standard deviations.

CHAPTER 4 USE OF DRY- GRIND ETHANOL DERIVED THIN STILLAGE TO PRODUCE EICOSAPENTAENOIC ACID (EPA) BY THE FUNGUS PYTHIUM IRREGULARE

Published in Bioresource Technology

Yi Liang, Xuefei Zhao, Megan Strait, Zhiyou Wen

ABSTRACT

The present study explored the use of thin stillage, a major byproduct in dry-grind corn-ethanol industry, for production of eicosapentaenoic acid (EPA) by the fungus *Pythium irregulare*. Thin stillage contained various compounds that were ideal for fungal growth. Thin stillage concentration and temperature played important roles in fungal growth and EPA production. When 50% thin stillage was used in a stepwise temperature shift culture process, the cell density reached 23 g/L at day 9 with 243 mg/L EPA yield and 27 mg/L·day productivity. The fungal biomass contained 39% lipid, 28% protein, 30% carbohydrate, and 3% ash. The fungal culture also generated a nutrient-depleted liquid by removing organic compounds in the raw thin stillage. A new use of thin stillage by feeding to the fungus *P. irregulare* for producing omega-3 fatty acids was demonstrated.

KEYWORDS: *Pythium irregulare*, Dry milling, Corn ethanol, Thin stillage, Fungal fermentation, Eicosapentaenoic acid

1. INTRODUCTION

Fuel ethanol production from corn in the United States has expanded rapidly over the past decades. According to the Renewable Fuel Association, the annual corn fuel ethanol production has reached 13 billion gallons in 2010 (http://www.ethanolrfa.org/pages/statistics). The majority of corn derived fuel ethanol is produced using a dry grind process. In this process, the residual ethanol-free slurry (whole stillage) after ethanol is distilled from fermented beer is separated into a solid fraction (wet distillers grains) and a liquid fraction (thin stillage) (Liu, 2011). While part of the thin stillage is recycled to slurry the ground corn at the beginning of the process, the majority of thin stillage is sent to an evaporator to be condensed into a syrup-like liquid with about 35% solids content. This condensed syrup is usually combined with the wet distiller's grains and dried into dried distillers grains with solubles (DDGS), which are primarily used as cattle feed. Evaporating the thin stillage is an energy-intensive process and a major cost to the ethanol plant. DDGS, as cattle feed, has only moderate value for the ethanol producers. Therefore, developing an efficient way to use thin stillage and increase product value will enhance the profitability of the dry-grind ethanol production.

Thin stillage from a dry-grind ethanol plant contains various unfermented components of the grain (e.g., fiber, oil and protein) and yeast cells (Kim et al., 2008). These compounds are ideal nutrients for microorganisms (Dowd et al., 1993; Kim et al., 2008). For example, thin stillage was used as a carbon source for growing the fungus *Ganoderma lucidum* to produce polysaccharides (Hsieh et al., 2005). van Leeuwen et al. (2010) used thin stillage to grow variety of fungi, which can be used as animal feeds, human food, and sources

of nutraceuticals. In particular, the group recently reported using thin stillage to grow oleaginous fungus *Mucor circinelloides* in an air-lift bioreactor, resulting a high mass concentration with high oil content (Mitra et al., 2002). Ahn et al. (2011) also reported using thin stillage to grow the bacterium *Clostridium pasteurianum* for butanol production. Using anaerobic digestion to treat thin stillage to improve water quality and energy efficiency in dry-grind ethanol plants was also investigated (Alkan-Ozkaynak and Karthikeyan, 2011).

Using thin stillage to produce eicosapentaenoic acid (EPA, C20:5, n-3) by fungal fermentation provides another outlet for this underutilized material. EPA is an important omega-3 polyunsaturated fatty acid that possesses many health-promoting properties such as prevention of human cardiovascular disease, cancer, schizophrenia, and Alzheimer's disease (Simopoulos, 1999). The omega-3 rich biomass is also an ideal aqua culture feed that can be used to substitute fish oil/fish meal. Currently, commercial microbial production of EPA faces the challenges of high production cost. As a result, fish oil is still the predominant commercial EPA source, despite various limitations associated with fish oil such as odor/taste problems, heavy metal contamination, and limited supply.

Less expensive agricultural byproducts for growing the fungus *Pythium irregulare*, which is a good EPA producer, have been used. These byproducts include crude soybean oil and soy protein isolate (Cheng et al., 1999), crude glycerol from a biodiesel plant (Athalye et al., 2009), and rendered animal proteins (Liang et al., 2011). However, these materials have only one function for microbial growth. For example, crude glycerol serves as a carbon source while rendered animal proteins serve as a nitrogen source for microorganism. There is still a need to supplement other nutrients in the growth medium, which may increase the medium cost. In contrast, thin stillage can be used as the sole nutrient source for the fungal

growth due to its complete nutrient composition for the microorganisms. The objective of the present work was to test the feasibility of using thin stillage as a nutrient source for EPA production by *Pythium irregulare*. The fungal culture process was also evaluated as an effective way of treating thin stillage to prepare nutrient-depleted "clean" water that can be recycled to produce additional ethanol to the fermenter.

