Utilization of oil crop residues for enhanced algae based production of lipids, polyunsaturated fatty acids, and protein

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Adviser: Dr. R. Roger Ruan

December, 2013

Acknowledgements

My deepest gratitude goes first and foremost to Professor Roger Ruan, my advisor, for his constant encouragement and guidance on my master study.

I am also greatly indebted to Professor Paul Chen and Professor Tonya Schoenfuss to be the committee members of my master degree defense. Without their illuminating instructions, this thesis could not have reached its present appearance.

I also would like to express my heartfelt gratitude to Doctor Joe Zhou and Professor Zata Vickers, who gave me a lot of useful suggestions, and led me to the world of professional English writing during master study. Moreover, I thank Professor Ernian Pan very much for his help in the process of this thesis revision. The officers of Center of Writing also helped me a lot in improving the quality of this thesis; therefore I want to thank them, too.

Last but not the least, my thanks would go to my beloved family for their fully support to me all through these two years. I also owe my sincere gratitude to my colleagues, friends and fellow classmates, who helped me solve my problems and gave me confidence all the times.

Dedication

This thesis is dedicated to my parents Yuqing Wang and Lingyun Qiao.

Abstract

Microalgae are capable of synthesizing many high value compounds, such as lipids, polyunsaturated fatty acids (PUFAs), and proteins, and therefore considered potential feedstock for production of biodiesel, nutraceuticals, and animal feeds. The synthesis and accumulation of lipids, PUFAs, and proteins are influenced by such factors as microalgae strain, trophic conversion mode of growth, nutrient supply, and light and temperature conditions. Regulation of these factors must take cost issue into account. The goal of this research was to investigate the use of oil crop biomass residue (OCBR), a low-cost material from oil extraction plants, to cultivate specific algae strains for targeted production of lipids, PUFAs, and proteins.

The specific objectives of present thesis were: 1) to optimize the conditions for acid hydrolysis of OCBR for best yield and desirable profile of nutrients; 2) to evaluate microalgae growth on different OCBR media; 3) to characterize the chemical compositions especially the lipid, protein and EPA contents in the harvested algal biomass as affected by culture media; 4) to study the effect of temperature and growth phase on algae EPA synthesis.

The one-factor-at-a-time experimental design was used to optimize the acid hydrolysis conditions based on the key nutrient level including the total nitrogen (TN), total phosphorus (TP), chemical oxygen demand (COD) and ammonia nitrogen (NH_4-N) in the hydrolysates. The optimal conditions were found to be using 3 % sulfuric acid and hydrolyzing residues at 90 °C for 20 hrs. The hydrolysates (OCBR media) produced

under the optimal conditions were used to cultivate two algae strains, namely UM258 and UM268. The results from 5 days of cultivation showed that the best OCBR media supported faster algae growth than artificial media, with maximal algal biomass yield of 2.7 g/L and 3 g/L, respectively. Moreover, the total lipids after 5 days cultivation for UM258 and UM268 were 54 % and 35%, respectively. The OCBR also promoted protein accumulation in UM 268 compared with artificial media. Temperature-time interaction-effect on EPA synthesis was observed. Temperature of 20 °C and time of longer than 6 days of cultivation (after algae reach stationary phase), were found optimal for EPA accumulation for UM258 with EPA reaching 18% in total fatty acids.

The results suggest that OCBR media are an excellent alternative for algae growth and have great potential for large scale productions of algae based ingredients for biodiesel, high value foods and animal feeds.

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Chapter 1 Introduction and Literature Review

1.1 Introduction of algae

1.1.1 Overview of algae

Algae are a group of natural organisms that have all or most of the following characteristics: photosynthetic, aquatic and simple vegetable structures. However, algae lack of many important cell organelles that characterize land plants, such as reproductive growth and protecting layer outside the cells. According to the above characteristics, algae are no longer regarded as a phylogenetic concept (Wehr et al., 2003). The composition of algae cell is shown in the following picture (Edwards, 2008).

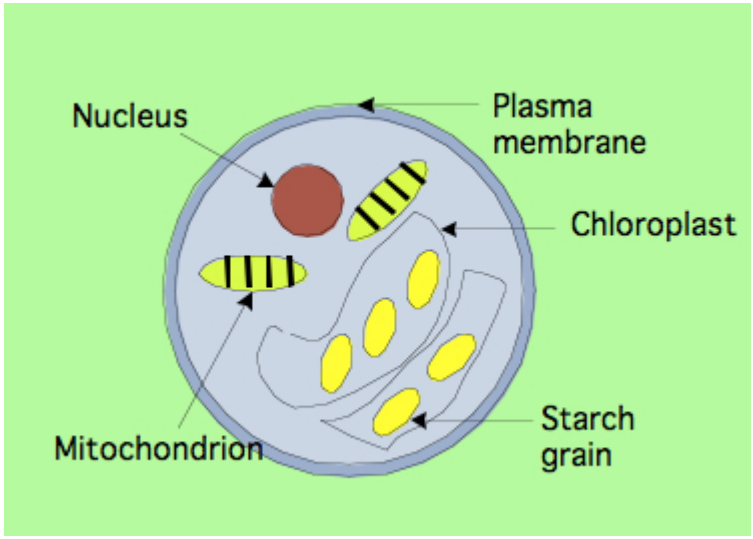


Figure 1.1 Algae cell composition

Algae can be classified into microalgae (unicellular microscopic organisms) and macroalgae (multicellular of great size). They have been utilized by humans for hundreds of years as food, feed, medicines and fertilizers. Ancient records show that people collected macroalgae for food as long ago as 500 B.C. in China and one thousand years

later in Europe. Microalgae such as *Arthrospira* have a history of human consumption in Mexico and Africa (Barsanti et al., 2005).

Currently, 42 countries have reports or research on commercial algae production. Microalgae biomass production for commercial use is limited to *Dunaliella, Haematococcus, Arthrospira* and *Chlorella* (Barsanti et al., 2005). Specifically, there are three significant attributes that contribute to the commercial and technical advantages of microalgae. First of all, they can incorporate the elements of carbon, nitrogen and hydrogen into their composition, and then transfer them into their diverse products. Secondly, the large size of unexplored group of algae provides an essentially unexploited source of algae byproducts. Thirdly, they are an alternative source of biodiesel and bioactive byproducts, since they are capable of synthesizing many high value compounds such as lipids, polysacharides, proteins pigments and vitamins. Since this source is more feasible and low-cost than others, algae have begun to be popular in the industries of nutraceuticals, pharmaceuticals, biofuel feedstock as well as animal feed (Radmer et al., 1994).

Microalgae contain more oil than macroalgae and can be grown more easily and quickly; thus, microalgae are widely used in the production of biodiesel (Hossain et al., 2008). Besides, some microalgae strains could produce large quantities of long-chain omega-3 and omega-6 fatty acids (LC-PUFAs), which are beneficial to human health. Therefore, microalgae are a great potential source of biofuel and LC-PUFAs for use in food products.

1.1.2 Algae serve as biodiesel feedstock

Biodiesel is chemically defined as a mixture of fatty acids methyl esters (FAMEs) produced from a reaction of triacylglycerols or TAGs (e.g., vegetable oils, animal fats, waste cooking oil, algae lipids, or other fatty acids) with alcohols, termed as transesterification in the presence of a catalyst (an acid, base, or an enzyme) (Shi et al., 2011). Biodiesel has many advantages as a renewable energy resource. Since it is derived from a renewable and domestic resource, it relieves human dependence on petroleum fuel. Furthermore, it is biodegradable and almost non-toxic.

Traditional petro-diesel has a lot of disadvantages for utilization. Firstly, it is non-renewable resource, and is predicted to be exhausted in the near future due to the high speed and large-size consumption. This goes against the worldwide requirement that energy resources should be renewable and sustainable. Secondly, the use of petro-diesel leads to some environmental problems. According to the data from U.S. Energy Information Administration, over 80% of the greenhouse gas released comes from energy-related CO2 releases in which petroleum is the largest fossil fuel source, contributing 42% of the total (U.S. Energy Information Administration. 2011). Thirdly, the price of petroleum oil becomes high, which is a big challenge for common use.

Biodiesel is better than the traditional petro-diesel because of its characteristics such as renewability, environmental friendliness, reduced emission, higher combustion efficiency, lower cost and higher safety. (Canakci and Sanli, 2008). Demirbas (2009) also summarized three aspects of the advantages of using bio-fuels, that is, environment, energy security, and economy. Therefore, biodiesel and biofuel provide a promising

alternative which has attracted increasing attention and great effort of researchers around the world.

Due to the advantages discussed above, the total world biodiesel demands and productions have been constantly increased. It was shown that there is a 16-fold increase over the past 10 years in the production, mainly produced in the European Union and the USA (Shi et al., 2011). Furthermore, new research centers with a fast increasing rate for biodiesel production are being built in US (National Biodiesel Board, 2011).

As stated above, the feedstock of biodiesel includes vegetable oils, animal fats, waste cooking oil, and algae lipids. When choosing the feedstock, there are two important factors should be concerned. The first one is if the feedstock is environmental friendly. It was reported that edible oils could lead to deforestation and destruction of ecosystems. Therefore, in some countries with strict forest resources, edible oil is not favorable as biodiesel feedstock (Demirbas et al., 2011). Another factor is the production cost. The feedstock cost constitutes approximately 70-95% of the overall cost of biodiesel production (Watts et al., 2008). Thus, due to these reasons, algae are increasing used as biodiesel feedstock since they are less land-use and cost less than other feedstocks, and thereby playing a fundamental role in energy research.

Using microalgae as a biodiesel feedstock with low cost has been seen with a sustaining growth. Microalgae produce lipids based on sunlight, air, and water, and can grow well all the year round. Besides, microalgae could use carbon dioxide in addition to sun light to complete autotrophic growth. Furthermore, algae could assimilate CO_2 photoautotrophically or mixtrophically, which could be a flawless candidate for CO_2

fixation and reduction (Wang et al., 2010). Additionally, algae have high production of biomass through the heterotrophic growth (Burrell et al., 1984). Therefore, higher biomass productions along with low-cost growth conditions over other energy feedstock are clearly demonstrated.

Like other plant-based biofuel resources, microalgae provide the mechanism for collection, conversion and storage of solar energy into the chemical form. For biofuel production, the major factors cited as determining economically viable production include productivity (i.e., strain selection, photosynthetic efficiency, and productivity of lipids), production and harvesting costs (Borowitzka, 1992). Photosynthetic efficiency is only relevant for autotrophic algae; utilization of sugars is more relevant for heterotrophically cultivated algae (Chisti et al., 2011; Brennan et al., 2010).

Therefore, in this thesis, two strains will be selected and productivity of lipids will be analyzed in order to prove their possibility for biodiesel application under particular cultivating conditions. More details will be presented in the Chapter 4.

1.1.3 Algae serve as animal feed

Algal proteins provide one kind of the common feedstock used as crude protein in animal feed. Proteins are composed of different amino acids, and thus the nutritional quality of a protein is determined basically by the content, proportion and availability of its amino acids. Data on the amino acid profile of various algae are collected in Table 1.1, which are recommended by WHO/FAO (1973), and are compared with some basic conventional food items and a reference of a well-balanced protein. It can be seen that the

amino acid pattern of almost all algae compares favorably with that of the reference and the other food proteins, showing that the algae protein has a good potential to supply animal with qualified protein feed (Becker, 2007).

Table 1.1 Amino acid profile of different algae as compared to conventional protein sources and the WHO/FAO (1973) reference pattern (g per 100 proteins)

Source	He	Leu	Val	Lys	Phe	Tyr	Met	
WHO/FAO	4.0	7.0	5.0	5.5	6.0			3.5
Egg	6.6	8.8	7.2	5.3	5.8	4.2	3.2	
Soybean	5.3	7.7	5.3	6.4	5.0	3.7	1.3	
Chlorella vulgaris	3.8	8.8	5.5	8.4	5.0	3.4	2.2	
Dunaliella bardawil	4.2	11.0	5.8	7.0	5.8	3.7	2.3	
Scenedesmus obliquus	3.6	7.3	6.0	5.6	4.8	3.2	1.5	
Arthrospira maxima	6.0	8.0	6.5	4.6	4.9	3.9	1.4	
Spirulina platensis	6.7	9.8	7.1	4.8	5.3	5.3	0.3	
Aphanizomenon sp	2.9	5.2	3.2	3.5	2.5	--	0.7	

Source	Cys	Try	Thr	Ala	Arg	Asp	Glu	Gly	His	Pro	Ser
WHO/FAO		1.0									
Egg	2.3	1.7	5.0	--	6.2	11.0	12.6	4.2	2.4	4.2	6.9
Soybean	1.9	1.4	4.0	5.0	7.4	1.3	19.0	4.5	2.6	5.3	5.8

Chlorella vulgaris	1.4	2.1	4.8	7.9	6.4	9.0	11.6	5.8	2.0	4.8	4.1
Dunaliella bardawil	1.2	0.7	5.4	7.3	7.3	10.4	12.7	5.5	1.8	3.3	4.6
Scenedesmus obliquus	0.6	0.3	5.1	9.0	7.1	8.4	10.7	7.1	2.1	3.9	3.8
Arthrospira maxima	0.4	1.4	4.6	6.8	6.5	8.6	12.6	4.8	1.8	3.9	4.2
Spirulina platensis	0.9	0.3	6.2	9.5	7.3	11.8	10.3	5.7	2.2	4.2	5.1
Aphanizomenon sp	0.2	0.7	3.3	4.7	3.8	4.7	7.8	2.9	0.9	2.9	2.9

In addition, algae protein is safe since it has undergone a series of toxicological tests for proving harmlessness. Based on the tests on the possible adverse effect and toxic properties of different algae strains so far, it can be declared that no algae stain shows any negative effect. Furthermore, there are no serious anomalies found during the algae short-term or long-term feeding research in the studies on acute or chronic toxicity (Chamorro, 1980). Hence, through numerous nutritional and toxicological evaluations, it can be demonstrated that the algae biomass is suitable to serve as an appreciated feed supplement, and should be a good potential substitute for traditional protein sources (soybean meal, fish meal, rice bran, etc.).

There are two major target markets for algal animal feed. One is the feed of poultry. Introduction of algae biomass into poultry rations offers a more commercial feeding cost, which has a promising prospect. Another growing market of microalgae is

the aquaculture use. It was reported that about 30% of the current world algal production was sold for animal feed application ((Becker, 2004).

Therefore, in the present thesis, two strains will be selected and productivity of protein will be analyzed in order to prove their possibility for animal feed application under particular cultivating conditions. More details will be dicussed in the following Chapter 4.

1.1.4 Algae serve as functional food

1.1.4.1 Brief introduction of functional food

It is well known that there are correlations between daily diet and healthy body. Therefore, people are interested in keeping or improving healthy body by taking the suitable food. Consequently, one of the main research fields of research in food science and technology is on the characterization and extraction of new natural functional ingredients in food with biological activity (e.g., antioxidant, antiviral, antihypertensive, etc.). This type of food that is able to promote human health has been defined as functional food (Plaza et al. 2008).

The concept of functional food was developed at the beginning of the 80s in Japan, which was defined as having functional effect to protect human health in a low-cost way and therefore consistent with high life expectation of humans (Arai, 1996). In this definition it is necessary to pay great attention to three significant and innovative parts: (a) the functional effect is different than the nutritious one; (b) the functional effect must be demonstrated satisfactorily; and (c) the benefit can improve physiological function

consistently or reduce the potential risk of pathological symptom. Besides, the functional food must have a quantity of additional characteristics, for instance, the need of efficiency in their beneficial action at the regular consumed amounts (Diplock et al., 1999).

