

UTILIZATION OF RICE BRAN BY *PYTHIUM IRREGULARE*
FOR LIPID PRODUCTION

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science in Biological and Agricultural Engineering

in

The Department of Biological and Agricultural Engineering

by

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December, 2002

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my major professor, Dr. Terry Walker, for offering me the opportunity to do this research under his guidance and support throughout my studies. Special thanks are extended to my committee members, Drs. Caye Drapcho and Donal Day, for their invaluable advice. I also thank the rest of the faculty and staff members of the Biological and Agricultural Engineering Department for their warm-hearted help during my staying at LSU.

Finally, I want to thank my mother, my brother and my sister, for their endless love, support and encouragement.

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ABSTRACT

This research investigated the feasibility of utilizing rice bran as feedstock for submerged fermentation of *Pythium irregulare* to produce fungal lipids, which contain polyunsaturated fatty acids (PUFAs) including arachidonic acid (AA, C20:4n6) and eicosapentaenoic acid (EPA, C20:5n3).

The fungal nutrient requirements, temperature characterization and growth modeling were determined in glucose-yeast extract media. Sixteen media combinations based on a 2⁴ factorial design with four levels of glucose and yeast extract ranged from 1% to 4% and 0.25% to 1%, respectively, and corresponding carbon/nitrogen ratio (C/N) ranged from 4 to 64, were examined. The suitable C/N for *Pythium irregulare* growth ranged from 12 to 24, while that for fungal lipid accumulation as well as EPA and AA synthesis was 32. The optimal medium for EPA and AA production was composed of 2% glucose and 0.25% yeast extract, with addition of 0.1% KH₂PO₄. Three constant incubation temperatures, 13 °C, 19 °C and 25 °C, were then tested for increasing the EPA and AA production with this medium. Biomass, substrate utilization, lipid and individual fatty acid yields during incubation were determined and the fungal growths were modeled. The optimal incubation temperature was 25 °C for EPA and AA production.

To meet the fungal nutrient requirements, a 5% (w/v) rice bran media was made and the cultures were incubated at 25 °C to produce fungal lipids containing EPA and AA. Biomass, substrate utilization, lipid, and individual fatty acid yields during incubation were determined. The results indicated rice bran could be utilized to produce EPA and AA by *Pythium irregulare*. After 7 days of fermentation, EPA and AA were synthesized by the fungus with yields of 207 mg/L and 70 mg/L, respectively. The

percentages of bioconversion from rice bran to EPA and AA by the fungus were 0.42% and 0.14%, respectively. Meanwhile, more than 73% COD in the rice byproduct medium was removed by the fungus.

CHAPTER 1

INTRODUCTION

1.1 Health Benefits of Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) are a group of fatty acids that have two or more double bonds on the carbon chain. Based on the location of the first double bond on the carbon chain, PUFAs can mainly be divided into ω -3 fatty acids and ω -6 fatty acids. ω -3 fatty acids are fatty acids whose first double bond is located three carbons away from the methyl end of the molecule, while ω -6 fatty acids' first double bond is six carbons away from the methyl end. Important representative PUFAs include linoleic acid (C18:2n6c), α -linolenic acid (ALA, C18:3n3), γ -linolenic acid (GLA, C18:3n6), dihomo- γ -linolenic acid (DGLA, 20:3n6), arachidonic acid (AA, C20:4n6), eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3). Structures of representative ω -3 fatty acid and ω -6 fatty acid are shown in Fig.1.1.

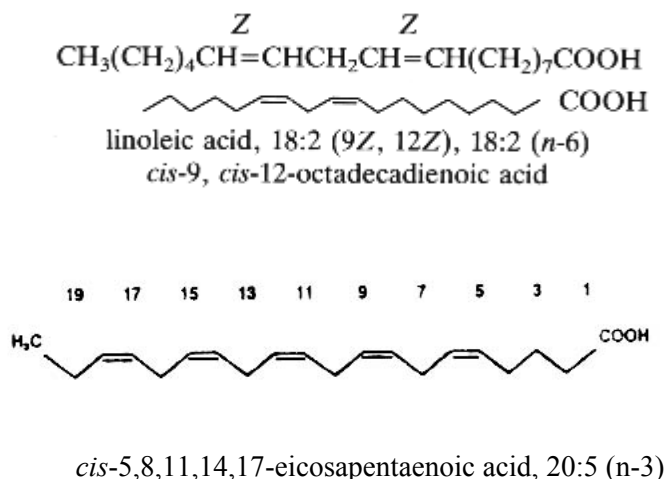


Fig. 1.1 Structures of linoleic acid and EPA, which are representatives of ω -6 and ω -3 fatty acids

PUFAs, with their unique structural and functional characteristics, are distinguished by two main functions in mammalian metabolism. The first function relates to their roles in regulating the architecture, dynamics, phase transition and permeability of membranes, and modulating the behavior of membrane-bound proteins such as receptors, ATPases, transport proteins and ion channels. In addition, PUFAs control the expression of certain genes and thus affect some processes including fatty acids biosynthesis and cholesterol transport in the body. The second function is that PUFAs serve as precursors of a wide variety of metabolites, such as prostaglandins, leukotrienes and hydroxy-fatty acids, which regulate critical biological functions. The various roles played by PUFAs make it apparent that they are required in every organ in the body in order to keep the organs functioning normally. Therefore, it is not surprising that PUFA deficiencies lead to abnormalities in the skin, nervous system, immune and inflammatory systems, cardiovascular system, endocrine system, kidneys, respiratory and reproductive systems. (Certik and Shimizu, 1999).