2. MATERIALS AND METHODS

2.1 Thin stillage

Thin stillage samples were obtained from Lincolnway Energy (Nevada, IA), a 50 million gal/year dry-grind corn ethanol plant. The samples were collected in 1-L Nalgene HDPE bottles and stored in a freezer at -20^{o}C. Prior to use, the frozen samples were thawed and homogenized. The thawed samples were characterized for total solids, COD (chemical oxygen demand), pH, total carbohydrate, reducing sugar, glycerol, lactic acid, acetic acid, nitrogen, and phosphorus contents.

2.2. Microorganism, media and culture conditions

The fungus *Pythium irregulare* (ATCC 10951) was used. The fungus was grown on agar plates (containing 30 g/L glucose and 10 g/L yeast extract) for 5 days at 25^{o}C. The agar plates were then washed with distilled water containing glass beads to dislodge the mycelium. The mycelium suspension was stored at 4^{o}C prior to use as inoculum. The inoculum size was 10% of the total culture medium. The formulation of 30 g/L glucose + 10 g/L yeast extract was used as the control medium. A formulation of 30 g/L glycerol + 10 g/L yeast extract was

tested as another control medium in the feasibility study (Section 3.2) because glycerol was the major carbon source in the thin stillage.

The fungal cells were grown in media containing different concentrations of thin stillage. The pH was adjusted to 7.0 before autoclaving the media at 121^{o}C for 15 min. The cells were grown in 250-mL Erlenmeyer flasks, each containing 50 mL medium and incubated in an orbital shaker at 200 rpm. The temperature was set to the desired levels based on experimental design. For each experimental condition, triplicates were used and the standard deviation was calculated.

2.3 Analyses

Cell dry weight: The fungal mycelium was harvested from each flask by filtering the spent medium through a stainless-steel wire mesh screen with a nominal size of 495 μm. The majority of the solid particles in thin stillage medium passed through the screen, while the large particles retained by the screen were manually removed from the screen. A small amount of particles remained attached on the biomass surface; distilled water was used to thoroughly wash the biomass to remove these particles. The washed biomass was then transferred to a pre-weighed tube and freeze-dried to determine the cell dry weight.

Thin stillage characterization: Total solids, total COD, total Kjeldahl nitrogen (TKN), nitrate, ammonium, total phosphorus, and orthophosphate were determined according to standard methods (APHA, 1995). Total carbohydrate was determined by using the phenol–sulfuric acid method (Dubois et al., 1956). Reducing sugars were determined by using the dinitrosalicylic acid method (Ghose, 1987). Glycerol was determined by using a free glycerol determination kit (Sigma Chemical, St. Louis, MO). Lactic acid and acetic acid were

analyzed by a Dionex ion chromatograph ICS 3000 system (Sunnyvale, CA) with an analytical column (Dionex IonPac®ICE-AS1 4×250 mm, P/N 064198). The column temperature was set at 19°C and the eluent (1.0 mM heptafluorobutyric acid) flow rate 0.120 mL/min. The concentrations were determined by comparing peak areas with standard samples.

***Proximate Analysis*:** The freeze-dried fungal biomass was subjected to proximate analysis. The lipids of the biomass were extracted and quantified according to the Folch method (Folch et al., 1957). The crude protein content was estimated by measuring the TKN and multiplying by the conversion factor of 6.25. The carbohydrate was estimated by subtracting lipid, protein, and ash contents from the dry biomass.

Fatty acid analysis: Freeze-dried fungal biomass and raw thin stillage were analyzed for their fatty acid compositions. The fatty acid methyl esters (FAME) were prepared according to previously described procedures (Pyle et al, 2008). The fatty acid profile was analyzed by a Varian GC-450 gas chromatograph (Sunnyvale, CA) equipped with a flame ionization detector and a SGE (Austin, TX) SolGel-Wax capillary column (30 m × 0.25 mm × 0.25 μm). The fatty acids were identified by comparing the retention times with those of standard fatty acids and quantified by comparing their peak areas with that of the internal standard (C17:0) (Liang et al., 2011).

2.4 Statistical analyses

Each experiment was conducted in triplicates and means and standard deviations were determined. Pair-wise comparison was also used to determine the differences of cell

growth and fatty acid production between experimental conditions. The probability value of <10% (P< 0.1) was defined as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Thin stillage characterization

The chemical characteristics of thin stillage indicated a variety of organic compounds were contained in the thin stillage (Table 1). Thin stillage contained around 6.5% total solids with 112 g/L total COD concentration of and 4.5 pH. The carbohydrate, nitrogen, and phosphorus were derived from corn kernel remnant and the residuals of yeast cells; while glycerol, acetic acid, and lactic acid were the metabolic byproducts of the yeast cells during ethanol fermentation. Table 1 indicates that thin stillage can provide important nutrients such as carbon, nitrogen, and phosphorus, for fungal growth. Glycerol is an ideal carbon source for the fungus *P. irregulare* (Athalye et al., 2009). Our earlier studies have also shown that yeast extract is a suitable nutrient source for supporting the growth of *P. irregulare* by providing not only nitrogen but also a variety of growth promoting factors such as B vitamins (Liang et al., 2011). Therefore, thin stillage should provide a similar function as yeast extract to support the growth of *P. irregulare* because some soluble compounds from dead yeast cells after fermentation are preserved in thin stillage.