As mentioned above, the functional effect of functional foods is the result of a series of ingredients that never or rare present in conventional food. Such ingredients are called functional ingredients, including: flavonoids, omega-3 fatty acids, soluble fiber, vitamins, etc. Selected examples of functional ingredients can be seen in Table 1.2.

Table 1.2 Some examples of functional foods and functional ingredients together with their possible effect on human health (Hasler, 2002).

Functional Food	**Functional ingredients**	**Possible health effect**
Chocolate	Flavonoids(procyanidins)	Reduce LDL cholesterol
Green tea	Catechins	Reduce risk of certain types of cancer
Tomatoes and processed tomato products	Lycopene	Reduce risk of certain types of cancer
Red wine	Polyphenolic compounds	Reduce risk of certain heart diseases
Fatty fish (n-3)	Fatty acids	Reduce risk of certain heart diseases
Fermented dairy products	Probiotics	Support intestinal tract health,boost immunity
Cruciferous	Glucosinolates,indoles	Reduce risk of certain types of cancer
Lamb,turkey,beef, dairy	Conjugated linoleic acids(CLA)	Reduce breast cancer

Cranberry juice	Proanthocyanidins	Reduce urinary tract infections
Fortified margarines	Plant sterol and stanol esters	Reduce total and LDLcholesterol
Fortified juice	Soluble fiber	Reduce total and LDLcholesterol
Garlic	Organosulfur compounds	Reduce total and LDLcholesterol

Furthermore, the content of functional ingredients in functional food can be added or enhanced via techniques. The foods that can be changed to contain or enriched with valuable microorganisms and thereby become beneficial to human health are called probiotics. Probiotics have the possibility to enhance the functions of intestinal tract and immune system. The common microorganisms that are added into food are generally lactic acid bacteria including *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus reuteri*, *Lactobacillus casei shirota*, among others. (Sanders, 1999)

Some functional foods could initially contain functional ingredients such as vitamins and/or minerals, e.g., vitamin C, vitamin E, folic acid, zinc, iron, and calcium (Sloan, 2000). The concentration of functional ingredients could be enriched in these foods, too. Such approaches can be seen in some previous literature that aimed to enrich the content of several micronutrients such as omega-3 fatty acids, linoleic acids, phytosterols and soluble fiber in foods for promoting human health and preventing diseases (Hasler, 1998; Sloan, 2002; Unnevehr & Hasler, 2000).

In the last two decades, there has been an increasing number of papers published relating to the extraordinary possibilities of functional foods to prevent chronic diseases and improve human health (Palanca et al., 2006; Roche, 2006). However, there is still unknown information on the range and mechanism of the functional ingredient and the corresponding food. Therefore, further research is necessary in this direction.

1.1.4.2 Polyunsaturated fatty acids (PUFAs)

Generally speaking, saturated fatty acids mainly exist in animal fats, while unsaturated fatty acids exist in plants and certain types of fish. Table 1.3 shows the chemical structure of major saturated and unsaturated fatty acids in the field of food science (Tapiero et al., 2002)

Table 1.3 The chemical structure of major saturated and unsaturated fatty acids in the field of food science (Tapiero, 2002)

Unsaturated Fatty acids
18:1 (11c) Oleic $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
18:2 (9c,12c) Linoleic $CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
18:3 (9c,12c,15c) α-Linolenic $CH_3(CH_2)CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$
18.3 (6c,9c,12c) γ- Linolenic $CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_4COOH$
$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
20:4 (5c,8c,11c,14c) Arachidonic
$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH)_3COOH$
20:5(5c,8c,11c,14c,17c) Eicosapentaenoic(EPA)
$CH_3(CH_2)CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$
22:6(4c,7c,10c,13c,16c,19c) Docosahexaenoic (DHA)
Saturated fatty acids
14:0 Myristic $CH_3(CH_2)_{12}COOH$

16:0 Palmitic $CH_3(CH_2)_{14}COOH$

18:0 Stearic $CH_3(CH_2)_{16}COOH$

Polyunsaturated fatty acids, which are abbreviated as PUFAs, are one kind of functional ingredients in food, and belong to bioactive compounds. PUFAs are fatty acids that contain two or more *cis* double bonds that are separated from each other in their structural backbone (Baggott, 1997). In PUFAs group, omega-3 fatty acids, omega-6 fatty acids and omega-9 fatty acids (methylene-interrupted) are the most essential ones.

Omega-3 fatty acids are one kind of polyunsaturated fatty acids that contains a double bond (C=C) at the third carbon atom from the end of the carbon chain. The fatty acids have two ends: the acid (-COOH) end, which is considered the start of the chain, is called "alpha"; and the methyl (CH_3) end, which is considered the "tail" of the chain, is called "omega". The nomenclature of the fatty acid is based on the location of the first double counted from the methyl/ omega (ω-)/ n- end on carbon bone (Clinic, 2012). Nowadays, humans have an increasing interest in n-3 PUFA, specifically eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3), due to their potential in improving human health. The term “EPA” refers to eicosapentaenoate, eicosapentaenoic acid, and/or derivatives including, but not limited to esters, glycerides, phospholipids, sterols, and/or mixtures. As used in the previous literature, the term “eicosapentaenoate” refers to all-cis-5, 8, 11, 14, 17-eicosapentaenoate (Figure 1.2) (Wu et al., 2010).

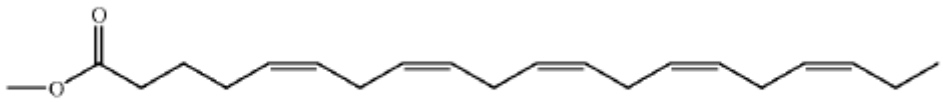


Figure 1.2 The structure of EPA (Wu et al., 2010)

In nature, PUFA, especially EPA is commonly found in marine animals and phytoplankton. The metabolic pathways of PUFAs and biosynthesis of eicosanoids from arachidonic acid (C20:4n-6) is illustrated in Figure 1.3 (Pratima Bajpai et al. 1993).

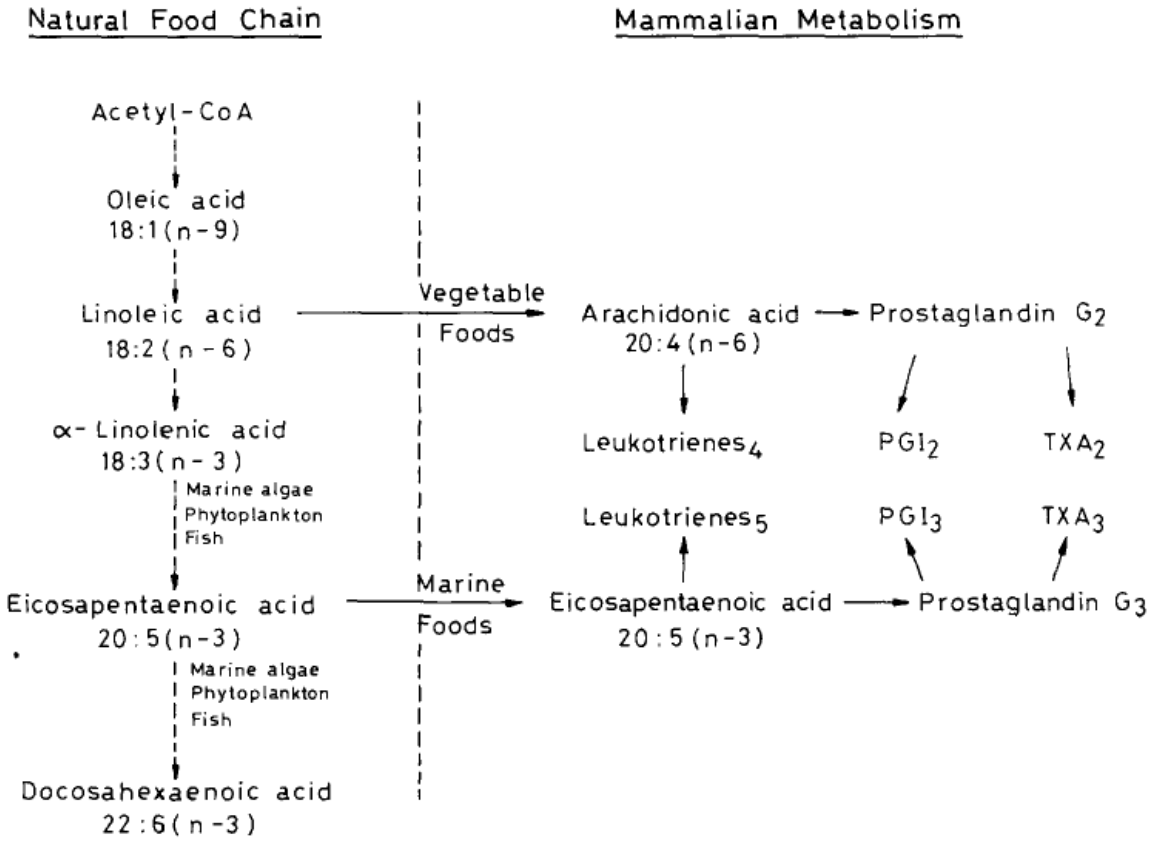


Figure 1.3 Origin and metabolic pathways of omega-3 and omega-6 fatty acids and biosynthesis of eicosanoids from arachidonic acid, 20:4(n-6) (Bajpai et al. 1993)

The metabolism of omega-3 fatty acids that benefit for prevention and treatment of human disorders and diseases is clear now: it is due to the eicosanoid changes in the human circulatory system. As showed in Figure 1.3, the eicosanoids are one kind of hormone like body substances including prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) (Weber et al., 1986). Arachidonic acid (AA) and EPA are precursors of eicosanoid compounds. The formation of thromboxane A_2 (TXA_2) derived from AA which is a platelet aggregator, is inhibited by EPA. At the same time, thromboxane A_3 (TXA_3) which is a weak aggregator is produced from EPA. Also, type 3 prostaglandin (PG_3) is further converted to prostacyclin 3 (PGI_3), an effective anti-aggregator and is formed from EPA along the wall of blood vessel (Fisher and Weber, 1984). Thus, omega-3 fatty acids are good in decreasing blood viscosity, and increasing membrane fluidity and thus improving its deformability. Studies showed that dietary fish oils, which contain EPA, can reduce blood viscosity with a percentage of 15% (Kobayashi et al., 1981). Consumption of fish oils also helps preventing high blood pressure (Singer et al., 1983). Besides, fish oils containing omega-3 fatty acids functions well for anti-inflammation (Higgs, 1986). It was also reported that omega-3 fatty acids could significantly relieve chronic migraine headaches. In addition, recent studies also indicated that certain LC-PUFAs have correlation with physical and mental enhancement to infants (Neuringer et al., 1987). A summary of pharmaceutical targets of omega-3 fatty acids is shown in Table 1.4, which mainly includes three aspects (Bajpai et al., 1993).

Table 1.4 Pharmaceutical targets of omega-3 fatty acids (Bajpai et al., 1993)

Heart and circulatory	Diseases class	
	Inflammatory	Cancers

Atherosclerosis	Asthma	Breast
Thrombosis	Arthritis	Prostrate
Hypertriglyceridaemia	Migraine headaches	Colon
High blood pressure	Psoriasis	
	Nephritis	
	Diabates	
	Graft rejection	

Unfortunately, most of the fish oil used for conventional omega-3 fatty acids production is hydrogenated and incorporated into margarine, which destroys the valuable omega-3 fatty acids inside. Moreover, limitations of fish oil such as peculiar taste, odor and stability problems contribute additional disadvantages of fish oil to serve as a source of omega-3 fatty acids. In addition, the costs of purifying these fatty acids from fish oil are also very high. Thus, the supply of omega-3 fatty acids from conventional sources such as fish oil is not adequate, and other new commercial sources have to be explored (Bajpai et al., 1993).

1.1.4.3 PUFA production by algae

Recently, several studies using lower fungi, marine algae and diatoms as alternatives to fish oil for PUFA (especially EPA) production have been reported. It has been proved that lower fungi could yield exceptionally high amount of EPA or AA when using specific species at particularly low temperatures. *Mortierella alpine* was detected as one potential source for commercial EPA production (Shimizu et al., 1988a). Besides, both microscopic and macroscopic marine algae were detected containing high levels of

EPA. On the contrary, there are only very few species of fresh water algae that contain significant amount of EPA. Among them Chlorophyceae perform quite as a good lipid production but the PUFAs are generally limited to various isomers of linolenic acid. So far, five species in class Chrysophyceae, that is, *Monochrysis luteri, Pseudopedinella sp.*, *Coccolithus huxleyi, Cricosphaera carterae* and *M. elongata* contain significant amount of EPA ranging from 17% to 28% of total fatty acids (Yongmanitchai & Ward, 1989). In diatoms such as Bacillariophyceae, a considerable amount of PUFAs was found to be EPA (Volkman et al., 1980).

As mentioned before, microalgae have been commonly used as biodiesel feedstock and animal feed due to their outstanding attributes of lipid and protein production. In recent two decades, humans also have paid their attention on its application on commercial functional food, since it has potential to provide a sustainable, renewable, toxic-free source of omega-3 fatty acids for improvement of human health. Experiments related to EPA production by algae mainly include four parts: microalgae strain selection, the effect of algal growth pattern, the effect of nutrient supply and the effect of environmental conditions during algae cultivation. This will be introduced in details as follows.

The first factor that has significant influence on algal PUFA production is microalgae strain. As stated before, not all kinds of algae strains are suitable for PUFA production. In general, marine algae perform better than fresh water algae. However, some species of fresh water algae, such as *Chlorella* minutissima, have a very high EPA content which is 45% (w/w) of total fatty acids, under their optimal culture conditions

(Seto et al., 1984). Besides, red algae have more potential than green algae for PUFA production. It was reported that one kind of red algae, *Porphyridium cruentum*, contains a considerable concentration of EPA and a large amount of AA (Ahern et al., 1983). It is critical to choose appropriate algae strain for EPA production, especially for large scale production, since the algae with higher PUFA concentration can reduce the cost of PUFA production, thereby have more commercial advantages.

Algal growth pattern also has an inevitable effect on the final PUFA production. Algae can grow by both photoautotrophic and heterotrophic ways. Usually, algae, which are in heterotrophic growth, utilizing the organic carbon substances as their sole carbon and energy source, have a higher accumulation of biomass and a faster growth rate, which is good for large scale production. It is remarkable that several approaches have been proposed to develop industrial-scale processes for the heterotrophic cultivation of microalgae. For example, in the late 1970s, two Chlorella species were cultivated heterotrophically in stainless steel tanks using glucose and acetic acid as carbon and energy sources for the production of health foods in Japan and Taiwan (Kawaguchi, 1980). Most recently, Martek Biosciences (Columbia, MD, USA) has set up an industrial heterotrophic cultivation process for the production of DHA-containing oil by *Crypthecodinium cohnii* according to general fermentation industry norms (Radmer et al., 1996). However, the cost of heterotrophic growth is much higher than conventional photoautotrophic one which only consumes free sunlight and carbon dioxide. It is still an important issue to concern about in current PUFA algae production.