In a cell, synthesis of PUFA is achieved by carbon chain elongation and desaturation. The metabolic pathways of important PUFAs are presented as Fig. 1.2. To accomplish these PUFA metabolic steps, enzymes such as D-6-desaturase, D-5-desaturase and elongase are involved. Due to a lacking of these enzymes, mammals cannot synthesize the long-chain PUFAs and must supplement them from diet. Thus, PUFAs deficiency is quite common in human beings unless diets contain a sufficient amount of marine fish, animal tissues or plant seed oils. Therefore, the market demand for the PUFAs is presently growing in the form of supplemental tablets.

Omega-3 route

α -linolenic acid (C18:3n3) → octadecatetraenoic (C18:4n3) →
 eicosatetraenoic (C20:4n3) → eicosapentanoic (C20:5n3) → eicosanoids

Omega-6 route

linoleic (C18:2n6) → γ -linolenic (C18:3n6) → dihomo- γ -linolenic (C20:3n6)
 → arachidonic (20:4n6) → eicosanoids

Fig. 1.2 Metabolic pathways of synthesis C₂₀ fatty acids by ω -3 and ω -6 routes from C₁₈ fatty acids, which result in the synthesis of eicosanoids

1.2 Current PUFAs Sources

PUFAs occur throughout the animal and plant kingdom, although the greatest diversity is encountered in microorganisms, especially algae, fungi and bacteria. In microorganisms, the fatty acids are typically present in storage oil and in membrane phospholipids (Gill and Valivety, 1997). The sources of some important PUFAs are summarized in Table 1.1.

Table 1.1 Sources of Important Polyunsaturated Fatty Acids

PUFA	Conventional sources	Microbial sources
γ -linolenic acid (GLA)	Plant (<i>Oenothera</i> , <i>Borago</i> , <i>Ornithogalum</i> spp.)	Fungi (<i>Mucor</i> , <i>Mortierella</i> , <i>Aspergillus</i> spp.), algae: (<i>Chlorella</i> and <i>Spirulina</i> spp.)
dihomo- γ -linolenic acid (DGLA)	Human milk, animal tissue, fish (<i>Scomber scombrus</i>),	Fungi (<i>Mortierella</i> , <i>Conidiobolus</i> , <i>Saprolegnia</i> spp.), mosses (<i>Ctenidium molluscum</i>)
α -linolenic acid (ALA)	Plant (<i>Brassica</i> , <i>Glycine</i> , <i>Juglans</i> , <i>Linum</i> spp.)	Algae: <i>Chlorella</i> spp.

Table 1.1 (continued)

arachidonic acid (AA)	Animal tissues, fish (<i>Brevoortia</i> , <i>Clupea</i> , <i>Sardina</i> spp.) mosses (<i>Ctenidium molluscum</i>)	Fungi (<i>Mortierella</i> , <i>Pythium</i> spp.), algae: (<i>Porphyridium</i> spp.), mosses (<i>Rhytidiadelphus</i> , <i>Brachythecium</i> , <i>Eurhynchium</i> spp)
Eicosapent aenoic acid (EPA)	Fish (<i>Brevoortia</i> , <i>Engraulis</i> , <i>Sardina</i> , <i>Scomber</i> spp.)	Fungi (<i>Mortierella</i> , <i>Pythium</i> spp.), algae: (<i>Chlorella</i> , <i>Monodus</i> , <i>Porphyridium</i> , <i>Nannochloropsis</i> , <i>Cryptoleura</i> , <i>Schizymeria</i> , <i>Navicula</i> spp.), mosses (<i>Brachythecium</i> , <i>Eurhynchium</i> , <i>Scleropodium</i> spp), bacteria (<i>Rhodopseudomonas</i> , <i>Shewanella</i> spp.)
docosahexa enoic acid (DHA)	Fish (<i>Brevoortia</i> , <i>Engraulis</i> , <i>Sardina</i> , <i>Scomber</i> spp.)	Fungi (<i>Thraustochytrium</i> , <i>Entomophthora</i> spp.), algae: (<i>Gonyaulax</i> , <i>Gyrodinium</i> , <i>Cryptoconidium</i> spp.), bacteria (<i>Rhodopseudomonas</i> , <i>Shewanella</i> spp.)

Sources: (Gill and Valivety, 1997; Certik and Shimizu, 1999)

Presently, the main commercial source for C₁₈ PUFAs is plant seed oils. The PUFAs above C₁₈ cannot be synthesized by higher plants owing to a lack of the required enzymes. AA is mainly obtained from animal tissues, such as pig liver. EPA and DHA are mainly obtained from marine fish oils. PUFA yields in agricultural and animal products are generally low and vary by season, climate and geographical location. Marine fish oils, which are the main source of commercial EPA and DHA, have disadvantages of objectionable taste and odors, high cholesterol and small amounts of potential toxic impurities that are difficult to remove. Therefore, the quantity and quality of conventional sources of PUFA may encounter problems meeting an increasing market demand.

1.3 Potential PUFA Sources

A variety of PUFAs have been detected in microorganisms including bacteria, fungi, algae and mosses. Microorganisms are thought to be very promising lipid

producers because of their high growth rate on simple media and the simplicity of their manipulation. The diversity of microbial species can facilitate the selection of strains producing lipids with the target fatty acids. Some oleaginous microorganisms have been intensively studied in recent years, as alternatives to agricultural and animal oil products (Certik and Shimizu, 1999).