3.2 Feasibility of using thin stillage for fungal culture

Media containing different compositions were used to support fungal growth. As shown in Table 2, in thin stillage only (TS-only) medium, thin stillage provided carbon, nitrogen, and various micro-nutrients. The sources of carbon to support fungal growth were

reducing sugars and glycerol. In this medium, glycerol was considered as the major carbon source for fungal culture due to its high content in the thin stillage (Table 1). In glycerol+TS medium, external glycerol was added to the medium to adjust the total glycerol to 30 g/L, which was similar to the controls in terms of C/N ratio. The glycerol+yeast extract (glycerol+YE) and glucose+yeast extract (glucose+YE) were two control media, in which glycerol and glucose were used as carbon sources, respectively; while yeast extract was used as a source of complex nitrogen, B vitamins, as well as other growth promoting factors. Comparison of glycerol+TS and glycerol+YE evaluated the function of thin stillage as a replacement for yeast extract. The concentration of thin stillage was such that the nitrogen concentration in the medium was at the same level as that in the yeast-extract containing medium.

Table 2 clearly indicates that thin stillage can be used either as a sole nutrient source or a replacement of yeast extract for fungal growth and EPA production. Both the TS-only media and glycerol+TS media resulted in significant cell growth, with cell dry weight higher than those of the two controls. The total fatty acid (TFA) contents of the thin stillage-derived biomass almost doubled that of the control biomass, while the EPA content was maintained at similar levels to the control. This result indicated the increased TFA in thin stillage culture was due to accumulation of the shorter chain fatty acid instead of EPA. The increase in EPA yield in the thin stillage culture was due to the increase in total amount of biomass.

The above results demonstrate the suitability of using thin stillage to produce EPA by the fungus *P. irregulare*. Thin stillage contained various nutrients, which play different functions in fungal culture. Although the 5-7 C/N ratio of the thin stillage (Table 1) was lower than 10-12, which was the value used in our previous studies (Liang et al., 2011),

increasing C/N ratio of the thin stillage medium by externally adding glycerol did not provide any further benefit in increasing fungal growth and EPA production. In the following studies, thin stillage was used as the sole nutrient source for *P. irregulare*.

3.3 Effects of thin stillage loading on fungal fermentation

The feasibility study showed that fungal cells can use thin stillage as the sole nutrient source for EPA production. However, the thin stillage concentration used in the feasibility study was arbitrary, therefore, the effects of thin stillage concentration on fungal EPA production were further investigated. As shown in Figure 1A, the cell dry weight increased as the thin stillage concentration from 5 to 70% and leveled off when thin stillage exceeded 70%. The EPA content showed an opposite trend of that for cell dry weight. For example, the EPA content of the fungal biomass maintained a high level (ca. 12 mg/g) as the thin stillage concentration ranged from 5 to 30%, and then decreased when thin stillage concentration exceeded 30% (Figure 1A). Similar to cell growth, the EPA yield increased with thin stillage concentration from 5 to 50%, leveled off at 50-70%, and then decreased with further increases in thin stillage (Figure 1A).

In addition to the effects of thin stillage on fungal growth and EPA production, use of fungal culture to treat thin stillage and prepared nutrient-depleted recyclable "clean" water was also evaluated. The initial thin stillage medium was a turbid and yellow liquid; after fungal fermentation, the medium became clear with less brown (data not shown). The COD, total nitrogen, and total phosphorus in this liquid decreased compared to the initial raw thin stillage solution. Figure 1B shows the removal efficiencies of total COD, total nitrogen, and total phosphorus at different thin stillage loading levels. The COD removal efficiency

gradually decreased with increasing thin stillage loading. The total nitrogen and total phosphorus removal efficiencies fluctuated within the range of 40-60% and 30-50%, respectively.

3.4 Effects of temperature and temperature shifts on fungal growth and EPA production

Temperature is another important factor influencing cell growth and lipid composition of many EPA-producing microorganisms. The effects of temperature on the fungal EPA production were investigated at 50% thin stillage concentration. Figure 2 shows the growth profiles of *P. irregulare* at different temperatures. At higher temperatures (e.g., 30^{o}C), the lag phase was shorter but the cell density was lower. Reducing temperature results in an opposite trend (i.e., longer lag phase but higher cell density). The growth kinetics and EPA production parameters are summarized in Table 3. Maximum cell density decreased as culture temperature increased; however, the time to reach the maximum cell density also declined. The specific cell growth rate of the culture at the exponential phase at each temperature setting was almost the same. The EPA and TFA contents, however, did not significantly change with culture temperature. As a result, the EPA and TFA yields have similar trends to maximum biomass density. The biomass, EPA, and TFA productivities were maintained within the range from 15 to 25^{o}C, and decreased to the lowest level when temperature was increased to 30^{o}C (Table 3).