The nutrient supply of algal PUFA production mainly includes two aspects, that is,

algal medium conditions and additives. Medium should provide algae with enough sources of nitrogen, carbon, phosphate and sometime salt for algae accumulation. It was reported that the most significant factor on PUFA production is the rate of nitrogen and phosphate in medium. Pinchetti et al. (1998) proved that PUFA could decrease to 27.7% in *Ulva rigida* under nitrogen-starvation period. Wen and Chen (2001) proved that the nitrogen sources also had an effect on heterotrophic production of EPA by the diatom *Nitzschia laevis*. Moreover, the pH of the medium is also an important factor. An initial culture of pH 6.0-7.6 was commonly used for EPA production in fungi and algae (Yongmanitchai and Ward, 1991; Bajpai et al., 1992a). In addition, some antioxidants, such as BHT, help protecting the double bond of PUFAs, which can also enhance algal PUFA accumulation.

As for the environment conditions for algal PUFA production, light and temperature should be the primary factors. In many photosynthetic algae, the absence of light was found to suppress the formation of omega-3 fatty acids particularly EPA but enhance the formation of omega-6 fatty acids (Erwin, 1973). For some green algae, such as *Chlorella minutissima* (Seto et al., 1984), and the red algae *Porphyridium cruentum* (Renzanka et al., 1987), high light intensity could enhance the formation of PUFA in them. However, the situation could be reversed in some other kinds of algae strains. In general, different algae species may have different tolerance to light intensity. As for the temperature effect on PUFA accumulation, it is also reported that low temperature was suitable for EPA production for some algae species. This issue will be discussed in Chapter 5 in details.

Therefore, algae have additional potential for providing omega-3 fatty acids and serving as functional food in industry. Besides, algae EPA production could be controlled and improved by a series of influencing factors. In this thesis, low temperature and algal growth time will be the two factors to be discussed under particular circumstance.

1.2 Media for algae cultivation

1.2.1 Current media for algae cultivation

As illustrated above, medium provides algae with necessary nutrient supply for accumulation. Particularly, in the heterotrophic growth, medium is indispensable nutrient sources for providing nitrogen, carbon, phosphate and sometimes salt for algal growth.

Currently, some commercial artificial heterotrophic media such as Tris-Acetate-Phosphorus (TAP) or BG-11 supplemented with glucose additions are used for microalgae heterotrophic culture for higher growth rate and larger algae biomass production as compared to phototrophic culture.

TAP (Harris, 1989) media contain the following chemicals: NH_4Cl (400 mg/L), $MgSO_4 \cdot 7H_2O$ (100 mg/L), $CaCl_2 \cdot 2H_2O$ (50 mg/L), K_2HPO_4 (108 mg/L), KH_2PO_4 (56 mg/L), Tris (hydroxymethyl) aminomethane (2420 mg/L), glacial acetic acid (1 mL/L), and trace elements solution (1 mL/L), consisting of Na_2EDTA 50 (g/L), $ZnSO_4 \cdot 7H_2O$ (22 g/L), $CaCl_2 \cdot 2H_2O$ (0.05 g/L), H_3BO_3 (11.4 g/L), $MnCl_2 \cdot 4H_2O$ (5.06 g/L), $FeSO_4 \cdot 7H_2O$ (4.99 g/L), $CoCl_2 \cdot 6H_2O$ (1.61 g/L), $CuSO_4 \cdot 5H2O$ (1.57 g/L), (NH4)6Mo7O24·4H2O (1.10 g/L), KOH (16 g/L). TAP media were also used to preserve the seeds of many algae species, such as *Chlorella* sp. and *Volvox carter*. (Wang et al.,

2010; Nedelcu, 2009), since the buffering agent in TAP media could keep algae seed active for a relatively long period.

BG-11 has been widely used since 1970s to cultivate algae strains, especially blue-green algae strains, such as *Gloeocapsa alpicola, Chlorogloea, Synechococcus, Aphanocapsa*, among others (Stanicr et al., 1971; Kenyon, 1972; Rippka, 1972). The composition of BG-11 can be seen in Table 1.5. Usually, it is necessary to add 1% sugar to serve as carbon source during algal heterotrophic growth. BG-11 was applied to enrich isolated microalgae strains from various environments (Chinnasamy, 2010).

Table 1.5 Composition of Medium BG-11* (Stanicr et al., 1971)

Compound	Amt (g/liter)
$NaNO_3$	1.5
K_2HPO_4	0.04
$MgSO_4 \cdot 7H_2O$	0.075
$CaCl_2 \cdot 2H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (disodium magnesium salt)	0.001
Na_2CO_3	0.02
Trace-metal mix A56	1mL/liter

*After autoclaving and cooling, pH of medium 7.1.

Recently, a new artificial algal medium was reported, which could promote algal growth at faster growing speed. The artificial media used as the baseline culture of algal in the study were composed of: KH_2PO_4 0.7 g/L, K_2HPO_4 0.3 g/L, $MgSO_4 \cdot 7H_2O$ 0.3 g/L, $FeSO_4 \cdot 7H_2O$ 3 mg/L, glycine 0.1 g/L, vitamin B1 0.01 mg/L, A5 trace mineral solution 1

mL/L. The trace metal mix A5 solution consist of H_3BO_3 2.86 g/L, $MnCl_2 \cdot 4H_2O$ 1.81 g/L, $ZnSO_4 \cdot 7H_2O$ 0.222 g/L, $NaMoO_4 \cdot 2H_2O$ 0.39 g/L, $CuSO_4 \cdot 5H_2O$ 0.079 g/L, and $CoCl_2 \cdot 6H_2O$ 0.05 g/L (Rippka et al., 1979; Hu et al., 2012). Glycine (5 g/L) was added to phototrophic culture broth as a nitrogen source; 20 g/L glucose was used as an organic carbon source for heterotrophic growth with glycine concentration up to 0.1 g/L (Xiong et al., 2010).This artificial medium is better suitable for the cultivation of *Chlorella* sp. However, the limitation of this fast algal medium is that the nutrients in it were also consumed faster than other media, thus each growth period would be shorten.

When it comes to the economic value of algal media for algal byproduct industry, the cost of ingredients in media should be as low as possible. In this case, adding glucose or some other additives to these artificial media makes them too expensive for large-scale heterotrophic cultivation. Furthermore, if algae are to be processed for animal or human consumption, the media must have no threat to the safety of the consumers. According to these requirements, a low-cost alternative of algae media is needed all over the world.

1.2.2 Oil crop biomass residue

1.2.2.1 Utilization Oil crops biomass residue

Several kinds of vegetable crops are widely used to produce biodiesel or edible oil, due to the high level of lipid content in their biomass, of which soybean and sunflower are two important types. The values for these high-oil vegetable crops mainly refer to the oil extracted from them (Vicente et al., 2009). The major oils used in

nowadays are shown in Table 1.6 (Gursche, 2008; Dean et al., 2011; Boland, 2011; Hansen, 2011; Bennett, 2003; Boland & Stroade, 2011).

Table 1.6 Major oils for worldwide fuel oil and edible oil production

Crop Oil Name	cooking oil use	fuel oil use	Comments
Coconut oil	×		medical applications
Corn oil	×		salad
Cottonseed oil	×		salad
Olive oil	×	×	soaps
Palm oil	×	×	Popular in West African and Brazilian cuisine
Peanut oil	×		Salad dressing
Rapeseed oil	×		
Safflower oil	×		Previously used in the paint industry
Sesame oil	×		
Soybean oil	×		
Sunflower oil	×	×	
Mustard oil	×		

However, after oil extraction, the residues of these oil crops are usually treated as waste. In fact, there are still a large amount of valuable compounds in the residues which could be recycled for use. Soybean and sunflower biomass are the residues after soybean or sunflower seeds being squeezed. The protein in soybean accounts for 35-40 % of total dry weight, which is one of important protein resources from plants. Currently, the soybean biomass is mainly used as livestock feed due to their high protein content. The polysaccharides in it was also commonly extracted for food use in industry,

Recent studies proved that they could become low-cost materials for continuing nutritional utilization. Soybean and sunflower residues from oil extraction operation can be hydrolyzed to produce media containing numerous amino acids and peptides which can serve as the nitrogen source to microorganism (Pan et al., 2007; Li et al., 2006). For instance, rapeseed meal hydrolysates were used as an alternative to beef extract, providing nitrogen for fermentation, in order to reduce the cost associated with L-lactic acid production (Pan et al., 2007). In a batch L-lactic acid fermentation study where yeast extract was substituted with corn steep liquor (CSL)-supplemented acid-hydrolysate of soybean meal (AHSM), the L-lactic acid yield was improved and the production cost was reduced by 75 % (Li et al., 2006). Thus, hydrolysates of crop residues provide a good nutritional source to various microorganisms.

Microalgae belong to microorganisms and they have a similar growth pattern as bacteria and fungi. Thereby nutrients derived from crop residues are also potential to be low-cost and safe media for algae culture. As mentioned above, even though a few reports on the use of crop residue-based media for bacteria culture were found in previous scientific literature, little has been done on algae.

1.2.2.2 Common hydrolysis methods

Common hydrolysis methods for oil crop residues include acid hydrolysis and enzymatic hydrolysis (Lee et al., 2001). Compared to enzymatic hydrolysis, acid hydrolysis is cheaper and easier to handle and therefore is commonly used for oil crop residues hydrolysis.

Acid hydrolysis of oil crop residues has been investigated for decades. The common acid includes sulfuric acid and hydrochloric acid. Acid hydrolysis treatment was found to break down the composition of soybean and help reflux extractor (80% EtOH) to extract isoflavone in soybean meal (Lee and Choung, 2011). Moreover, Lee et al. (2001) reported that hydrolysates produced from defatted soybean flour (DSF) through mild-acid treatment and enzymatic hydrolysis contained a lot of free amino acids, dipeptides and tripeptides which are valuable for continuing utilization. Acid concentration (HC), hydrolysis time (HT), and reaction temperature (RT) were identified as the three key process variables of acid hydrolysis, which should be optimized for yield and targeted products. For example, in the study on soybean isoflavons extraction which is done by Chiang et al. (2011), the optimal acid hydrolysis conditions were obtained by evaluation of these three key factors including HCl concentration (HC=0-6.0 N), hydrolysis time (HT=0-350 min) and reaction temperature (RT=5-95 ℃). Therefore, the specific optimization of acid hydrolysis on oil crops residues is one of the objectives in this thesis, for the sake of achieving top nutrient supply to algal growth.

As mentioned above, in the algae production, algal growth and lipid content are affected by the medium composition, especially by the carbon source, nitrogen content and C/N (ratio of carbon source/nitrogen source) in the medium (Cheng et al., 2009). The nitrogen deficiency and corresponding carbon sufficiency could enhance lipid accumulation in algae (Converti et al, 2009; Ruangsomboon, 2012). Therefore in this thesis, algal lipid yield as a function of carbon and nitrogen level and C/N in the oil crop residue hydrolysate media were also investigated.

1.3 Objectives and prospective

The objectives of the present study are:

a) To optimize the acid hydrolysis conditions of soybean and sunflower biomass residues for top nutrient supply;

b) To evaluate the feasibility of using acid hydrolysis pretreated soybean and sunflower biomass residues as cultural media for microalgae growth;

c) To clarify the growth pattern of microalgae growing in hydrolyzed OCBR media

d) To prove that enhanced algal lipid production could be obtained and EPA could be accumulated by using hydrolyzed OCBR as media;

e) To prove that low temperature has positive effect on algal EPA production;

f) To illustrate the effect of algal growth stage on algal EPA production during the whole cultivation process.

The prospective of this thesis is to develop new low-cost alternative for edible algae production with high level of byproduct accumulation especially in biofuel and food industry, and to seek the best environmental temperature and harvest time for such edible algae, while at the same time to maintain/improve its ability to produce EPA for promoting human health.

Chapter 2 Optimization of acid hydrolysates of Oil crops biomass residue (OCBR)

2.1 Introduction

Oil crops biomass residue should be a good nutrient supply to microorganisms. In this chapter, the objective is to evaluate the potential of oil crops biomass residue to serve as nutrient (i.e., nitrogen, carbon, phosphate, etc.) supply to microorganisms.

As mentioned before, among the hydrolysis treatments of oil crops residues, acid hydrolysis has lower cost than enzymatic hydrolysis, due to that enzymatic hydrolysis usually need specific enzyme with high price in general during its process. As many articles reported, under the specific rate of oil crop residue to acid, there are three main factors that could affect the acid hydrolysis pretreatments of oil crops residues, that are, acid concentration, hydrolysis time, hydrolysis temperature (Li et al., 2006). Therefore, in this chapter, these three factors will be studied when the rate of oil crop residue to acid is 1:4 (v/v) (got from preliminary research), in order to draw out the optimal conditions for maximal nutrient output of oil crop residues.

2.2 Materials and methods

Four Oil crop biomass residues (OCBR) provided by South Dakota State University, Brookings, SD (wastes after oil extraction under different processing conditions) were used in this study. The four OCBR include NANTC/low sugar/soybean (sample 1), low tripsin Inhibitor/non-heated/cold-pressed/soybean (sample 2), low tripsin Inhibitor/heated/cold-pressed/soybean (sample 3) and cold-pressed/sunflower (sample 4).

These four OCBR contain lipid content of 22%, 20%, 26% and 23% in composition after oil extraction preprocessing (indicated in the preliminary experiment).

In order to determine the optimal acid hydrolysis pretreatment conditions, the one-factor-at-a-time method was used to design the experiments to evaluate the effects of three key factors, namely acid concentration, hydrolysis time and hydrolysis temperature (Chiang et al., 2001), on hydrolysis for the purpose of optimizing the processing conditions (Kumon et al., 2005); Choudhari and Singhal, 2008) (Table 2.1). The experiments were conducted in the sequence (A through C) indicated in Table 2.1. Each sequence was designed to study one variable. Once an optimal value was determined for this variable, the value would be used in the next sequence.