In the past several years, research on microbial PUFA production was basically aimed at improving economic competitiveness of microbial lipids compared to plant and animal-derived lipids. Emphasis was placed on screening for more efficient strains, increasing the product value, using inexpensive substrates, and reducing the processing steps necessary for lipids recovery from the cells. Two basic processes have been developed for microbial production of PUFAs: solid-state fermentation (Jang, 2000; Conti, 2001) and submerged fermentation. However, due to technical problems such as difficulties in contamination control, scaling-up and lipid extraction, solid-state fermentation has received less attention than submerged fermentation.

Most microorganisms produce a group of PUFAs, instead of a single PUFA. For example, the microorganisms that can produce AA can also produce a certain amount of EPA. However, yield of individual fatty acid vary by culture condition and media composition. Therefore, the environmental factors or media composition may be adjusted to obtain optimal target PUFAs. *Mortierella* and *Pythium* species are the most intensively studied fungal microorganisms that can produce high amounts of GLA, AA and EPA (Certik and Shimizu, 1999; Cheng, 1999; Aki, 2001; Hong, 2002).

O'Brien (1993) reported that 24.9 mg EPA/g dry biomass was achieved while using sweet whey permeate as fermentation substrate in a fed-batch culture of *Pythium irregulare* at 14 °C. An AA yield of 5.3 g/L was achieved by *Mortierella alpina* in glucose-yeast extract media with 100g/L glucose in the initial media (Li, 1995). The yield of 11.1 g/L was achieved with same strain growing on similar medium in fed-batch culture (Singh, 1997). In Cheng's research, the greatest EPA yield was 1.4 g/L in the medium composed of 4% soybean oil and 1% soymeal waste at 12 °C and that of AA was 2 g/L in the media composed of 4% soybean oil at 18 °C by *Pythium irregulare* fermentation (Cheng, 1999).

The PUFA profile and yield vary depending on species and in many cases culture conditions, particularly temperature. Much of the literature showed that lower temperature (normally 12-14 °C) could enhance PUFA production in fungal fermentation (Stinson, 1991; O'Brien, 1993; Cheng, 1999). The mechanism explaining PUFA enhancement at lower temperature is still not clear. There are two hypotheses. One is that increasing solubility of molecular oxygen at low temperature increases available intracellular molecular oxygen, which would be required by oxygen-dependent enzymes catalyzing the desaturation of long chain unsaturated fatty acids (Brown and Rose, 1969). The second hypothesis is that when the growth temperature is lowered, the proportion of unsaturated fatty acids to saturated fatty acids tends to increase as a result of increased membrane fluidity as an adaptation to the cold environment (Suutari, 1994).

Based on the finding of lowering temperature for the enhancement of PUFA production, a technique called "temperature shift" was applied to fermentation process to shorten the fermentation span and lower the production maintenance cost (Stinson, 1991;

O'Brien, 1993; Cheng, 1999). In this technique, the culture is incubated at the optimal growth temperature (normally 25 °C for fungi) for several days to accumulate biomass followed by incubation at lower temperature (12-14 °C) to accumulate target PUFAs.

Regardless of the achievement in culture conditions to enhance the PUFA production, much work is needed to explain the temperature phenomena. Also, the relationships among fatty acids production, biomass formation, nutrient utilization and some culture parameters, which are very important for fermentation optimization and control, are rarely reported. Important kinetic parameters of the fungi growth and PUFAs production are still unknown, but such information is essential for engineering design and optimization of the fungal lipid production system.

The demand of reducing the process cost coupled with the environmental pressure caused by the release of agricultural/industrial byproduct and waste has resulted in the need for utilization of byproduct/waste as fermentation feedstock. Normally, the composition of such byproduct/waste is very complex, which may result in the substrate not being directly suitable for certain microorganisms. The known nutrient requirements of the microorganism could serve as guideline to adjust the media composition, which would ideally be mainly composed of the byproduct or waste.

1.4 Rice Bran

Rice bran is an important byproduct in rice milling industry, which occupies about 10% of rough rice weight. According to USDA, the rough rice production of the United States is about 9 million tons in recent years (USDA Annual Reports, 1996-2000). That means, about 0.9 million tons rice bran is produced every year. Rice bran is a complex substrate containing 12-14% protein, 19-22% lipids, 50% carbohydrates (21%

fiber) and high levels of vitamins and essential trace minerals (see Table 1.2). In the United States, the predominant use of rice bran has been an ingredient in livestock feed. Therefore, the value of rice bran is low.

Attracted by its potential value, value-added processing technologies for rice bran have been sought by researchers. One example was utilizing rice bran to extract rice bran oil (Orthofer, 1996). However, the PUFAs contained in rice bran oil are mainly composed of C₁₈ fatty acids, which are not as valuable as the C₂₀ group for human health.

Table 1.2 Crude Rice Bran Composition (source: www.nal.usda.gov)

Nutrient	Units	Value per 100 grams of edible portion	Sample Count	Std. Error
Proximates				
Water	g	6.13	10	1.111
Energy	kcal	316	0	
Energy	kJ	1322	0	
Protein	g	13.35	4	0.528
Total lipid (fat)	g	20.85	6	1.563
Ash	g	9.98	5	0.659
Carbohydrate, by difference	g	49.69	0	
Fiber, total dietary	g	21.0	0	
Minerals				
Calcium, Ca	mg	57	5	6.106
Iron, Fe	mg	18.54	5	3.556
Magnesium, Mg	mg	781	5	150.607
Phosphorus, P	mg	1677	5	236.516
Potassium, K	mg	1485	5	198.479
Sodium, Na	mg	5	5	1.175
Zinc, Zn	mg	6.04	5	0.928
Copper, Cu	mg	0.728	5	0.095
Manganese, Mn	mg	14.210	2	
Selenium, Se	mcg	15.6	1	
Vitamins				
Vitamin C, total ascorbic acid	mg	0.0	0	
Thiamin	mg	2.753	6	0.511
Riboflavin	mg	0.284	4	0.073