The effects of temperature on cell growth and omega-3 fatty acid synthesis have been widely reported in various algal/fungal culture systems. In general, high temperature results in faster cell growth rate and higher cell density, while low-temperature stress increases the

unsaturated fatty acid content (Jiang and Chen, 2000; Wen and Chen, 2001) because the cells need to maintain membrane fluidity at lower temperature by increasing the degree of fatty acid unsaturation (Wen and Chen, 2003). In the present work, however, high temperature reduced the maximum cell density level (Figure 2 and Table 3). Perhaps, high temperature led to reduced dissolved oxygen which in turn limited cell growth. The thin stillage medium was a viscous liquid with a high solids content, which attached to the biomass and thus impeded oxygen transfer. With rising temperature, this oxygen limitation would be severe and thus reduce cell growth.

Table 3 also shows that temperature change did not significantly affect EPA content, which is contrary to previous findings that low temperature significantly increased EPA content (Jiang and Chen, 2000; Wen and Chen, 2001). Probably, EPA and other polyunsaturated fatty acids are not exclusively located in cell membranes of *P. irregulare*, and thus, the temperature effect on the membrane fluidity is not a significant factor in increasing EPA content.

A "temperature shift" strategy was developed to achieve higher cell density within a shorter period of time. As shown in Figure 3, the culture temperature was 30^{o}C for the first 4 days and decreased stepwise during the culture period. The maximum cell density (23.4 g/L) in temperature shift experiment (Figure 3) was similar to the culture when 15^{o}C was used for the entire culture period (Figure 2). However, cells grown in the temperature shift experiment took a shorter time (9 days) to attain similar maximum cell density (Figure 3) as compared to that at constant 15^{o}C (Figure 2). The kinetic parameters obtained from the temperature shift are summarized in Table 3. The maximum cell density, and EPA and TFA contents, and the EPA and TFA yields from the temperature shift were similar to those obtained at constant

15°C. However, the temperature shift shortened the growth period, and thus, increased the biomass and EPA productivities (Table 3).

3.5 Characterization of fungal biomass and fungal-treated thin stillage liquid

The fungal biomass obtained with the 50% thin stillage medium in the temperature shift study as well as the control medium (30 g/L glucose and 10 g/L yeast extract) were characterized for potential nutritional value. Table 4 shows the proximate analysis of the biomass. Compared to the biomass derived from the control medium, the 50% thin stillage-derived biomass had higher lipid content, but lower crude protein content. The carbohydrate contents of the two types of biomass were similar.

The fatty acid profiles of the fungal biomass were characterized. The fatty acids in the raw thin stillage were also determined to provide insight into fatty acid synthesis by *P. irregulare*. As shown in Table 5, the raw thin stillage mainly contained C16:0, C18:1 and C18:2 fatty acids, with minor amount of C18:0 fatty acids. When 50% thin stillage was used for fungal fermentation, the resulting biomass had higher content of TFA, higher proportions of C18:1 and C18:2 than those of the biomass grown in the control medium. The proportion of EPA, however, decreased in the thin stillage-derived biomass, while the EPA cellular contents of the two types of biomass were similar. These results indicated that the fatty acids contained in the raw thin stillage were absorbed by the fungal biomass; however, the absorbed shorter chain fatty acids were not elongated into long-chain fatty acids.

In addition to EPA production by *P. irregulare*, the fungal culture also showed great capability of reducing organic compounds and preparing "clean" water from turbid, viscous raw thin stillage slurry. Compared with the raw thin stillage, the spent medium had a much

less total solids and COD contents (Table 1). The fungal culture also reduced almost each type of nutrient such as carbons (sugars, glycerol, and organic acids), nitrogen (TKN, nitrate and ammonia), and phosphorus (total phosphorus and orthophosphate). This result further confirmed the complete utilization of the thin stillage compounds by *P. irregulare*. Using fungal culture as a way of removing nutrients and preparing "clean" water is another benefit of growing *P. irregulare* on thin stillage medium.

4. CONCLUSIONS

Thin stillage from a dry-grind corn ethanol plant can be a potential nutrient source for growing the fungus *P. irregulare* to produce high-value omega-3 fatty acid. Cell growth and EPA production were highly dependent on the concentration of thin stillage and the temperature used to grow the fungal culture. The thin stillage-derived fungal biomass had higher lipid content but lower protein content. In addition to EPA production from thin stillage, the fungal culture also resulted in "clean" water with less nutrient load, which enables much higher setback levels and water use in ethanol production. The results in this work provide an alternative for utilizing the large amounts of thin stillage generated in dry-grind ethanol plants. A thorough study on the process development and optimization (such as fungal culture reactor design, characterization and extraction of EPA rich oil, animal feeding trials, EPA-fortified food development) is needed in order to lead to commercial application of this process.