Table 2.1 Fixed and Different Variable acid hydrolysis conditions for optimization

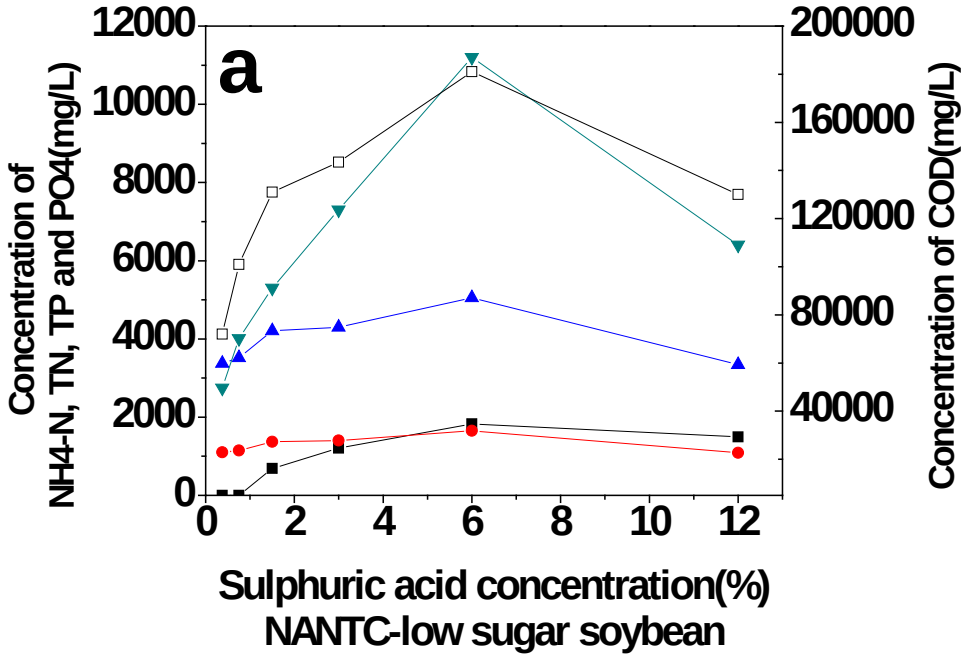
Sequence	Fixed Acid Hydrolysis Conditions			*Variable Range						
	Acid Conc. (%)	Time (h)	Temperature (℃)							
A	a*	20	90	a	0.0375	0.75	1.50	3	6	12
B	3	b*	90	b	1	2	5	10	20	30
C	3	20	c*	c	30	50	70	80	90	10^2

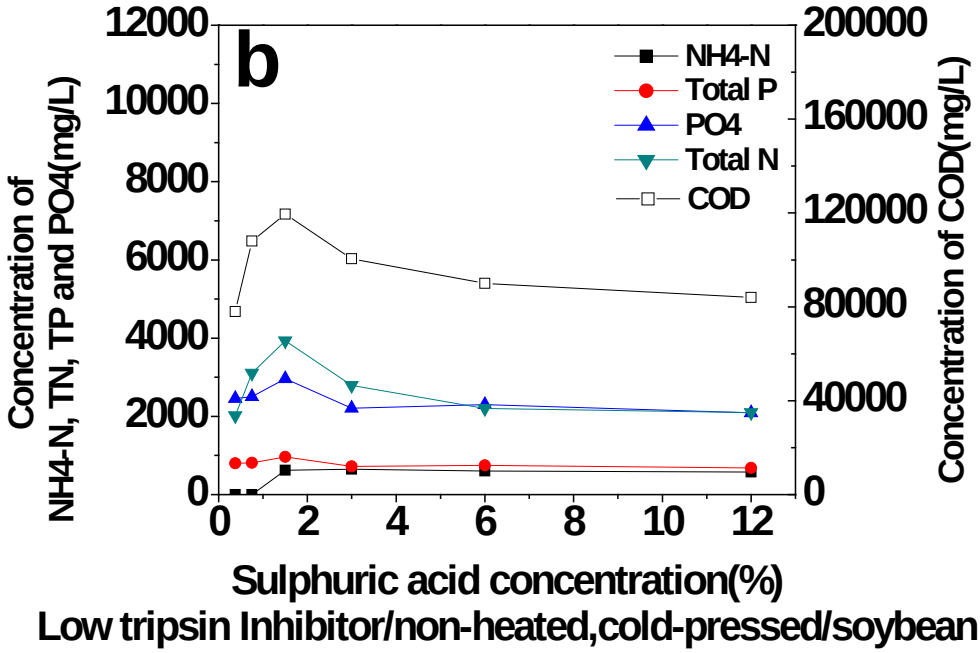
The hydrolysis temperature was controlled by incubating the mixtures of crop residue and sulfuric acid contained in sealed glass tubes submerged in a water bath set to a designated temperatures. After hydrolysis with 4:1 (v/w) ratio of sulfuric acid to residue (determined in the preliminary experiment), the mixtures were cooled for 1.5 h, and centrifuged for 15 min before filtration. The soluble ammonium (NH_4-N), total nitrogen (TN), total phosphorus (TP) and chemical oxygen demand (COD) in supernatant were measured immediately after filtration.

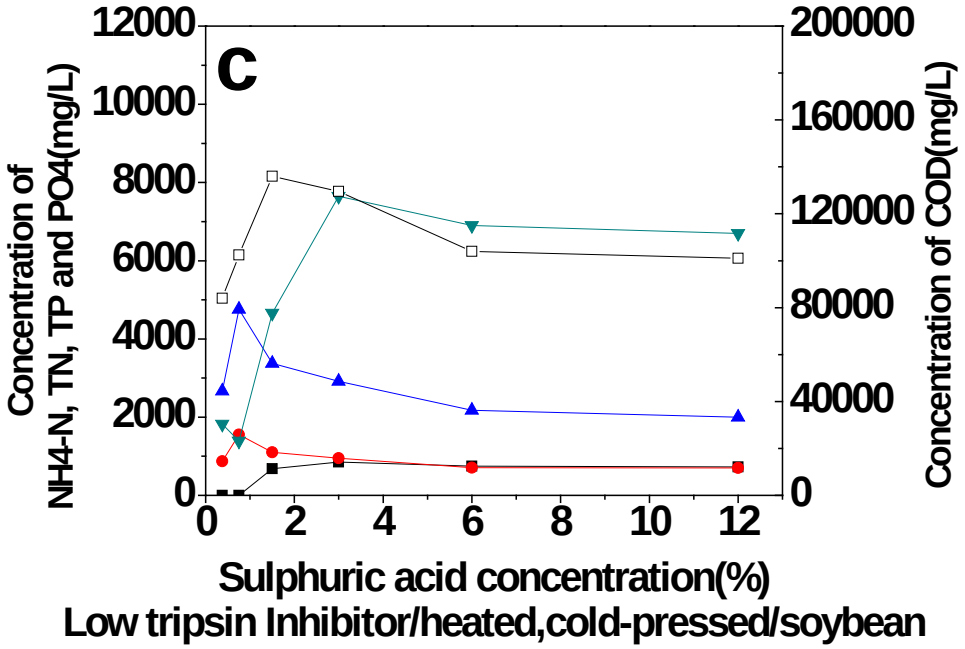
During the nutrient analysis of acid hydrolysates of four OCBRs, the supernatant obtained from the centrifuge of hydrolysis mixture was subsequently filtered through a 0.45-μm filter. The filtrate was then centrifuged at 5000 rpm for 15 min and supernatant was collected and properly diluted for analysis of NH_4-N, COD, TN and TP by following the Hach DR 5000 Spectrophotometer Manual (Hach Procedure Manual, 2008).

2.3 Results and discussion

The effect of the sulfuric acid (SA) concentration on hydrolysates nutrient profile at the range of 0.0375 % - 12 % is shown in Figure 2.1. In general, too low concentration for sulfuric acid caused much less nutritional profiles for tested samples, and too high concentration for sulfuric acid did not help residues release much additional nutrients. Figure 2.1 a and Figure 2.1 d show that biomass residues 1 and 4 had the highest nutrient concentrations at 6 % SA, and Figure 2.1 band Figure 2.1 c show that biomass residue 2 and 3 had the highest nutrient concentrations at 1.5 % SA, indicating that a reasonable nutrient profile level could be obtained at 1.5 % - 6 % SA. Thus the medium SA concentration of 3 % was chosen among the optimal concentration ranged from 1.5 % to 6 % for studying the effect of time and temperature for all four test samples as described below.


a
Concentration of NH4-N, TN, TP and PO4(mg/L)
Concentration of COD(mg/L)
0
2000
4000
6000
8000
10000
12000
40000
80000
120000
160000
200000
0
2
4
6
8
10
12
Sulphuric acid concentration(%)
NANTC-low sugar soybean


b
NH4-N
Total P
PO4
Total N
COD
Concentration of NH4-N, TN, TP and PO4(mg/L)
Concentration of COD(mg/L)
0
2000
4000
6000
8000
10000
12000
0
40000
80000
120000
160000
200000
0
2
4
6
8
10
12
Sulphuric acid concentration(%)
Low tripsin Inhibitor/non-heated,cold-pressed/soybean


c
Concentration of NH4-N, TN, TP and PO4(mg/L)
Concentration of COD(mg/L)
0
2000
4000
6000
8000
10000
12000
40000
80000
120000
160000
200000
0
2
4
6
8
10
12
Sulphuric acid concentration(%)
Low tripsin Inhibitor/heated,cold-pressed/soybean

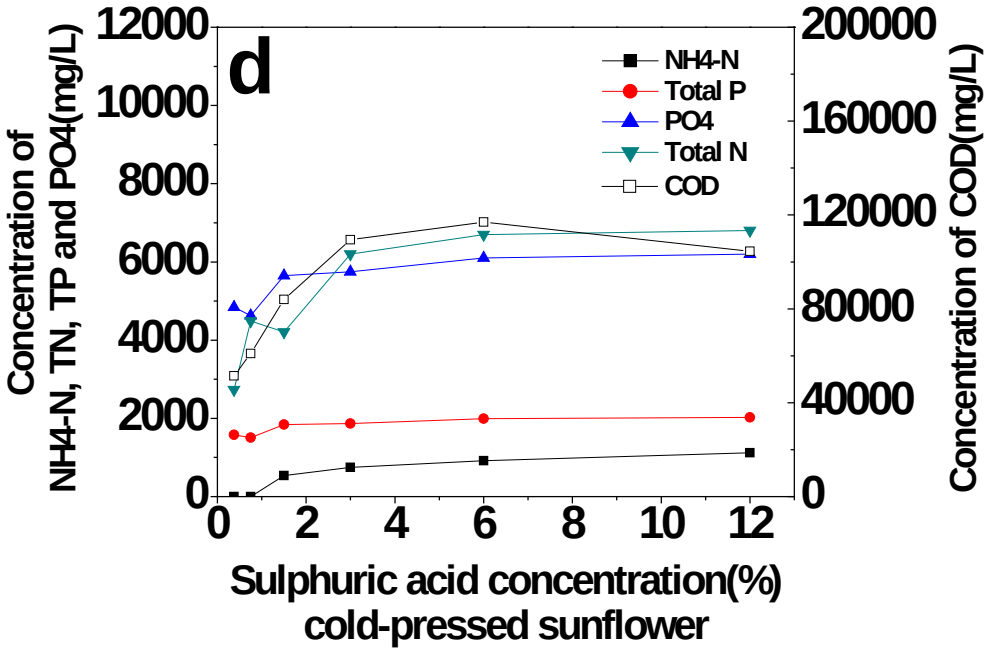
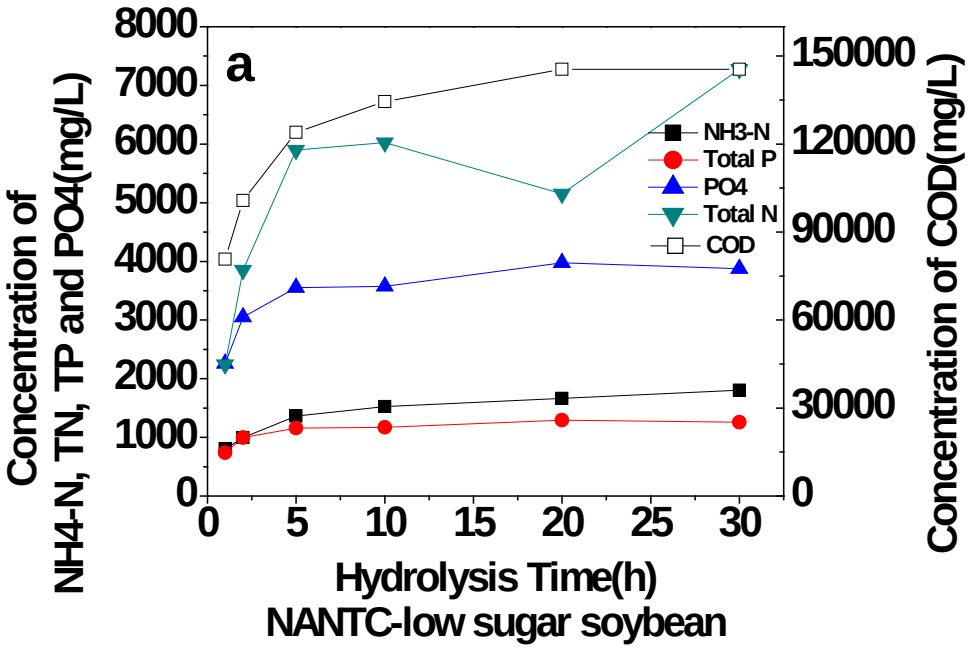
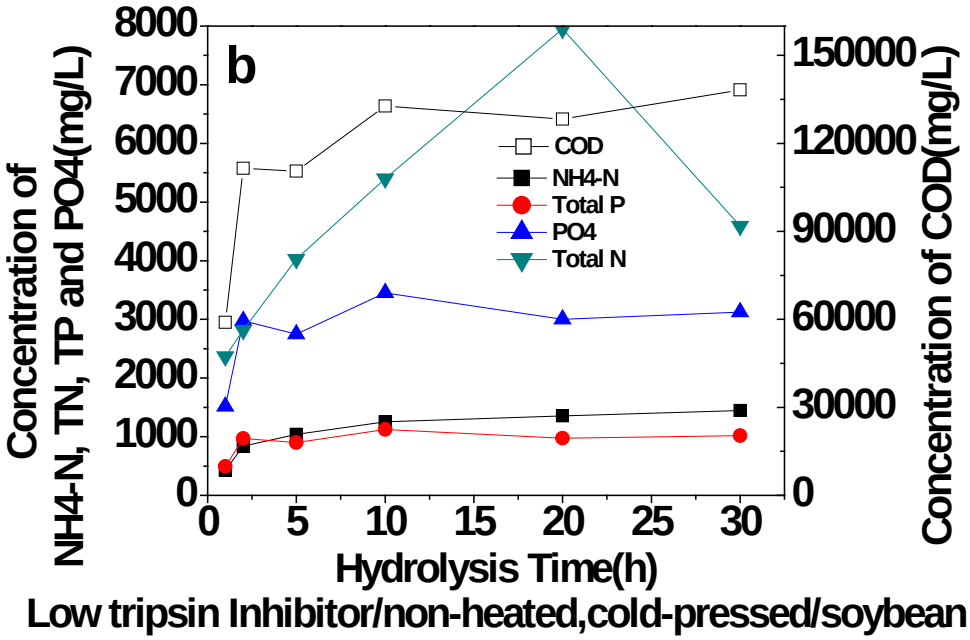

d
NH4-N
Total P
PO4
Total N
COD
Concentration of NH4-N, TN, TP and PO4(mg/L)
Concentration of COD(mg/L)
0
2000
4000
6000
8000
10000
12000
40000
80000
120000
160000
200000
0
2
4
6
8
10
12
Sulphuric acid concentration(%)
cold-pressed sunflower

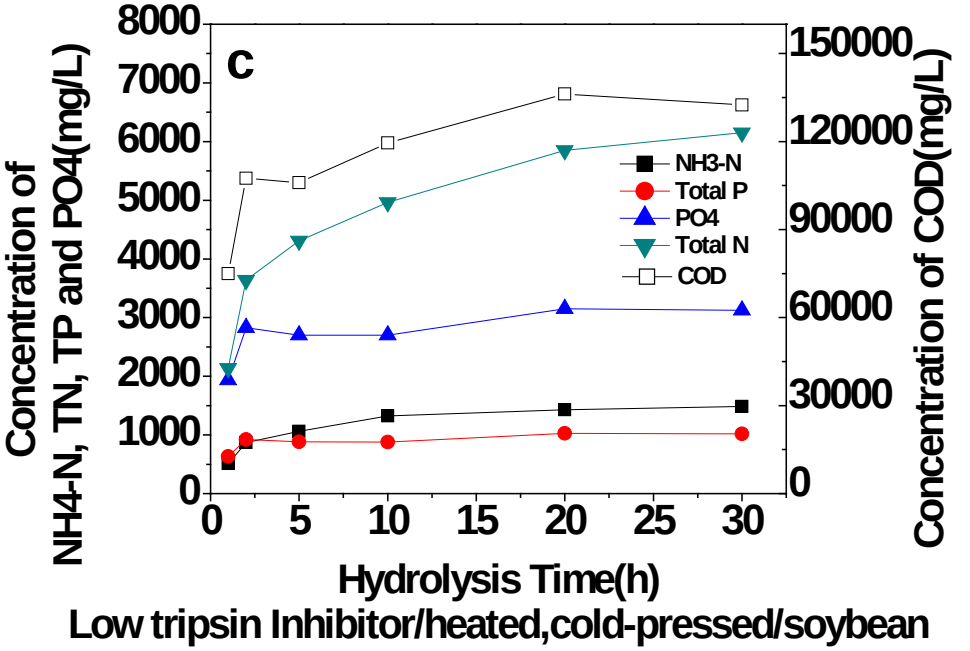
Figure 2.1 The nutrient profiles - total nitrogen (TN), total Phosphorus (TP), chemical oxygen demand (COD) and Ammonia nitrogen (NH_4-N) in 4 kinds of residues (a, b, c and d) under different sulfuric acid concentrations

The effect of the hydrolysis time on hydrolysates nutrient profile at the range of 1 h - 30 hrs is shown in Figure 2.2. Noticeable increases were observed before 10 hrs; thus it was reasonable to conclude that the nutritional compounds cannot be released in a short time span. It should be noting that during 10 hrs to 30 hrs, the concentration of nitrogen, phosphorous and COD changed within a slight range, and no appreciable increase was observed after 20 hrs. Therefore, 20 hrs was chosen among the optimal time range of 10 hrs - 30 hrs for experiments in the next section.