Table 1.2 (continued)

Niacin	mg	33.995	4	9.119
Pantothenic acid	mg	7.390	4	2.219
Vitamin B-6	mg	4.070	4	0.277
Folate, total	mcg	63	2	
Folic acid	mcg	0	0	
Folate, food	mcg	63	2	
Folate, DFE	mcg_DFE	63	0	
Vitamin B-12	mcg	0	0	
Vitamin A, IU	IU	0	0	
Retinol	mcg	0	0	
Vitamin A, RAE	mcg_RAE	0	0	
Vitamin E	mg_ATE	6.050	0	
Lipids				
Fatty acids, total saturated	g	4.171	0	
4:0	g	0	0	
6:0	g	0.000	0	
8:0	g	0.000	0	
10:0	g	0.000	0	
12:0	g	0.019	73	
14:0	g	0.078	73	
16:0	g	3.557	73	
18:0	g	0.373	73	
Fatty acids, total monounsaturated	g	7.549	0	
16:1 undifferentiated	g	0.075	73	
18:1 undifferentiated	g	7.475	73	
20:1	g	0.000	0	
22:1 undifferentiated	g	0.000	0	
Fatty acids, total polyunsaturated	g	7.459	0	
18:2 undifferentiated	g	7.143	73	
18:3 undifferentiated	g	0.316	73	
18:4	g	0.000	0	
20:4 undifferentiated	g	0.000	0	
20:5 n-3	g	0.000	0	
22:5 n-3	g	0.000	0	
22:6 n-3	g	0.000	0	
Cholesterol	mg	0	0	

Regarding its constitution, rice bran contains the main nutrients for microbial growth. Therefore, it may serve well as a substrate for microbial fermentation to add value to rice bran. Some successes using rice bran as solid-state fermentation substrate

have been reported (Costa, 1998; Feng, 2000). No published report was found that stated that rice bran was applied in submerged fermentation. Submerged fermentation has some advantages over solid-state fermentation, such as easiness of process control and scale-up.

Most of the rice bran constituents are insolvable in water, which is the main reason for its limited application in submerged fermentation. However, some microorganisms, such as the soil-borne fungi that contain cellulases and other enzymes are capable of utilizing rice bran in submerged fermentation if the rice bran amount in medium is not too high to affect mass transport.

Rice bran has a relatively high amounts of α -linolenic acid (C18:3n3, 0.32 g/100 g rice bran) and linoleic acid (18:2n6, 7.14 g/100 g rice bran), which could be advantageous for the microbial production of EPA and ARA, because α -linolenic acid and linoleic acid are metabolic precursors of EPA and ARA, respectively (Holman, 1998, see Fig. 1.2). Research has shown that addition of oils containing these fatty acids to media could greatly increase EPA and ARA yields (Shimizu, et al, 1989; Bajpai, et al, 1991).

1.5 Objectives of This Research

In this research, *Pythium irregulare*, which is one of the most promising fungal strains to produce EPA and AA, was selected to investigate the feasibility of utilizing rice bran to produce fungal lipids containing essential PUFAs. Specific objectives include:

1. Investigate the nutrient requirements of the fungus including media composition and C/N ratio on glucose-yeast extract media for lipid production.
2. Characterize and model the temperature effect using the optimized medium from Objective 1.

3. Investigate the utilization of rice bran as a potential substrate for fungal lipid production in submerged fermentation.

Fig. 1.3 shows an overview of the experimental plan to complete the three objectives. Chapter 2, 3, and 4 are arranged as separate papers addressing each objective, respectively. Chapter 2 illustrates the optimization of C/N and media composition to achieve high EPA and AA production for Objective 1. Chapter 3 focuses on Objective 2 for optimization of temperature conditions. Chapter 4 focuses on Objective 3 for utilization of rice bran as a potential feedstock to produce fungal lipids. In all the three chapters, the fungal biomass, nutrient utilization, lipid content, individual fatty acids profiles were investigated and their relationships were discussed. Fig. 1.4 is a general flowchart showing the experimental procedure for the three studies.