REFERENCES

Ahn, J.H., Sang, B.I., Um, Y., 2011. Butanol production from thin stillage using *Clostridium pasteurianum*. Bioresour. Technol. 102, 4934-4937.

Alkan-Ozkaynak, A., Karthikeyan, K.G., 2011. Anaerobic digestion of thin stillage for energy recovery and water reuse in corn-ethanol plants. Bioresour. Technol. 102, 9891-9896.

Athalye, S.K., Garcia, R.A., Wen, Z.Y., 2009. Use of biodiesel-derived crude glycerol for producing eicosapentaenoic acid (EPA) by the fungus *Pythium irregulare*. J. Agric. Food Chem. 57, 2739-2744.

Cheng, M.H., Walker, T.H. Hulbert, G.J., Raman, D.R., 1999. Fungal production of eicosapentaenoic and arachidonic acids from industrial waste streams and crude soybean oil. Bioresour. Technol. 67, 101-110.

Dowd, M.K., Reilly, P.J., Trahanovsky, W.S., 1993. Low molecular weight organic composition of ethanol stillage from corn. Cereal Chem. 70, 204-209.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356.

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Bio. Chem. 226, 497-509.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure. Appl. Chem. 59, 257-268.

Hsieh, C., Hsu, T.H., Yang, F.C., 2005. Production of polysaccharides of *Ganoderma lucidum* (CCRC36021) by reusing thin stillage. Proc. Biochem. 40, 909-916.

Jiang, Y., Chen, F., 2000. Effects of temperature and temperature shift on docosahexaenoic acid production by the marine microalga *Crypthecodinium cohnii*. J. Am. Oil Chem. Soc. 77, 613-617.

Kim, Y., Mosier, N.S., Hendrickson, R., Ezeji, T., Blaschek, H., Bien, B., Cotta, M., Dale, B., Ladisch, M.R., 2008. Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage. Bioresour. Technol. 99, 5165-5176.

Liang, Y., Garcia, R.A., Piazza, G.J., Wen, Z.Y., 2011. Nonfeed application of rendered animal proteins for microbial production of eicosapentaenoic acid by the Fungus *Pythium irregulare*. J. Agric. Food Chem. 59, 11990-11996.

Liu, K.S., 2011. Chemical composition of distillers grains, a review. J. Agric. Food Chem. 59, 1508-1526.

Mitra, D, Rasmussen, M.L., Chand, P., Chintareddy, V.R., Yao, L., Grewell, D., Verkade, J.G., Wang, T., van Leeuwen, J., 2012. Value-added oil and animal feed production from corn-ethanol stillage using the oleaginous fungus *Mucor circinelloides*. Bioresour. Technol. 107, 368-375.

Pyle, D.J., Garcia, R.A., Wen, Z.Y., 2008. Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition. J. Agric. Food Chem. 56, 3933-3939.

Simopoulos, A.P., 1999. Essential fatty acids in health and chronic disease. Am. J. Clin. Nutr. 70, 560S-569S.

van Leeuwen, J., Khanal, S.K., Pometto, A.L., Rasmussen, M.L., Mitra, D., 2010. Fungi cultivation on alcohol fermentation stillage for useful products and energy savings. US patent application publication. Pub. No.: US 2010/0196994 A1.

Wen, Z.Y., Chen, F., 2001. Application of statistically-based experimental designs for the optimization of eicosapentaenoic acid production by the diatom *Nitzschia laevis*. Biotechnol. Bioeng. 75, 159-169.

Wen, Z.Y., Chen, F., 2003. Heterotrophic production of eicosapentaenoic acid by microalgae. Biotechnol. Adv. 21, 273-294.

Table 1. Characterization of raw thin stillage and *P. irregulare* culture medium

Characteristics	Thin stillage	Spent medium [b]
Total solids, %, w/v	6.53±0.19	1.06±0.08
Total COD, g/L	112.0±2.8	4.0±2.5
pH	4.5±0.2	6.8±0.4
Total carbohydrates, g/L as glucose	20.5±2.0	2.5±0.3
Reducing sugar, g/L as glucose	2.1±0.1	0.23±0.04
Glycerol, g/L	17.5±0.8	0.02±0.01
Total Kjeldahl nitrogen (TKN), g/L	2.5±0.1	0.12±0.06
Nitrate, mg/L as N	44.0±0.6	8.3±1.8
Ammonia, mg/L as N	35.0±3.0	7.5±3.2
Total phosphorus, g/L as P	1.6±0.1	0.11±0.03
Ortho-phosphate, g/L as P	0.99±0.10	0.30±0.06
Lactic acid, g/L	2.23±0.03	Not detected
Acetic acid, g/L	0.65±0.04	Not detected
C/N ratio [a]	5 - 7	-