Low tripsin Inhibitor/non-heated,cold-pressed/soybean



Low tripsin Inhibitor/heated,cold-pressed/soybean

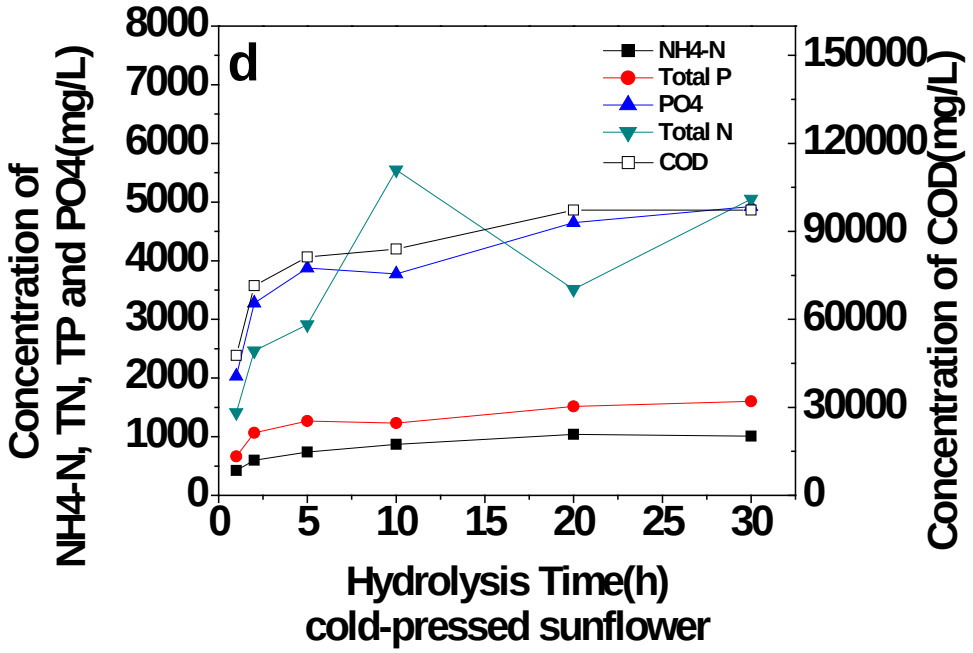
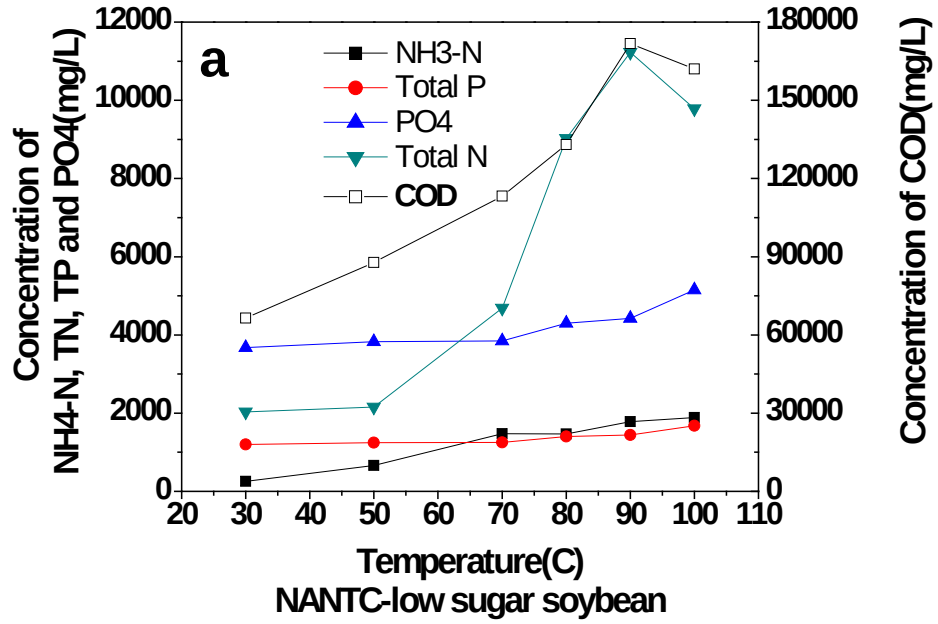
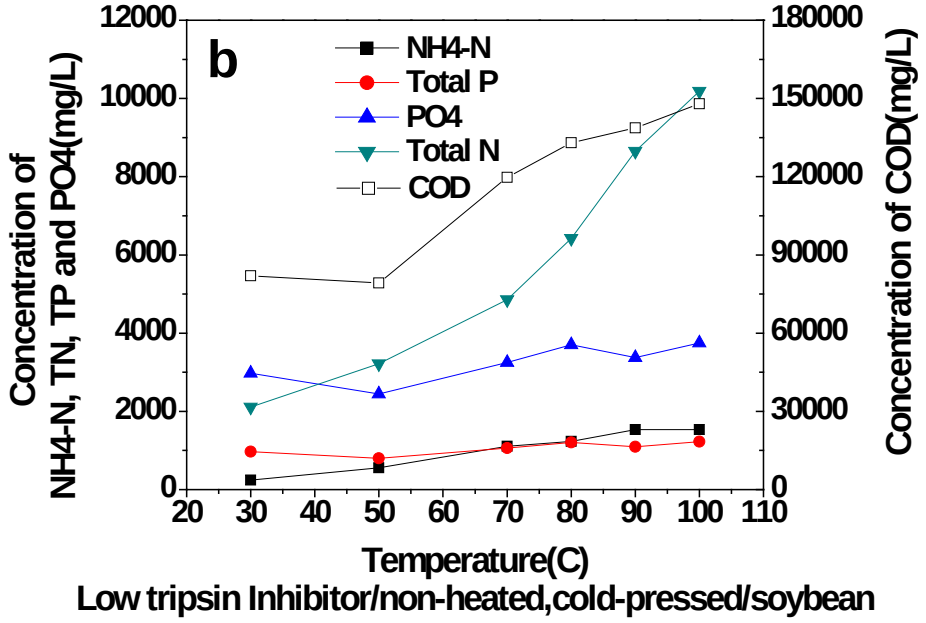
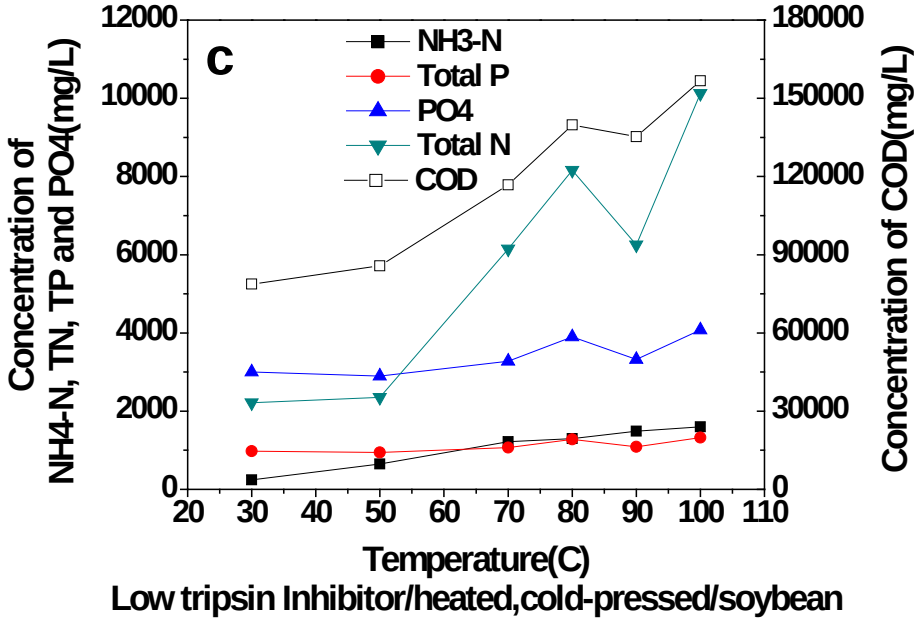


Figure 2.2 The nutrient profiles (TN, TP, COD and NH_4-N) in 4 kinds of residues (a, b, c and d) under different acid hydrolysis time

The effect of the hydrolysis temperature on hydrolysates nutrient profile at the range of 30 °C - 100 °C is shown in Figure 2.3. The nutrient levels increased with temperature increase. COD and TN increased faster than other nutrients. Between 30 °C and 100 °C, the nutritional compounds level was proportional to the temperature increase. 100 °C was not considered as the optimal temperature for hydrolysis in this study since at which water was evaporated. It was observed that the results of nutrient profiles were almost equally high when temperature was controlled at 80 °C and 90 °C, respectively.


a
NH3-N
Total P
PO4
Total N
COD
Concentration of NH4-N, TN, TP and PO4(mg/L)
Concentration of COD(mg/L)
Temperature(C)
NANTC-low sugar soybean


b
NH4-N
Total P
PO4
Total N
COD
Concentration of NH4-N, TN, TP and PO4(mg/L)
Concentration of COD(mg/L)
Temperature(C)
Low tripsin Inhibitor/non-heated,cold-pressed/soybean


c
NH3-N
Total P
PO4
Total N
COD
Concentration of NH4-N, TN, TP and PO4(mg/L)
Concentration of COD(mg/L)
Temperature(C)
Low tripsin Inhibitor/heated,cold-pressed/soybean

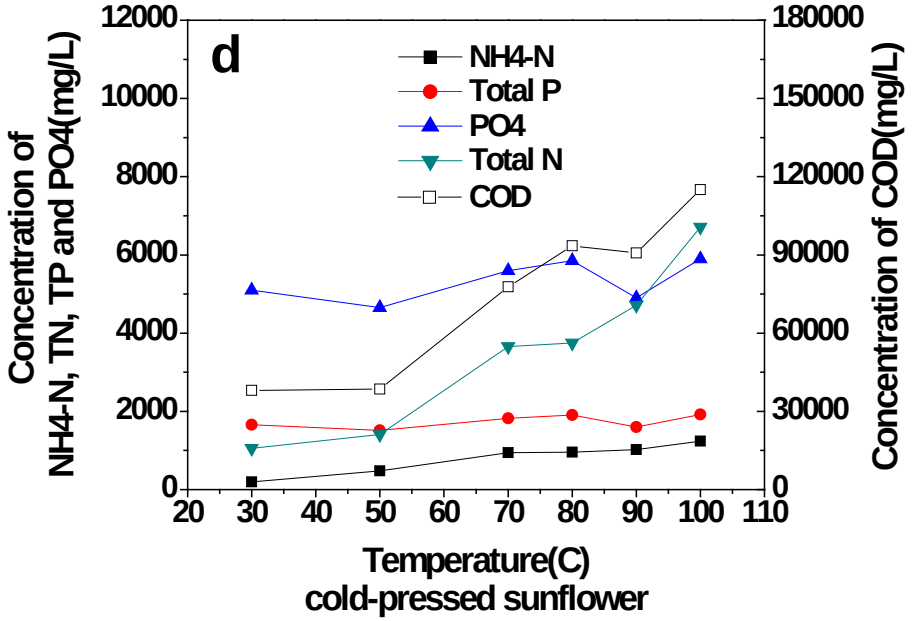

d
NH4-N
Total P
PO4
Total N
COD
Concentration of NH4-N, TN, TP and PO4(mg/L)
Concentration of COD(mg/L)
Temperature(C)
cold-pressed sunflower

Figure 2.3 The nutrient profiles (TN, TP, COD and NH_4-N) in 4 kinds of residues (a, b, c and d) under different acid hydrolysis temperatures

The data points at 90 °C in Figure 2.3 represent the optimal hydrolysis conditions (i.e., sulfuric acid concentration at 3 %, hydrolysis time at 20 hrs, hydrolysis temperature at 90 °C), which was consistent with results reported by Li et al. (Li Z et al, 2006). Under above optimal hydrolysis conditions, the NANTC/low sugar/soybean derived hydrolysates, denoted as OCBR medium 1 had better nutrient profiles than other three media. The low tripsin inhibitor/ heated/cold-pressed/soybean derived hydrolysates denoted as OCBR medium 3, and the low tripsin inhibitor/non-heated/cold-pressed/soybean derived hydrolysates denoted as OCBR medium 2, were ranked second and third. The nutrient profile of the cold-pressed/sunflower derived hydrolysates, denoted as OCBR medium 4 was the lowest among four test samples, performed worse than soybean biomass medium (medium 1-3). OCBR media 1-3 have higher COD and TN, while medium 4 has higher TP, which may be related to the difference between compositions of biomass residues. Figure 2.3 b and Figure 2.3 c showed the heating preprocessing of crop residues had no salient influence on subsequent nutrient profiles. OCBR media 1 and 3 share similar nutrient profiles (TN, TP, COD and NH4-N were around 8,000 mg/L, 1,000 mg/L, 150,000 mg/L and 1,500 mg/L, respectively). Moreover, C/N of OCBR media was 18.75, which indicated OCBR media had potential to grow microalgae with high lipid content (Cheng Y et al, 2009). Therefore, OCBR media 1 and 3 were considered as the best media candidates to stimulate high lipid accumulation for microalgae due to their nutrient profiles.

2.4 Conclusions

OCBR media 1 and 3 were considered as the best media candidates to stimulate high lipid accumulation for microalgae due to their highest nutrient levels under the optimal acid hydrolysis pretreatment conditions, i.e., sulfuric acid concentration at 3 %, hydrolysis time at 20 hrs, hydrolysis temperature at 90 °C.

Chapter 3 Algae Cultivation on OCBR media

3.1 Introduction

As indicate in Chapter 2, oil crop biomass residue (OCBR) could be a good source of nitrogen, carbon and phosphate to microorganism culture after acid hydrolysis pretreatment. After hydrolyzing OCBR with 3 % sulfuric acid at 90 °C for 20 hrs, the nutrients was best released from OCBR for utilization.

Previous researches have proved that these nutrients released from OCBR after acid pretreatment could be used as media for various microorganisms. However, there is little research on its potential as media for algae culture. In this part of the study, the first objective was to prove OCBR acid hydrolysates could serve as the media for algae culture; then, which fold of dilution of OCBR hydrolysates was better for algal biomass accumulation. The second objective was to detect whether the culture of algae in OCBR media was heterotrophic culture or not.

3.2 Materials and methods

The microalgal strains used in this study included green microalgal strains UM258 (*Scenedesmus* sp.) and UM268 (*Chlorella* sp.), which were isolated from the wastewaters in a Minnesota municipal wastewater treatment plant. Both of them have been proved that they could grow in phototrophic and heterotrophic ways. The detailed information about the two strains was described previously (Zhou et al, 2011). All of microalgae strains were axenically used in this study.