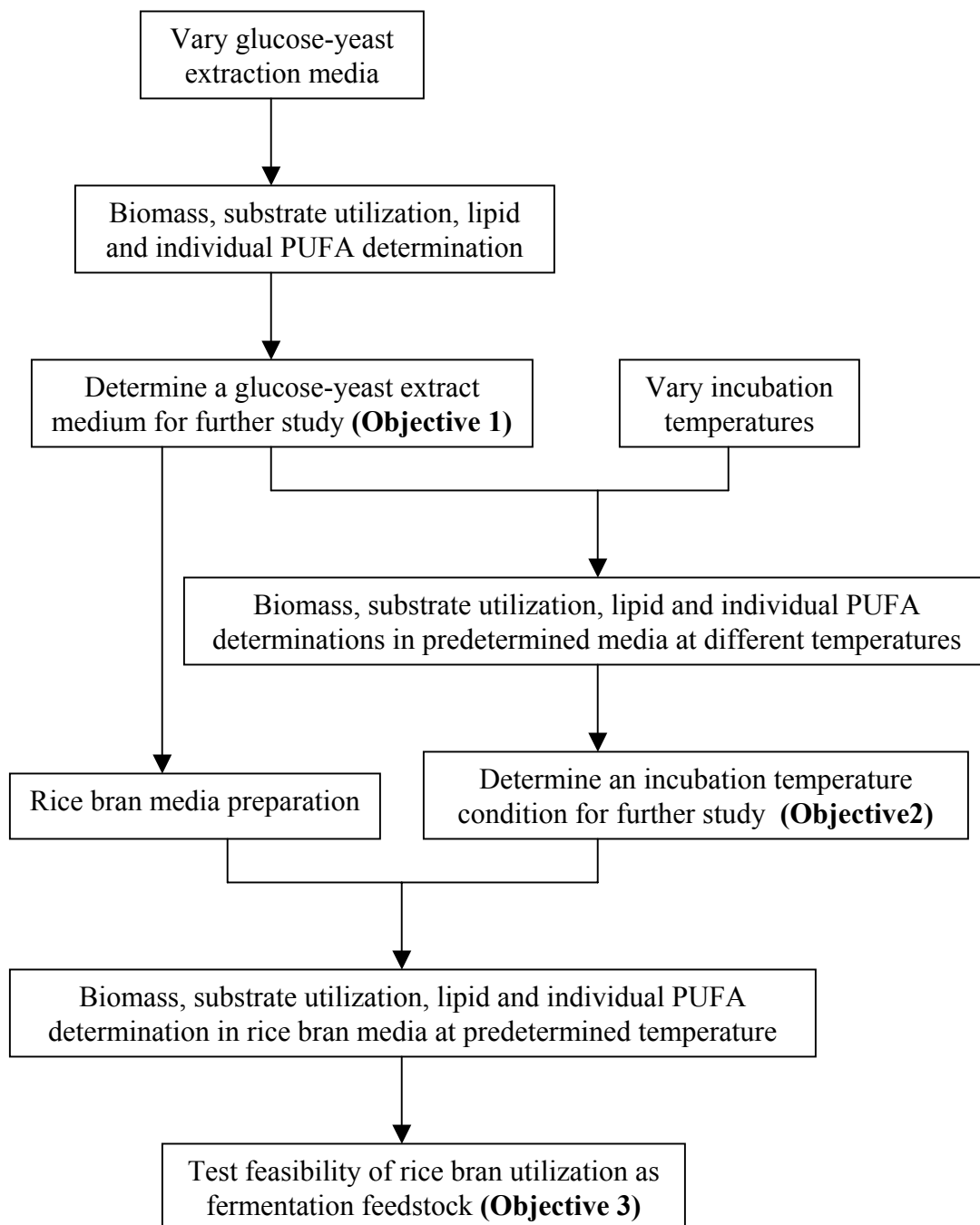


Fig. 1.3 Flowchart of an overview experimental plan

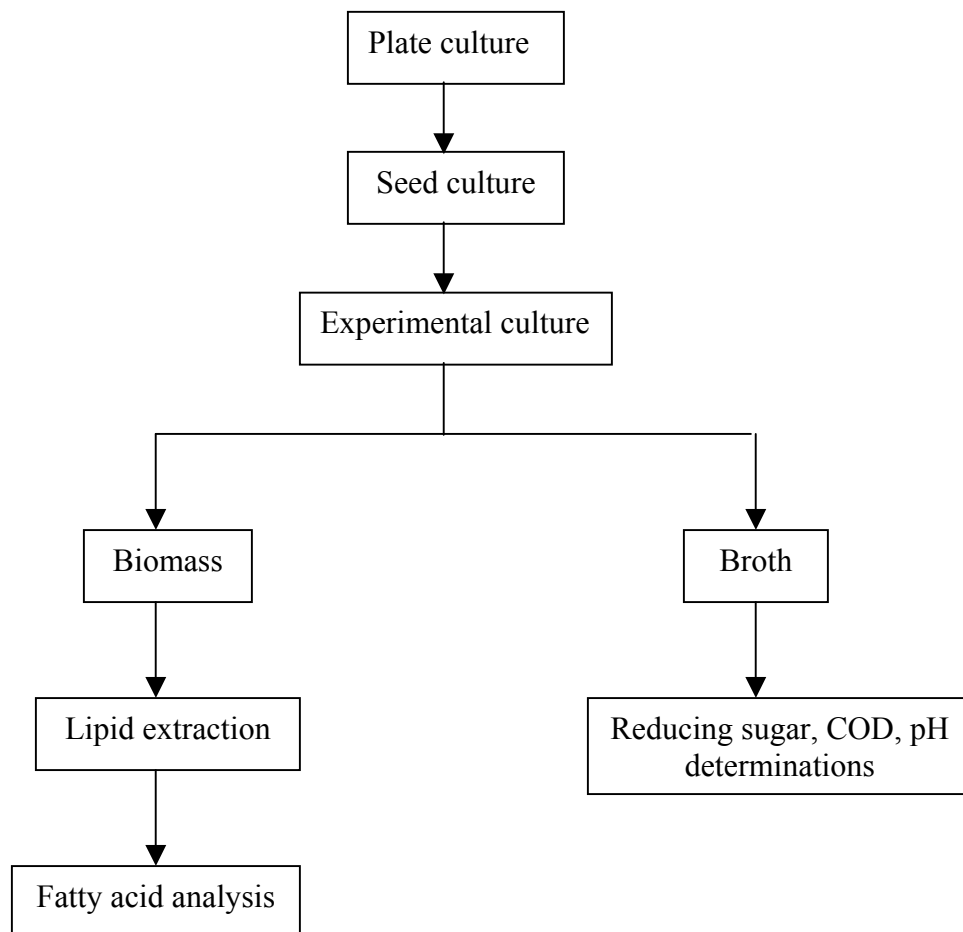


Fig. 1.4 General experimental procedure of the proposed research

CHAPTER 2

EFFECTS OF MEDIA COMPOSITION AND C/N RATIO ON POLYUNSATURATED FATTY ACID PRODUCTION

2.1 Introduction

Growing interest in health benefits of polyunsaturated fatty acids (PUFAs) has focused attention on providing suitable sources of these compounds. Isolation of highly efficient oleaginous microorganisms and development of related fermentation technologies may lead to fermentation as an alternative to agricultural and animal processes (Certik, 1999). Some microbial species produce high yields of certain PUFAs, which include *Mortierella alpina* (Li, 1995; Higashiyama, 1998; Hoike, 2001), *M. enongata* (Bajbai, 1991), *Pythium irregulare* (Stinson, 1991; O'Brien, 1993; Cheng, 1999; Hong, 2002) *P. ultimum* (Gandhi, 1991, Stredansky, 2000) and *Entomophthora exitalis* (Kendrick, 1992). *Pythium irregulare* is regarded as one of the most promising microbial species for possible commercial production of eicosapentaenoic acid (EPA) due to its high EPA yield. This microorganism can also produce a significant amount of another important PUFA, arachidonic acid (AA) (Stinson, 1991; O'Brien, 1993; Cheng, 1999).

Research has indicated that culture conditions, such as medium composition, oxygen availability, pH and temperature, could influence microbial lipid accumulation as well as PUFA composition (Stinson, 1991; Hoike, 2001). Due to the nature of the individual microorganism, the optimal conditions may differ from strain to strain.

Some of the culture conditions for *Pythium irregulare* have been optimized (Stinson, 1991; O'Brien, 1993; Cheng, 1999). Glucose and yeast extract are considered as the preferred carbon and nitrogen sources for this microorganism (Stinson, 1991). Nitrogen limitation is commonly believed to have significant effect on lipid accumulation in microbial fermentation (Ykema A, 1988). However, the optimal C/N ratio of *Pythium irregulare* for the target PUFA synthesis is not known and few published studies have addressed this issue with other species. Therefore, optimizing medium composition and C/N ratio will be important for target PUFA production and process control.