[a] C/N ratio was only estimated for the raw thin stillage. [b] The culture medium refers to the liquid after *P. irregulare* biomass was harvested from the broth at day 9 of the temperature shift study (Figure 3). Data are means of triplicates ± standard deviations

Table 2. Growth and EPA production of *P. irregulare* grown on thin stillage containing medium and control medium

Medium composition [a]	C/N ratio [b]	Cell dry weight (g/L)	TFA content [c] (mg/g)	EPA content (mg/g)	EPA yield (mg/L)
TS (40%) only	5-7	12.3±0.5	203.3±22.4	9.70±1.73	119.2±15.8
Glycerol (23 g/L) + TS (40%)	11-12	15.1±0.6	236.1±32.4	7.93±2.40	119.8±21.8
Glycerol (30g/L) + YE (1%)	10-12	4.8±0.8	131.4±30.9	8.40±1.36	40.3±6.5
Glucose (30g/L) + YE (1%)	10-12	10.4±0.5	124.7±11.7	9.65±1.24	100.3±13.4

[a] TS and YE are abbreviations for thin stillage and yeast extract, respectively. The concentration of TS was based on wet weight (w/v). [b] N content for TS was based on total Kjeldahl nitrogen. N content of commercial YE ranged from 10.0 to 12.5% as indicated by the manufacturer. [c] TFA: total fatty acid. Data are means of triplicates ± standard deviations

Table 3. Effects of temperature at single setting and temperature shift on growth kinetics and EPA production by *P. irregulare* grown on thin stillage medium [a]

Parameter	Unit	Temperature (single setting)				Temperature Shift
		15°C	20°C	25°C	30°C	
Maximum cell density	g/L	24.3±1.8	18.0±1.3	13.9±0.8	9.8±0.4	23.4±1.7
Days [b]		12	9	7	6	9
Maximum specific Growth rate	day^{-1}	0.45±0.04	0.46±0.05	0.52±0.04	0.44±0.06	0.32±0.03
Biomass productivity	g/L·day	2.03±0.15	2.00±0.14	1.99±0.11	1.63±0.07	2.60±0.19
EPA content	mg/g DW	8.71±0.73	8.18±1.12	8.38±1.00	7.58±1.74	10.38±0.47
EPA yield	mg/L	211.7±22.0	147.2±20.2	116.5±13.9	74.3±17.1	243.1±29.4
EPA productivity	mg/L·day	17.6±1.8	16.4±2.2	16.6±2.0	12.4±2.8	27.0±3.3
TFA content	mg/g DW	253.9±25.3	266.7±32.4	232.8±10.6	269.6±49.4	291.9±34.5
TFA yield	g/L	6.17±0.61	4.80±0.58	3.24±0.15	2.64±0.48	6.83±0.72
TFA productivity	mg/L·day	514.1±50.8	533.4±64.8	462.3±21.0	440.3±80.7	758.9±80.2

[a] Thin stillage concentration was 50% (w/v). [b] Refers to the day when maximum cell dry weight was achieved. Data are means of triplicates ± standard deviations

Table 4. Proximate analysis of freeze-dried fungal biomass grown on thin stillage medium and control medium

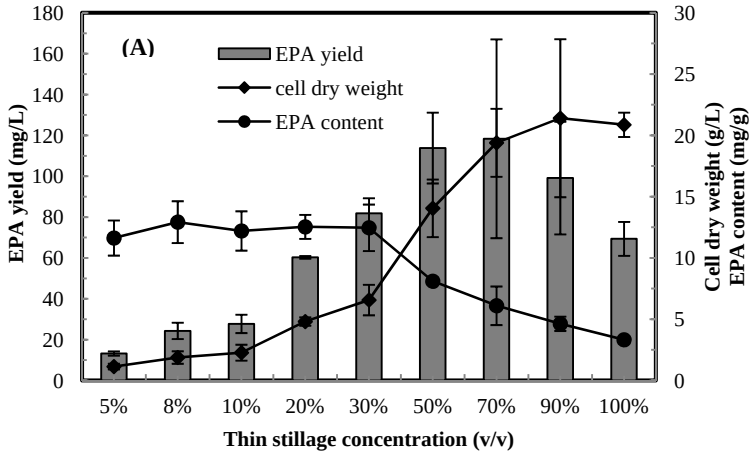
Component (% dry biomass)	Fungal biomass	
	Thin stillage medium [a] (50% thin stillage)	Control medium [b] (30 g/L glucose + 10 g/L yeast extract)
Lipid	38.52 ± 3.61	18.27 ± 1.61
Crude protein	28.39 ± 3.40	43.69 ± 9.31
Carbohydrate	29.97 ± 3.01	34.67 ± 1.49
Ash	3.12 ± 0.42	3.36 ± 0.30

[a] The biomass in thin stillage medium was harvested at day 9 of the temperature shift study (Figure 3).
[b] 30g/L glucose and 10g/L yeast extract were used. Data are means of triplicates ± standard deviations