The artificial media described in Xiong et al. (2010), was used as the baseline culture media as well as the positive control in algal growth comparison in this study. Glycine (5 g/L) was added to phototrophic culture broth as a nitrogen source.

The media used in this part of the study were obtained from different dilutions of hydrolysates of OCBRs 1, 2, 3, and 4 (described in Chapter 2), which were named OCBR 1, OCBR 2, OCBR 3 and OCBR 4, respectively. The hydrolysates produced under the optimal acid hydrolysis conditions (sulfuric acid concentration, hydrolysis time and temperature) determined through above described experiments were used to culture algae.

To study the effect of dilution ratio of hydrolysates on algae growth, algae strain UM268 was cultured on 2-fold and 10-fold dilution of hydrolysates which were obtained by adding appropriate amount of double distilled water to hydrolysate stock solution. The choice of dilution ratio was based on the difference between the nutrient profiles of OCBR media and previous culture media (Zhou et al., 2011). Additional experiments were conducted to compare growth performance of UM258 and UM268 on 10-fold dilution media. Double distilled water and the artificial medium were used as a blank control and a positive control, respectively. The artificial medium was used to maintain UM258 and UM268 seeds as described previously (Zhou et al, 2011). The seed was cultivated in the artificial medium for 7 days under daylight lamp in 250 ml flask, and then inoculated into 100 ml OCBR media. The culture temperature was maintained at 25 °C with continuous shaking at 150 rpm in an orbital shaker (Zhou et al, 2012a). Samples

without algal cells were used as negative controls, for which the same treatments were taken.

Algal growth is expressed as “number of cells” and “total volatile suspended solids (TVSS). Algal number was monitored daily by cell counting through microscope. The TVSS was determined using a standard method (APHA et al, 1995). All of the experiments were conducted in triplicates. Usually the growth rate of the cells is proportional to the biomass of cells during the exponential growth phase. Therefore, the growth rate is calculated using the following equation:

$R = Ln (TB_t) – Ln (TB_0)/t$

where R is growth rate based on TVSS. TB_t and TB_0 are the TVSS at day t and day 0. t is the time interval (days) between TB_t and TB_0 (Zhou et al, 2012b).

After proving that these two algae strains could grow well on OCBR media, further study was carried out to determine if these algae strains experienced phototrophic or heterotrophic growth. UM258 was chosen as an example of algae, and OCBR 1 was used in this experiment. Since the Total Organic Carbon (TOC) concentration of medium was very high, OCBR 1 was diluted to 50-fold before experiment, the TOC of four OCBR media was monitored in before and during the cultivation. BG 11 medium was used as control. The TOC measurement was performed in duplicate each two days. The TVSS measurement was performed in triplicate each day during the whole process of six days.

Similar to previous nutrient analysis, during the TOC analysis of acid hydrolysates of four OCBRs, the supernatant obtained from the centrifuge of hydrolysis

mixture was subsequently filtered through a 0.45-μm filter. The soluble TOC in supernatant were measured immediately after filtration. The filtrate was then centrifuged at 5000 rpm for 15 min and supernatant was collected and properly diluted for analysis of TOC by following the Hach DR 5000 Spectrophotometer Manual (Hach Procedure Manual, 2008).

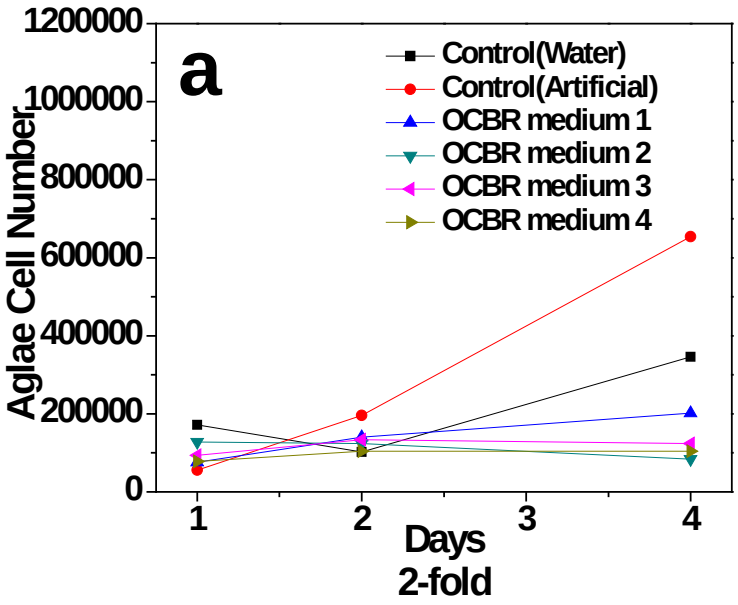
3.3 Results and discussion

Effect of dilution on UM268 growth

UM268 grew better in 10-fold diluted solutions than in the artificial medium and 2-fold diluted solution (Figure 3.1). In fact, UM268 showed no apparent growth in 2-fold diluted media after 4 days cultivation, which was similar to algae grown on water and much lower than on the artificial medium (Figure 3.1 a). The main reason may be that very concentrated OCBR media restrained the algae growth. Figure 3.1 b shows that after 4 days, the algal growth was in the decreasing order of 3>2>artificial≥1>4>water, which had positive correlation with nutrient profiles of OCBR media. UM258 also had good growth on 10-fold diluted solution. The growth of UM258 on 10-fold diluted OCBR solution was shown in Figure 3.2 and 3.3, and would be discussed later in this chapter. Therefore, 10-fold dilution of OCBR media was more suitable than 2-fold dilution for algal growth, and was used in subsequent experiments.

The results indicate that algae exhibited reasonable growth on all four OCBRs. Since the trend of algal growth in OCBR media 1 and 3 always had equal or better performance when compared with the artificial medium. OCBR medium 1 was chosen as

the example to compare growth performance of UM258 and UM268 on 10-fold dilution media.



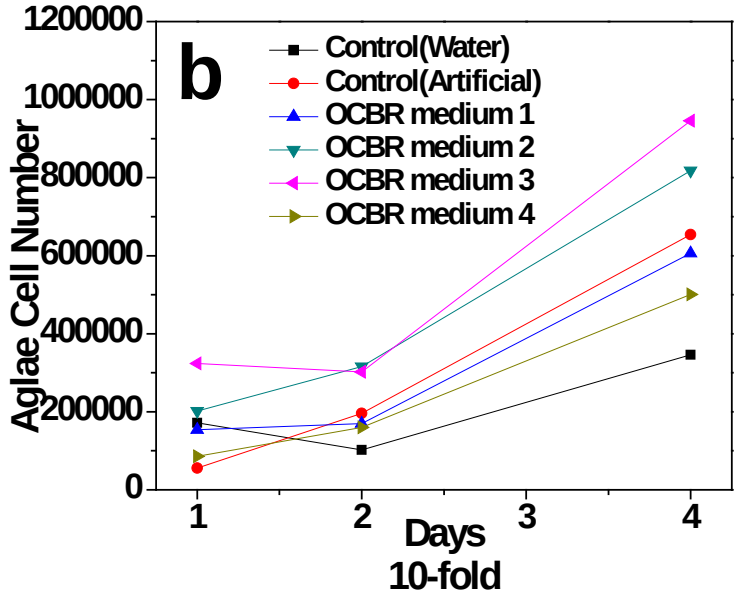


Figure 3.1 Growth of algae UM268 within 4 days in water, artificial medium and 4 kinds of OCBR media a) 2-fold dilution of residue hydrolysates; b) 10-fold dilution of biomass hydrolysates.

Comparison between UM258 and UM268 grown on 10-fold diluted OCBR 1

The growth profile of UM258 and UM268 on 10-fold OCBR medium 1, water and artificial medium within 6 days are shown in Figure 3.2. Both algae UM258 and UM268 showed fast growth. UM258 grew faster on OCBR medium 1 than on the artificial medium and water (Figure 3.2 a); UM268 showed similar growth performance when compared with that grown on the artificial medium, and much better than that grown on water (Figure 3.2 b). UM258 grew much faster than UM268. The difference in growth performance between two strains may be due to the strains specific and different uptake efficiency of OCBR media nutrients for two strains.

This is a preliminary research in present thesis. The process of cultivation were not completed in Figure 3.1 and Figure 3.2, and several algae cells may be still in logarithmic growth phase, but the experiment was ended within 4 or 6 days, because this series of experiments was aimed to indicate the effect of different hydrolysate dilutions on algae UM268 growth under optimal acid hydrolysis pretreatment conditions. According to Figure 3.1 and Figure 3.2, the growth comparisons in high and low dilutions for two algae strains were already achieved, and we would also provide additional complete growth profiles of two strains with three triplicate experiments in the following experiments. Therefore, it is not necessary to show the whole process of cultivation in Figure 3.1 and Figure 3.2 in this part for dilution comparison.

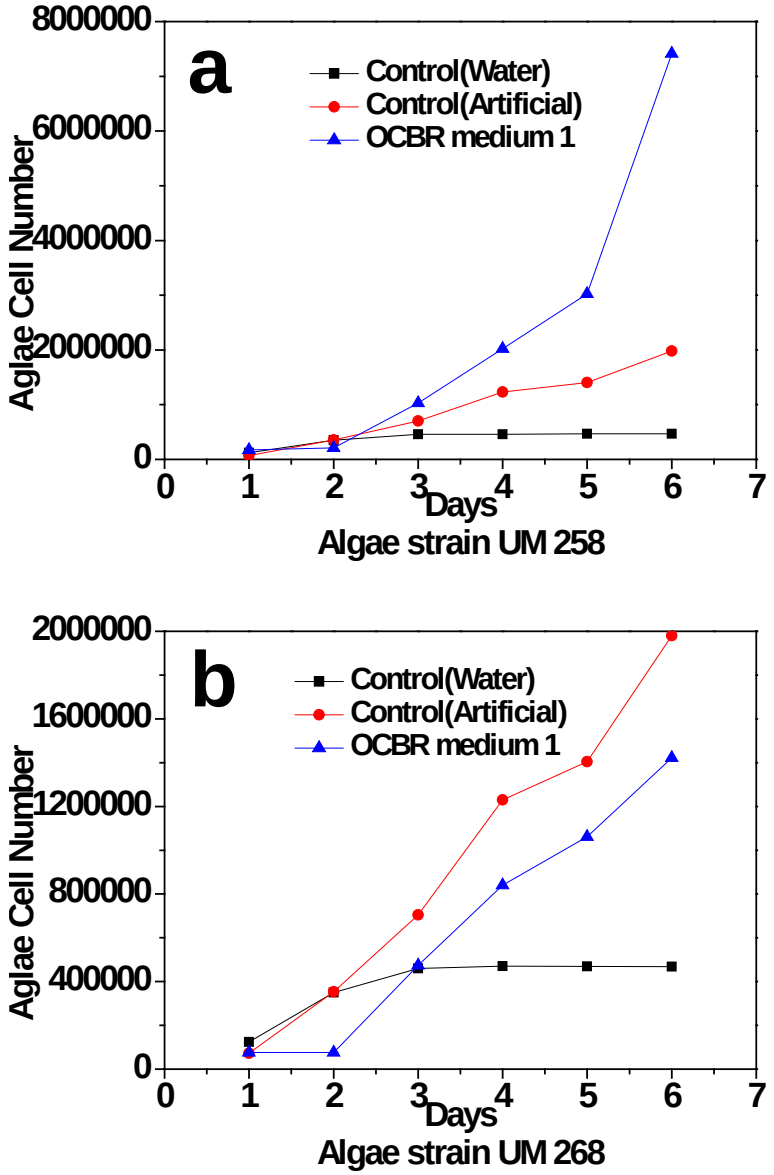
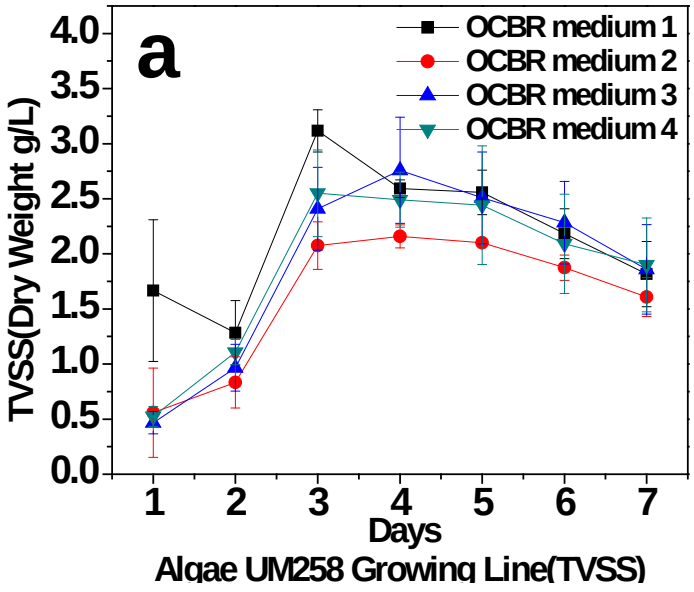


Figure 3.2 Algae growth within 6 days in water, artificial medium and 10-fold OCBR medium 1 a) Algae strain UM258; b) Algae strain UM268

The complete growing profiles (with whole 7-day cultivation) of UM258 and UM268 obtained through triplicate experiments for further algal composition analysis are shown in Figure 3.3. The concentration of UM258 reached maximum, which was 2.7 g/L

of dry weight on day 5, then decreased subsequently. Besides, UM268 grew slower than UM258, but reached much high accumulation of 3 g/L after 6 days cultivation, then decreased subsequently. It is also proven again that OCBR media 1 and OCBR media 3 performed better than the other two in terms of algae growth.



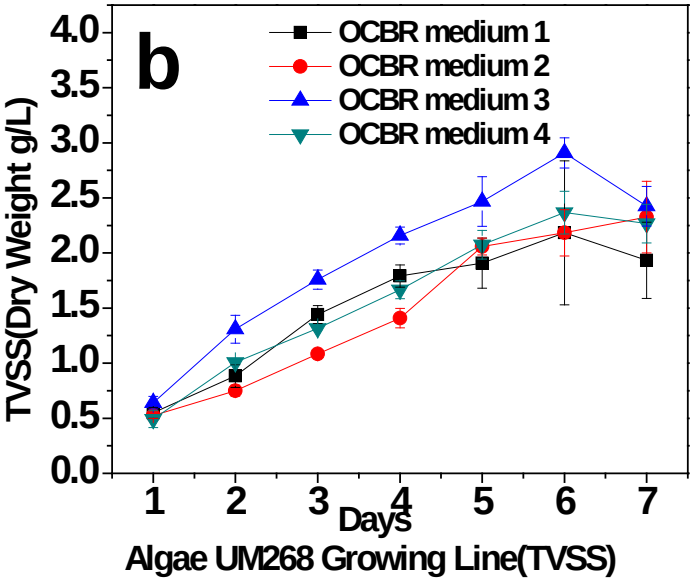


Figure 3.3 TVSS of algae UM258 and UM268 grown on OCBR media in one week (triplicate) a) UM258; b) UM268

It was shown that the cell number of UM258 and UM268 were low in Figure 3.1 and Figure 3.2, but the dry weight (TVSS) of algae UM258 and UM268 grew on OCBR media reached high values in Figure 3.3. The main reason is, in Figure 3.1 and Figure 3.2, we used low inoculum concentration, in order to find the algal growth more easily and show it obviously. While in Figure 3.3, we repeated a series of algal cultivation with much higher inoculum concentration of 0.5 g/L at the very beginning, because only in this case, the dry algae sample could be enough for the following lipid analysis. However, no matter how much inoculum concentration at the beginning, we keep the rate of initial algae inoculum to media solution at the same level (1:9) in Figure 3.1, Figure 3.2 and Figure 3.3; therefore, the growing trend should be similar in these figures.