The objectives of this part of research were determining suitable medium composition and C/N ratio for *Pythium irregulare* EPA and AA production. Specifically the effects of C/N ratio on biomass formation, lipid accumulation and EPA and AA synthesis were investigated. The relationships among substrate utilization, biomass formation, lipid accumulation and the fatty acids synthesis were discussed.

2.2 Materials and Methods

The microorganism used was *Pythium irregulare* ATCC 10951. Approximately 1 square centimeter culture from PDA (potato dextrose agar, Fisher Scientific) plate was inoculated to 100 ml media, which consisted of 2% glucose, 0.5% yeast extract (Fisher Scientific) and 0.1% KH_2PO_4 with natural pH (about 6.5), incubated at 25 °C with shaker speed of 150 rpm for 2 days at orbital shaker (KC-25D, New Brunswick Scientific). The culture was then blended with a polytron homogenizer at high speed for 10 seconds, 5 ml of the blended culture was inoculated to 45 ml of media in 250 ml culture flask.

A 2⁴ factorial design (Kuehl, 2000) was conducted for media composition. Glucose (Fisher Scientific) and yeast extract (Fisher Scientific) were added in the amount

of 1%, 2%, 3%, 4% and 0.25%, 0.50%, 0.75%, 1%, respectively. In addition, 0.1% KH_2PO_4 was added to all media and the pH was adjusted to 6.5. Then 45 ml of medium was added to 250 ml flask and autoclaved at 121 °C for 15 min. The cultures were incubated at 25 °C in an orbital shaker (KC-25D, New Brunswick Scientific) with speed of 150 rpm. Triplicate samples of each treatment were harvested after 5 days of incubation for analysis.

The cultures were vacuum filtered with Whatman No.1 filter paper to separate biomass and broth. The mycelium was freeze-dried at -40 °C and 0.15 Mbar vacuum (Labconco) until constant weight was obtained. Biomass-free broth was used to determine COD and reducing sugar concentration. COD determination followed the APHA method (1995) and reducing sugar determination adopted the DNS method (Mandels, 1976).

The method of Lepage (1984) was modified and used to extract lipids. A 10ml hexane-isopropanol solution (3:2, v/v) was added to the dried mycelium in a 45 ml centrifuge tube, homogenized with the polytron for 1 min, and centrifuged at 5000 rpm for 10 min. This extraction procedure was repeated twice for the residue and the three supernatants were combined. Ten ml of 0.47 M sodium sulfate was added to the supernatant to break the emulsion. The upper phase containing purified lipid was transferred to another tube and evaporated to dryness under a stream of nitrogen in a 45°C water bath. The dry weight of the residue was determined as the weight of total lipids.