Table 5. Fatty acid profiles of thin stillage and fungal biomass grown on thin stillage medium and control medium

Fatty acid	Unit	Thin stillage [a]	Fungal biomass	
			Thin stillage medium [b]	Control medium [c]
C 14:0	%TFA	-	2.94 ± 0.67	10.10 ± 2.16
C 16:0	%TFA	17.32 ± 3.21	21.00 ± 1.25	28.33 ± 6.11
C 16:1	%TFA	-	2.38 ± 1.10	6.90 ± 2.09
C 18:0	%TFA	2.18 ± 0.12	2.68 ± 0.06	2.87 ± 0.44
C 18:1	%TFA	26.90 ± 2.44	20.91 ± 0.74	16.19 ± 2.99
C 18:2 (n-6)	%TFA	53.60 ± 3.58	42.34 ± 2.88	17.83 ± 3.40
C 18:3 (n-3)	%TFA	-	1.47 ± 0.13	1.60 ± 0.24
C 20:4 (n-6)	%TFA	-	2.70 ± 0.52	6.66 ± 1.48
C 20:5 (n-3)	%TFA	-	3.56 ± 0.31	8.24 ±1.15
EPA content	mg/g of DW	-	10.38 ± 0.47	9.52 ± 1.14
TFA content	mg/g of DW	133.41 ± 10.23	291.9 ± 34.5	115.55 ± 16.11

[a] Thin stillage slurry was freeze-dried before fatty acid analysis by GC. [b] Biomass was harvested at day 9 of the temperature shift experiment (Figure 3). [c] 30g/L glucose and 10g/L yeast extract were used. Data are means of triplicates ± standard deviations



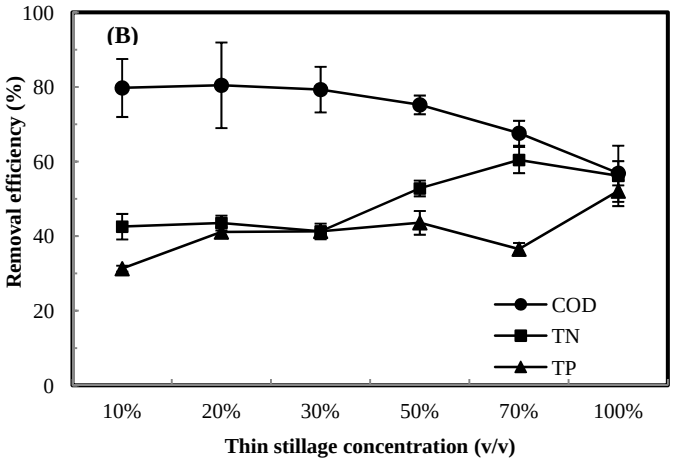


Figure 1. Effects of thin stillage concentration on *P. irregulare* cell growth, EPA content, and EPA yield (**A**) and COD, total nitrogen (TN) and total phosphorus (TP) removal efficiencies (**B**). Culture temperature was 25^{o}C. Data are means of triplicates and error bars show standard deviations.

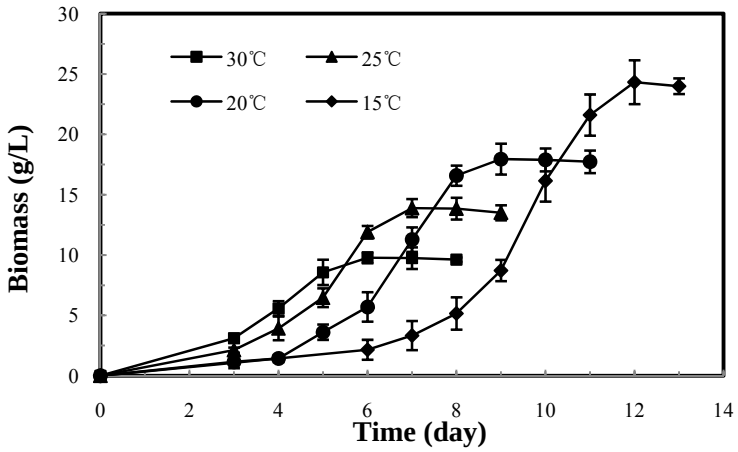


Figure 2. Growth of *P. irregulare* on thin stillage medium under different temperatures. Data are means of triplicates and error bars show standard deviations.