The high growth yields may be because OCBR media provided algae with a mixtrophic growth mode that had a high carbon/COD supplement (15,000 mg/L), which yield higher accumulation rate than phototrophic growth (Zheng et al., 2012). All of OCBR media showed good performance on the culture of UM258 and UM268. Besides, algae grown on OCBR medium 3 could reach the maximal biomass accumulation compared with other three OCBR media. According to literature review (Vazhappilly et al.; Bajpai et al., 1992b), the lipid reached to high level during algae fast growth phase. Therefore, the algae samples on day 3 and day 5 were chosen for algal lipid analysis and other related analysis in next Chapter 4.

Trophic Conversion Mode

Figure 3.4 and Figure 3.5 show that the TOC in the OCBR medium deceased dramatically during the first 3 days of cultivation (From 1045 mg/L to 302 mg/L) and the algae concentration increased accordingly. Therefore, it is reasonable to conclude that the cultures of UM258 in this study should be heterotrophic rather than phototrophic. Besides, as mentioned in "Materials and methods" section in this manuscript, UM258 is suitable for phototrophic and heterotrophic cultivation according to the referenced literature (Zhou et al., 2011).

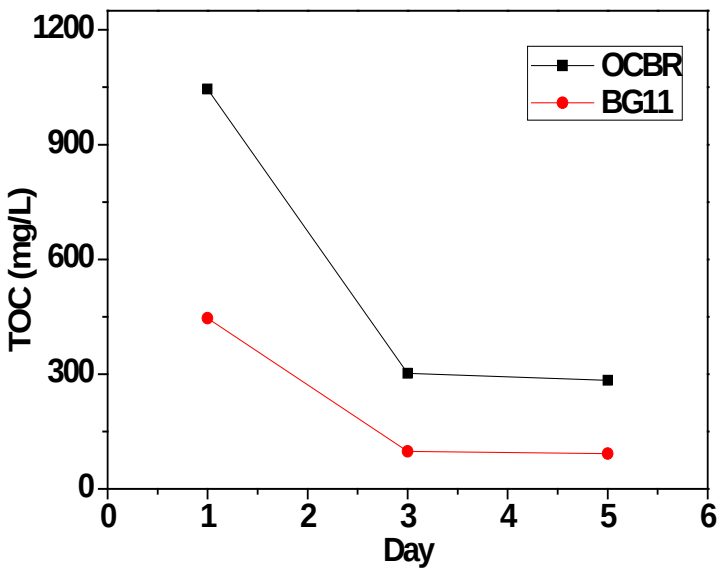


Figure 3.4 TOC concentration of culture of UM258 grown on OCBR media (use OCBR media 1 for example) and BG 11 media during five days

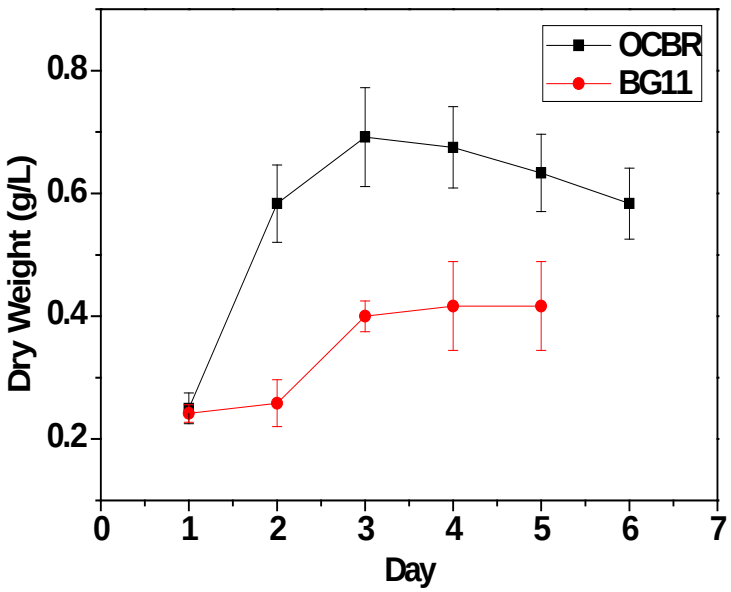


Figure 3.5 UM258 biomass concentration grown on OCBR media (use OCBR media 1 for example) and BG 11 media during five days

3.4 Conclusions

The dilutions of acid hydrolysates of oil crop biomass residue could serve as a good algae culture media. Due to high concentration of nutrients in OCBR media, dilution is necessary. It was found that 10-fold dilution was better than 2-fold dilution in terms of algae growth. Moreover, OCBR media 1 and OCBR media 3 performed better than the other two, which agrees with the level of nutrient (i.e. nitrogen, phosphate and carbon) in them. With the 10-fold dilution of OCBR media 1 and 3, the algae biomass accumulation could arrive 2.7 g/L and 3 g/L for UM258 and UM268, respectively. In addition, the level of organic carbon is high in OCBR media, and the algae could utilize the organic carbon very well in OCBR media, which proved that the algae growth in OCBR media was heterotrophic.

Chapter 4 Composition of Algae Cultivated on OCBR media

4.1 Introduction

The composition of algal feedstock is of great importance to its application potential. For example, the quantity and profile of lipids and proteins in algal biomass determine its value in manufacture of biodiesel, nutraceuticals, and feeds. The composition of algal feedstock is often a function of algae strains and culture conditions. Culture medium is an important factor. It not only affects the growth rate but also determines the growth mode (trophic conversion mode) and nutrient deficiency conditions which have strong influence on lipid accumulation and profile.

Therefore, in this chapter, the contents of lipid, protein and PUFAs in total algae biomass as affected by OCBR media will be reported and the potential of OCBR based biomass to serve as biodiesel feedstock, animal feed and functional food ingredients will be discussed.

4.2 Materials and methods

Total lipid analysis

Algae cells were harvested through centrifugation (2044 g for 15 min) and dried in a freeze dryer (Savant Instruments Inc., USA). Lipids were extracted using the one-step extraction method described by Folch et al. (1956) with modifications. Briefly, ca. 0.1 g dried algae powder was accurately weighed into clean, 25 ml screw-top glass tubes and 10 ml of 2: 1 chloroform - methanol (v/v) mixture was added. Extraction was carried out in a 30°C water bath (Cole-Parmer, USA) for 30 min with 100 rpm rotation speed.

Upon completion of the reaction, the algal solid residues were removed by passing the suspension through a Whatman 934-AH glass fiber filter (Whatman Inc., USA). The filtrate was transferred to another clean, 25 ml screw-top glass tube containing 2 ml 0.9 % NaCl solution to wash out water-soluble components. After centrifugation at 2044 g for 15 min, a biphasic system without any interfacial fluff was obtained. The volume of the lower phase containing essentially all the algal lipids was measured, and 3 ml of the lower phase was transferred into a weighed, clean, 5 ml glass tube, and the organic solvent was removed under a N-EVAP Analytical Nitrogen Evaporator (Organomation Associates, Inc., USA). Duplicate lipid determination was made for every sample. The lipid content of the dry material was calculated according to the following formula:

$LW\ (g/g) = (m_2\text{-}m_0) \times V/ (3 \times m_1)$

where LW stands by "lipid content based on dry weight", m_1 is the weight of the algae powder, m_0 is the weight of the empty 5 ml glass tube, m_2 is the weight of the 5 ml tube with the dried lipids, and V is the total volume of the lower phase after washing (Zhou et al, 2012a).

Fatty Acid Profile Analysis

Algae cells were harvested through centrifugation and then dried with a freeze dryer (Savant Instruments Inc., USA) before analysis. The fatty acid profiles of the dried algae were analyzed by following two successive steps including preparation of fatty acid methyl ester (FAME) and GC–MS analysis. FAME was prepared by a one-step extraction–transesterification method described by Indarti et al. (2005), with minor

modification. Dried algae samples (about 100-150 mg) were weighed into clean, 25-mL screw-top glass bottles, to which 10 mL mixture of methanol, concentrated sulfuric acid, and chloroform (4.25:0.75:5) were added. Transesterification was carried out in a 90 °C water bath (Cole-Parmer) for 90 min. Upon completion of the reaction, the chloroform layer containing FAME was carefully collected and subjected to gas chromatography/ mass spectrometer (GC/MS) analysis. Agilent 7890–5975C GC/MS with a HP-5 MS capillary column was used. The oven temperature was set at 80 °C, and held for 5 min, then raised to 290 °C at a rate of 4 °C/min, and held at 290 °C for 5 min, while the injector and detector temperature were set at 250 °C and 230 °C, respectively. The carrier gas (helium) was controlled at 1.2 mL/min. Chromatographic data were recorded and integrated using Agilent data analysis software. The compounds were identified in the NIST Mass Spectral Database and quantified by comparing the peak area with that of the external standard (C18:2; Sigma-Aldrich, MO) (Zhou et al, 2011).

Protein Content Analysis

The carbon (C), hydrogen (H), oxygen (O), and nitrogen (N) contents in the freeze-dried algae cells were analyzed, using an elemental analyzer (Vario EL III, Elementar Analysen Systeme, USA). The crucial protein content was derived from nitrogen content using an official nitrogen-to-protein conversion factor 6.25 suggested by Salo-väänänen and Koivistoinen (1996).

4.3 Results and discussion

Lipids

Algae were harvested on Day 5 (the stationary of algae growth) in the 7-day cultivation for total lipid determination. The average total lipid content in algae grown on the 4 different OCBR media was 54 % and 35 % for UM258 and UM268, respectively (Table 4.1). The highest total lipid content was observed in algae samples grown on OCBR medium 1.

Table 4.1 Lipid content in algae UM 258 and UM268 grown on OCBR media on Day 5

Biomass Medium	Algae	Average Total Lipid (Duplicate)	STDEV	Average Algal Lipid* (in 4 biomass medium)	STDEV
1	UM258	59%	0.053	54%	0.048
2		50%	0.048		
3		50%	0.044		
4		57%	0.028		
1	UM268	38%	0.035	35%	0.032
2		33%	0.017		
3		31%	0.036		
4		37%	0.036		

*Estimated lipid content, may be not as high as the estimated since extraction solvent may also extract other compositions (i.e. pigments, sugars, hydrophobic proteins, chlorophyll) (Grima et al., 2013; Piorreck et al., 1984)

We reported previously that UM258 and UM268 accumulated 25.7 % and 26.8% lipids, respectively, when grown on highly concentrated municipal wastewater (Zhou et al, 2011). Therefore, OCBR media significantly enhanced lipid accumulation in UM258 and UM268. Ten-fold dilution of OCBR media used in this experiment (TN, TP, COD

and NH_4-N were around 800 mg/L, 100 mg/L, 15000 mg/L and 150 mg/L) had similar concentrations of TN, TP and NH_4-N but much higher concentration of COD than the municipal wastewater as described previously (TN, TP, COD and NH_4-N were around 930 mg/L, 212 mg/L, 2350 mg/L and 91 mg/L) (Zhou et al, 2011). Algae grown on high-carbon OCBR media showed better performance on lipid accumulation than wastewater, which was consistent with the result in a study that high concentration of carbon in media could enhance the lipid content of *Chlorella* sp. (Moheimani, 2013). In addition, higher lipid content was obtained on OCBR media with a high C/N ratio of 18.75 compared with that on wastewater (C/N ratio of 2.53), which was also consistent with the result of Cheng et al. (Cheng et al, 2009).

Omega-3 Fatty Acids

In our previous report, it was demonstrated that UM258 and UM268 were able to accumulate high-value unsaturated fatty acid (EPA, C20:5), which accounted for 7.8 % of total fatty acid, when grown in municipal wastewater (Zhou et al, 2011). EPA is one of important omega-3 polyunsaturated fatty acids which plays an important role in the prevention of various human diseases, such as high blood pressure, arthritis and sleep disease (Wu et al., 2010). However, wastewater could not be used as medium materials for cultivating algae for human consumption. The OCBR media, on the other hand, could fill the gap. In this study, omega-3 fatty acid was further examined and found to be 3% and 3.2% in UM258 and UM268, respectively, when grown on the 10-fold dilution OCBR medium. Moreover, the characteristic of high-oil of the algae grown on media

with nutrient supplement provided by OCBR hydrolysates were demonstrated. Thus, the algae grown on OCBR media have a potential to be functional ingredients for human and animal consumption to promote health, and at the same time provide good feedstock for biofuels and bioproducts application.

Protein

Since UM268 had the relatively low lipid content with OCBR medium when compared with UM258, further study was conducted in this chapter on the protein content of strain UM268, which had not been done before. The additional work and data on protein of UM258 will be provided in next chapter. The comparisons of contents of isotopes C, H and N and corresponding protein contents of dry weight in algae UM268 are shown in Table 4.2. In general, there was higher nitrogen or protein accumulated in the algae in OCBR medium than in artificial medium. The samples on day 3 had the higher nitrogen level and lower carbon level than that on day 5, which means the nitrogen/protein content could be affected by the algae age. The protein content was higher on day 3 even though the algae accumulation reached its maximum on day 5-day 6.

Furthermore, on OCBR medium 1 it showed the best nitrogen content, which was approx. 9.2 %, respectively on day 3. When compared with samples from the artificial medium, it was apparent that the protein content of algae in OCBR medium 1 was approx. 10.3 % higher than that in the artificial medium, containing approx. 57 % protein content on day 3.

Therefore, OCBR medium increased the protein content in UM268 significantly but the lipid accumulation was not enhanced as much as for UM258. Previous literature mentioned before (Zhou et al, 2011) had demonstrated that the maximum lipid content was obtained at the lowest N: P (1:1) and the maximum crude protein content was obtained at the highest N: P (35:1). Since the OCBR medium in this research had a ratio of N: P (8:1), it could either enhance or restrain the lipid and protein contents in algae, which mainly depended on the specific algae strain. Further study was necessary to investigate the accurate correlation between the nutrient profiles of OCBR medium and the lipid and protein contents of algae.

Therefore the high-protein algae strain UM268 could be a good protein resource for livestock feeds.

Table 4.2 Nitrogen/Protein contents in algae UM268 with 4 kinds of biomass media as well as artificial medium on Day 3 and Day 5

Medium		Day	N %	Average N%	Protein Content* %	Average Protein* %	N Increasing Rate
Biomass residues	**1**	3	9.228	.8.883	**57.7**	55.5	**110.28 %**
	2		8.462		52.9		101.12 %
	3		9.146		57.2		109.30 %
	4		8.696		54.4		103.92 %
	1	5	8.976	8.764	**56.1**	54.8	**107.27 %**
	2		8.291		51.8		99.08 %
	3		9.117		57.0		108. %
	4		8.672		54.2		104.60 %
Artificial		N/A**	8.367	8.367	**52.3**	52.3	**100.00 %**

*Estimated protein by nitrogen on the basis of elemental analysis, multiplied by a factor of 6.25 (Salo-väänänen and Koivistoinen, 1996), may be not as high as the estimated since algae may include other nitrogen composition (i.e. chlorophyll (Piorreck M et al., 1984))

**N/A: there was no day condition applied on artificial medium

4.4 Conclusions

This study shows that the OCBR media significantly enhanced lipid accumulation when compared with the wastewater media (Zhou et al., 2011). UM258 was able to accumulate much more lipids than UM268, making UM258 an excellent feedstock for lipid based applications. The data for UM268 shows that OCBR also promoted protein accumulation compared with artificial media. The high protein content in UM268 makes it a suitable feedstock for animal feed formulation. Both of UM258 and UM268 have detectable omega-3 fatty acids in their composition; however, the level of omega-3 fatty acids was still low compared with 7.8 % which reported in our previous research (Zhou et al., 2011). Therefore, we believed that there was room for omega-3 fatty acids accumulation improvement, which is the focus of next chapter.