A rapid transmethylation method based on the procedure outlined by Christie (1982) was applied in this study. Twenty μl of methyl acetate was added to 1 mL of

sample to suppress any competing hydrolysis and then 20 µl of sodium methoxide (1M in dry methanol) acted as a basic catalyst to form fatty acids methyl esters (FAME). The mixture was then vortexed and allowed to react for 5 min. Then 30 µl of acetic acid (1M in dry methanol) was added to stop the reaction. The reacted sample was placed in a 2 mL vial for GC analysis. The FAMEs were determined with capillary gas chromatograph (Shimazu GC-17A) equipped with a FID detector and a 30 m×0.25 mm CP wax 52 CB capillary column (Chrompack Inc, Netherlands). The GC programming is shown in Appendix A. FAME-37 mixture and nonadecanoic acid (C19:0) methyl ester (Supelco) were used as the standard and internal standard, respectively. The quantification of FAME were accomplished using the internal standard method as follows:

$$C_s = \frac{C_{is} * A_s * RR_f * D_f}{A_{is}}$$

where

C_s = concentration of sample

C_{is} = concentration of internal standard

A_s = area of sample

A_{is} = area of internal standard

RR_f = relative response area for individual fatty acid to internal standard (see appendix B)

D_f = dilution factor

2.3 Results and Discussion

2.3.1 Media Composition and C/N Ratio

Glucose contains 40% carbon and yeast extract contains approximately 10% nitrogen by weight. The 16 media (glucose and yeast extract combinations with addition

of 0.1% KH₂PO₄) based on 2⁴ factorial design and their corresponding C/N ratio are listed in Table 2.1. The C/N ratio was calculated as follows:

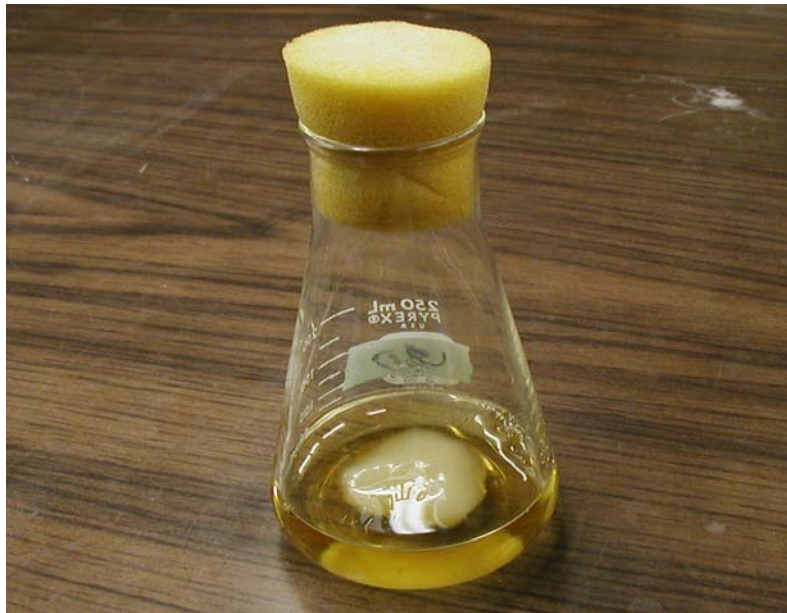
$$C/N = \frac{GlucoseMass * 40\%}{YeastExtractMass * 10\%}$$

Table 2.1 The Media Composition and the Corresponding C/N Ratio

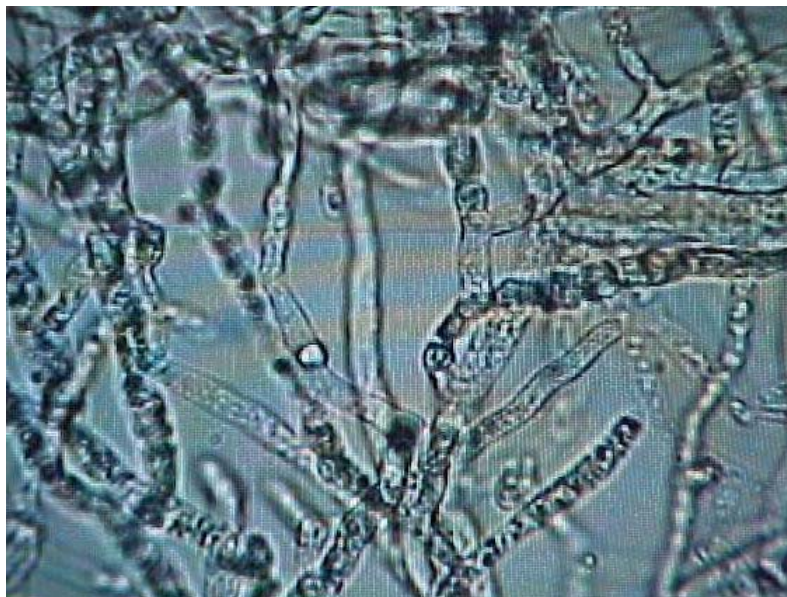
Medium No.	Glucose % (w/v)	Yeast extract % (w/v)	C/N	Medium No.	Glucose % (w/v)	Yeast extract % (w/v)	C/N
1	1	0.25	16	9	3	0.25	48
2	1	0.50	8	10	3	0.50	24
3	1	0.75	5.3	11	3	0.75	16
4	1	1.0	4	12	3	1.0	12
5	2	0.25	32	13	4	0.25	64
6	2	0.50	16	14	4	0.50	32
7	2	0.75	10.7	15	4	0.75	21.3
8	2	1.0	8	16	4	1.0	16

2.3.2 Biomass and Substrate Utilization Rates

A 2-day *Pythium irregulare* culture and its mycelium structure were shown at Fig. 2.1 (a) and (b). The fungal mycelium did not form well-shaped pellets, which are usually seen in filamentous fungal submerged culture. Similar to some other reports, the mycelium could be described as flocculus. However, the mycelium structure under microscope was very typical as filamentous fungus. The biomass, reducing sugar and COD in the broth, lipid content in the biomass, and fatty acids were determined after 5 days incubation for all the cultures.