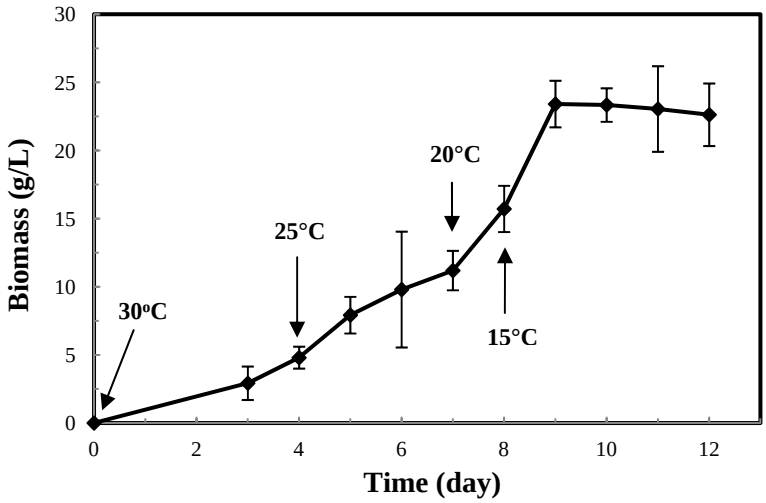


Figure 3. Cell growth of *P. irregulare* with stepwise temperature shift from 30 to 15°C. Data are means of triplicates and error bars show standard deviations.

CHAPTER 5 GENERAL CONCLUSIONS

1. GENERAL DISCUSSION

The present body of work focused on using agriculture byproducts and plant materials to produce valuable compounds. The general strategy was based on the bio-renewable concept, and was to test the feasibility of supplying carbon with fractionated bio-oil to the microalga *Chlamydomonas reinhardtii*, and feeding rendered animal proteins and thin stillage to EPA-producing fungus *Pythium irregulare*.

The acetic acid-rich bio-oil fraction was demonstrated to be a viable carbon source for microalgae cultivation with the potential of producing lipid-based biofuel. Complete separation or purification of bio-oil was not easy to achieve because bio-oil consists of many compounds, which strongly inhibit algal growth. Activated carbon adsorption and development of toxicity-tolerant strains are economically accessible tools, which make biological utilization of bio-oil possible. The present work identifies a great opportunity for fermentation for fuels and chemicals by using bio-oil derived substrates.

Rendered animal proteins was found to be a potential nutrient source for growing microorganisms, providing the proteins are appropriately hydrolyzed into smaller molecules. Enzymatic hydrolysates were able to replace yeast extract as the nitrogen source to support growth and EPA production in *P. irregulare*. The growth performance, EPA production, and proximate composition in *P. irregulare* when using enzymatic protein hydrolysates are similar to cultures using commercial yeast extract as the nutrient source. The present work

provides a new alternative outlet for the large amounts of animal proteins produced by the rendering industry.

Thin stillage from a dry-grind ethanol plant was an effective nutrient source for growing the fungus *P. irregulare* to produce high-value omega-3 fatty acids. The cell growth and EPA production were highly dependent on the concentration of thin stillage and the temperature used to grow the fungal culture. The thin stillage-derived fungal biomass had higher lipid content but lower protein content. In addition to EPA production from thin stillage, the fungal culture also resulted in "clean" water with less nutrient load. Our results provide an alternative outlet for utilizing the large amounts of thin stillage generated in dry-grind ethanol plants.

2. RECOMMENDATIONS FOR FUTURE RESEARCH

Much additional work must be done in order to commercialize these processes. The following recommendations will enable others to further advance production of bio-based chemicals and fuels.

The acetic acid-rich fraction of pyrolytic bio-oil can be fed to microalgae for producing lipid after adopting a directed evolution strategy and activated carbon treatment to mitigate the toxicity of the bio-oil fraction. Additional insight on the tolerance mechanisms of the adapted strains is needed. Evolved strains need to be isolated and the genes responsible for enhanced tolerance need to be identified. Once these genes are identified, they can be incorporated into other microorganisms for utilizing pyrolytic bio-oil based chemicals.

The feasibility of using rendered animal protein hydrolysates to produce EPA by fermentation has been demonstrated. In the future, more fermentation tests using different industrial microorganisms are needed to determine the breadth of applicability of the protein hydrolysates. The hydrolytic process will also need to be optimized to prevent the loss of growth-promoting factors and improve cost-effectiveness.

Thin stillage was tested for the potential to be a sole nutrient source for supporting *P. irregulare* growth. In the future, a thorough study on the process development and optimization (such as fungal culture reactor design, characterization and extraction of EPA-rich oil, animal feeding trials, EPA-fortified food development) are needed in order to enable commercial application of this process.

ACKNOWLEDGEMENT

First of all, I would like to express my gratitude to my POS committee, Drs. Lawrence Johnson, Ruth MacDonald, Tong Wang, Buddhi Lamsal, and Shihwu Sung for their tremendous support during my study at Iowa State University. Their guidance and encouragement help me to grow each step of the way.

I would also like to thank all the professors that I met at Iowa State University. I appreciate all the lessons that I learned and all the knowledge that I obtained. With the valuable experience, I have been able to grow.

My sincere thanks go to all my colleagues and friends at Iowa State University, in particular, Min Liu. With her help and care, I survived all the difficult situations.

My deepest gratefulness goes to my family in China. Their unconditional love and support have accompanied me all the way through this process. I am so blessed with the courage and strength that they give me, and I believe these gifts will benefit me for the rest of my life.