Chapter 5 Effect of temperature and time on EPA accumulation in algae

5.1 Introduction

Polyunsaturated fatty acids (PUFAs) include arachidonic acid (20:4(n-6), ARA), eicosapentaenoic acid (20:5(n-3), EPA) and docosahexaenoic acid (22:6(n-3), DHA), which are considered as treasured components that promote human health. Therefore, there are considerable interests in PUFAs (Funk, 2001; Hong et al., 2003; Crawford et al., 1997; Benatti et al.,2004; Spector, 1999). Among above PUFAs, EPA, which has been used in pharmaceutical applications for its favorable influence on the circulatory system, has been anticipated for the prevention and cure of illnesses such as thrombosis and atherosclerosis (Yongmanitchai & Ward, 1989). Microalgae have been proven to have the capability to produce protein and lipid, especially some functional fatty acids in an industrial scale. Besides, it can serve as a crucial supplement to the cultivation of some marine species (Watanabe et al., 1983) as well as human body. Therefore, there is great potential to use microalgae as the feedstock of marine species as well as the healthy food.

The accumulation of EPA in microalgae is affected by such factors as algae species, media composition, light intensity, cultivation period, environmental variations and temperature (Bajpai et al., 1993). It is reported that the key environmental factors associated with lipid yields are temperature (Marr et al., 1962; Bajpai et al., 1991a; Baipai., 1991b; Baipai et al., 1992), light intensity (Seto et al., 1984; Erwin, 1973; Leu et al., 2006; Liang et al., 2006; Guihéneuf et al., 2010) and algal cultivation time (Shimizu et al., 1989; Bajpai et al., 1991c).

It has been reported that lower growth temperature leads to accumulating higher EPA in microorganisms (Shimizu et al., 1988a; Shimizu et al., 1988b; Yongmanitchai & Ward, 1991). Even though PUFAs are usually rare in blue-green algae, it has been observed that lower temperatures could increase the synthesis of unsaturated fatty acids in blue-green algae (Yongmanitchai & Ward, 1989). Different algae species have different optimal temperature for EPA synthesis. For instance, 20°C produced large amounts of EPA with Chlorella *minutissima* (Seto et al., 1984), while the optimum temperature for EPA production in marine diatom *P. tricornutum* was 21.5°C (Yongmanitchai & Ward, 1991). In other cases, low temperature cannot promote synthesis of PUFAs in some strains such as *Mucor* and *Rhizopus* species (Wassef, 1977). Right now, there is no specific research focusing on the effect of low temperature on algae strains which are originated from cold climate regions such as Minnesota.

The cultivation time also has an interaction-effect with temperature on the final EPA production. In many microorganisms, unsaturated fatty acids decreased as a function of time (Erwin, 1973). However, in other species such as *P. tricornutum* (Yongmanitchai & Ward, 1989) and some *Motierella* species (Shinmen et al., 1989), unsaturated fatty acids increased considerably with increase in cultivation time. The typical algae growth curve can be divided into four growth stages, that is, lag phase, exponential phase, stationary phase and decline phase. EPA can be easily accumulated in the early stationary phase in *I. galbana* (Fidalgo et al., 1998; Lin et al., 2007), but may be not in other strains. So far, a limited number of studies on the interaction-effect of temperature and cultivation time have been reported.

Therefore, in the present study, in order to evaluate the effect of temperature and time on algal EPA production, one locally isolated microalgal strain, *Scenedesmus sp.* was used, and optimal conditions for EPA accumulation in OCBR medium (Wang et al., 2013) were to be established.

5.2 Materials

The green microalgal strain UM258 (*Scenedesmus* sp.) isolated from the wastewaters in a Minnesota municipal wastewater treatment plant was axenically used in this study. The detailed information was described previously (Zhou et al., 2011).

Oil crop biomass residues (OCBR) media described in Chapter 3 were used in this study. Our previous result showed that 50-fold dilution of this OCBR medium was suitable for the growth of UM258.

5.3 Temperature treatment

UM258 was grown at 5 different temperatures (10°C, 15°C, 20°C, 25°C and 29°C inside climate chambers. Temperatures 10°C and 29°C were chosen as two extreme control during the experiment design (Mitchell et al., 2004), depending on climate extremes for algae growth on different seasons (Mitchell & Lampert, 2010).

5.4 Cultivation time treatment

The algae strain UM258 was inoculated in OCBR media with a rate of 1:10 (v/v), and then was cultivated for 10 days under a daylight lamp in a 250 ml flask, with 150 ml

OCBR media in each. The flasks containing the culture broth were placed on orbital shaker operated at a fixed rotating speed of 150 rpm (Zhou et al., 2012). The light intensity inside the climate chambers was set to a fixed level at 80 µmol PAR photons/ (m^2s) using a light intensity detector. Algae cells were harvested on day 3, day 6 and day 9 (the last day) in order to compare the effect of cultivation time on algal EPA accumulation. Triplicate experiments were conducted on every series of cultivation.

Dry weight (TVSS), fatty acids and protein contents were measured by standard methods which were described previously in Chapter 3 and Chapter 4.

5.5 Results and Discussion

Growth profiles of algae grown on different temperature

The growth of algae UM258 on OCBR media at five temperatures (10°C, 15°C, 20°C, 25°C and 29°C) for ten days are illustrated in Figure 5.1. UM258 was able to grow from 10°C to 29°C, and reached maximum algal biomass concentration of 0.6 – 0.8 mg/L on 50-fold diluted OCBR media. At 10°C, UM258 grew slower than at the higher temperatures, and reached its maximum concentration on day 6, probably because the physiological activity of this strain was depressed in such low temperature environment. The growth of UM258 increased with increasing temperature from 10°C to 29°C. The biomass concentration of UM258 at 15°C, 20°C and 25°C reached peak on day 3, maintained steady from day 3 to day 6, and then decreased. UM258 at 29°C increased significantly in the first several days, but decreased afterwards, probably due to the exhaustion of media nutrients during the algal growth at 29°C.

In general, UM258 on 20°C reached a relatively high biomass concentration in the stationary phase, which was around 0.8 mg/L. Besides, it also had a relatively fast growth rate to reach its maximal accumulation (usually within seven days), which suggested that this temperature is more suitable for algae growth in large-scale than others.

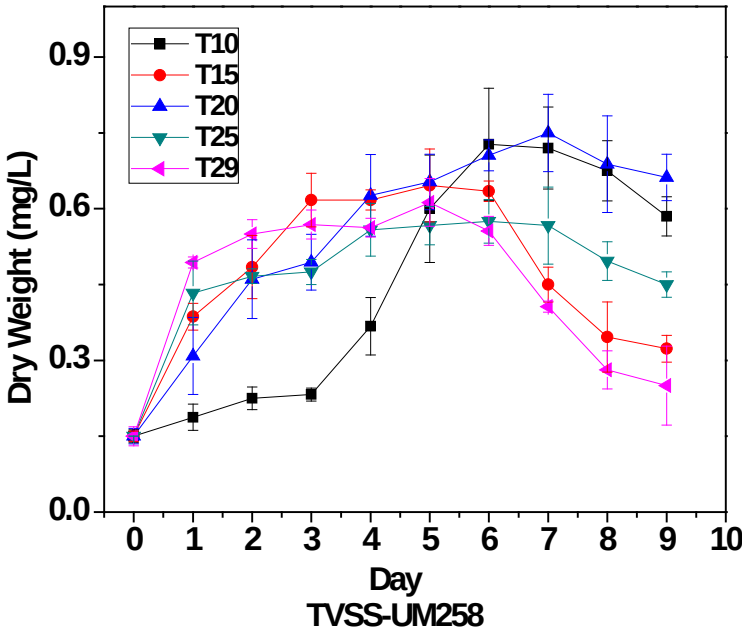


Figure 5.1 Growth profiles of algae UM258 under five temperatures (10°C, 15°C, 20°C, 25°C and 29°C) in 10 days

Protein Content

The protein contents of algae UM258 on OCBR media on day 3, day 6 and day 9 during the 10-day cultivation are depicted in Figure 5.2. The algal protein content varied with culture temperature. At 10°C and 15°C, the protein content increased from day 3 up to day 6 and then maintained steady while at 20°C, 25°C and 29°C, the protein content decreased slightly from the beginning to the end. Finally UM258 had similar protein

content of 45% - 52% at all five temperatures after ten days. However, the reason why it had less nitrogen content at the beginning of growth when grown at lower temperatures is still unknown. More studies are necessary to understand the underlying mechanisms.

The protein contents were estimated by the nitrogen level in UM258 on the basis of elemental analysis, multiplied by a conversion factor of 6.25 (Salo-väänänen and Koivistoinen, 1996). Therefore, the actual protein contents may not be as high as estimated, since algae may have other nitrogen-containing components such as chlorophyll (Piorreck et al., 1984). Interpretation of the protein content results must use cautions.

Our results show that 20°C was the best for algal protein production among all the cultivation temperatures.

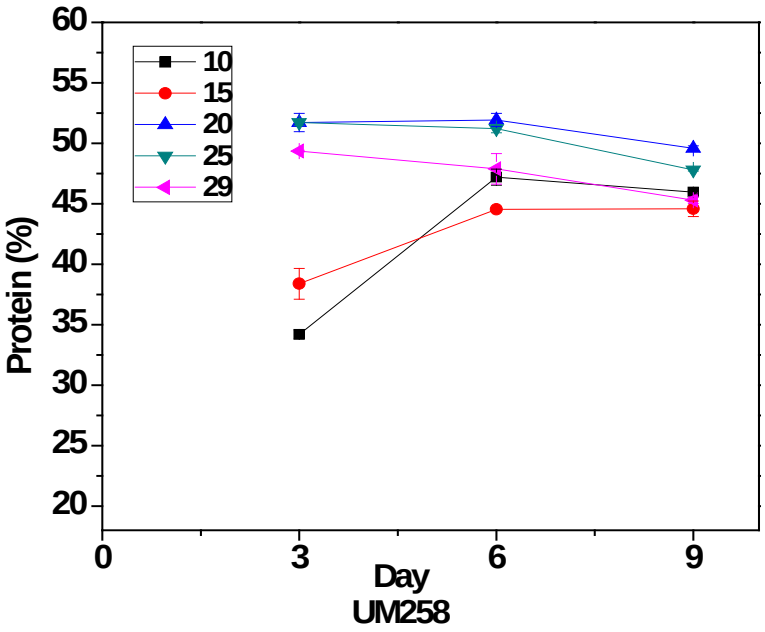


Figure 5.2 Protein content of algae UM258 under five temperatures (10°C, 15°C, 20°C, 25°C and 29°C) at day 3, day 6 and day 9

EPA Production

The trends of EPA production of UM258 on OCBR media corresponding at different cultivation times (day 3, day 6 and day 9) at different temperatures (10°C, 15°C, 20°C, 25°C and 29°C) are illustrated in Figure 5.3. All data were generated with duplicate experiments.

It was observed that the percentage of EPA in total fatty acids increased with cultivation time. The EPA production was the lowest at the exponential growth stage (day 0 – day 4) followed by a consistent increase during the stationary phase (day 4 – day 7). The EPA continued to rise even when the total algal biomass was in the decline phase (after day 7). Therefore, in order to produce algal EPA in a more effective way, it is better to choose algae when it has arrived at the top biomass accumulation stage.

The EPA increased from 4% to 18% when temperature was increased from 10°C to 20°C. However, it decreased from 18% to 12% when temperatures continued to rise after 20°C. Therefore, 20°C should also be a desirable temperature for EPA production in algae UM258. However, using 20°C for commercial algal EPA production may lead to higher energy cost than using room temperature (25°C).

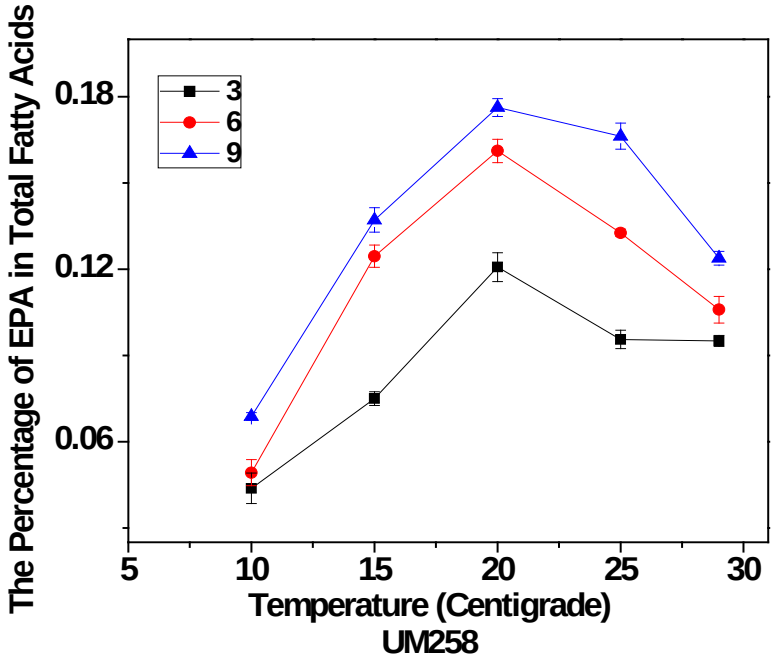


Figure 5.3 Trend of EPA productions corresponding with temperature during 10 days

5.6 Conclusions

The biomass yield in UM258 reached 0.8 mg/L after 4 to 7 days cultivation on OCBR media at optimal temperature of 20°C. In addition, the EPA accumulation of UM258 reached 16% during the same period, and 18% at the end of 10-day cultivation. Temperature 20°C proved to be the optimal temperature for algal biomass accumulation, EPA and protein accumulation. Therefore, there is great potential to use the 20°C to enhance algal EPA productivity.

Chapter 6 Conclusions and future work

Soybean and sunflower biomass residue hydrolysates proved to be excellent media to stimulate algae growth. Under the optimal acid hydrolysis conditions, soybean and sunflower biomass residues can be converted to culture media with high level of carbon content and high ratio of C/N (e.g., greater than 18). OCBR media performed better than artificial medium in terms of algal biomass accumulation and lipid content. The maximal algal biomass concentration of 3 g/L was achieved under optimal conditions. Moreover, algae on OCBR media produced high lipids and high value omega-3 fatty acids at the same time. At 20°C, algae strain UM258 showed outstanding EPA production of 18% and maintained a high-level of protein composition after arriving maximum biomass accumulation.

The results presented in this thesis demonstrate that acid hydrolyzed oil crop biomass residue (OCBR) media have the great potential to replace the artificial media for large scale cost-effective algal biofuel, protein and omega-3 fatty acids production. Besides, relatively low temperatures, such as 20°C, and algae in the stationary phase or decline phase, such as one cultivated for more than 6 days, can enhance the EPA production of algae effectively. This research should be a good guide for industrial algal EPA production.

Further research is necessary to understand the relationship between the chemical compositions of OCBR media and lipid accumulation, and to further optimize and develop OCBR media using multi-factor orthogonal experiment design. In addition, the

impacts of other factors such as light intensity on high-PUFA algae growth also deserve further investigation.

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