(a)



(b)

Fig. 2.1 (a) A *P. irregulare* ATCC10951 culture incubated for 2 days in glucose-extract medium
(b) Photomicrograph of the mycelium of *P. irregulare* ($\times 1000$)

The biomass yields are shown as Fig.2.2 (a). The biomass yields were affected by the initial carbon and nitrogen concentrations. Statistically, the interaction of glucose and yeast extract had significant effect on biomass formation ($P < 0.0001$, Table 2.2), which indicates that the C/N ratio played a predominant role for biomass synthesis. Because of this interaction, the main effects (glucose treatment and yeast extract treatment) may not be meaningful, even if both main effects were also significant ($P < 0.0001$). Therefore each level of treatment was considered separately. For a fixed glucose level, i.e. 1%, 2%, 3% or 4%, biomass yield increased when yeast extract level increased from 0.25% to 1%. A similar trend was seen for glucose when yeast extract level was fixed.

Table 2.2 Statistical Effects of Glucose, Yeast Extract and Their Interaction on Biomass

Source	Degree of freedom	Type III SS	Mean Square	F Value	Pr > F
Glucose	3	139.42	46.47	168.09	<0.0001
Yeast extract	3	44.70	14.90	53.89	<0.0001
Interaction	9	21.01	2.33	8.44	<0.0001

* The SAS code is presented in Appendix C.

At lower glucose levels (1% and 2%), except the one with a C/N of 32 (media No. 5), the glucose utilization rates were higher than 98%, along with 74-84% COD removal rates. When the glucose level increased to 3% and 4%, the glucose utilization and COD removal dropped significantly (Fig. 2.2 (b)). Normally, fungi are very sensitive to osmotic pressure in media. This low glucose utilization and COD removal rates possibly due to high osmotic pressure caused by the high substrate concentration thus inhibition of fungal growth.

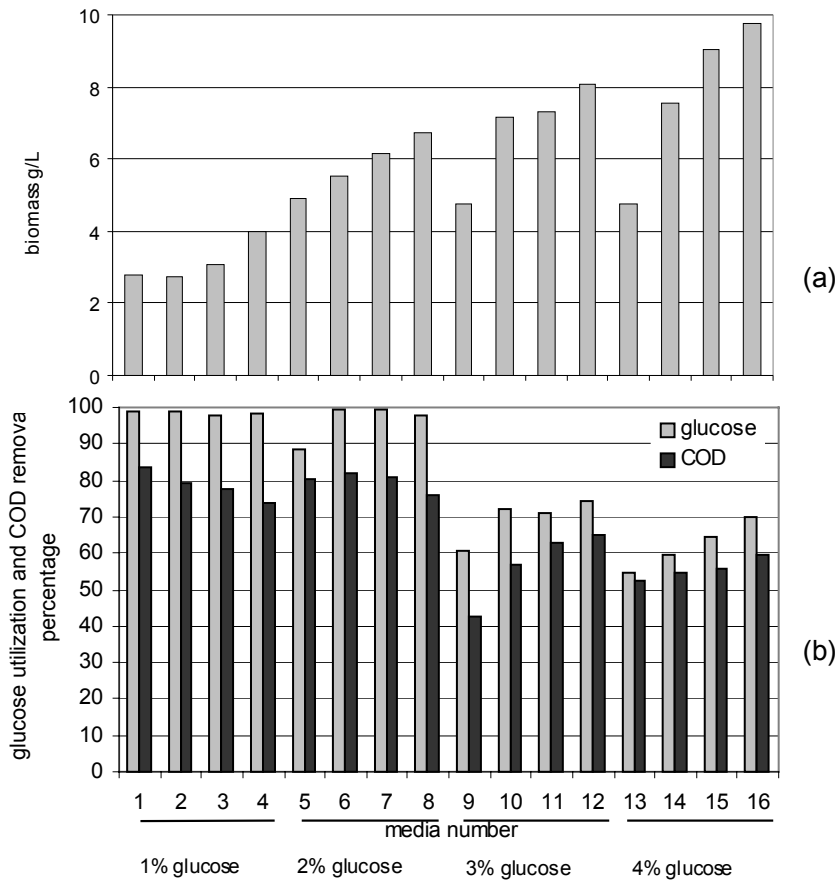


Fig. 2.2 (a) Biomass yield per volume broth (b) Percentages of glucose utilization and COD removal in different media after 5 day incubation

Since the total amount of substrate was different in each combination of media, the percentages of glucose utilization and COD removal were compared by grouping the media by glucose level or yeast extract level. For the 1% glucose group, of which the C/N ranged from 4 to 16, both percentages increased with an increase in C/N ratio. For the 2% glucose group, where the C/N ranged from 8 to 32, both percentages were the greatest at a C/N of 16. For the 3% and 4% glucose groups, where the C/N ranged from 12 to 48 and 16 to 64, respectively, both percentages decreased when the C/N ratio

decreased. These trends illustrated the nutrient presence for *Pythium irregulare* growth: carbon limitation (or nitrogen excess) occurred at C/N of 4-12 and carbon excess (or nitrogen limitation) occurred at C/N of 24-64.

The biomass yields to utilized glucose and removed COD, which reflected the efficiency of substrate bioconversion rate to biomass, are shown over C/N ratio (Fig. 2.3). The C/N from 12 to 24 formed a plateau for biomass yields to substrates. Therefore, the optimal C/N ratio for *Pythium irregulare* growth would be located in this range. For many filamentous fungi, the optimal C/N is around 20 (Zhu, 1999). Similar results were obtained by Yasuhisa on *Mortierella alpina* (2001).

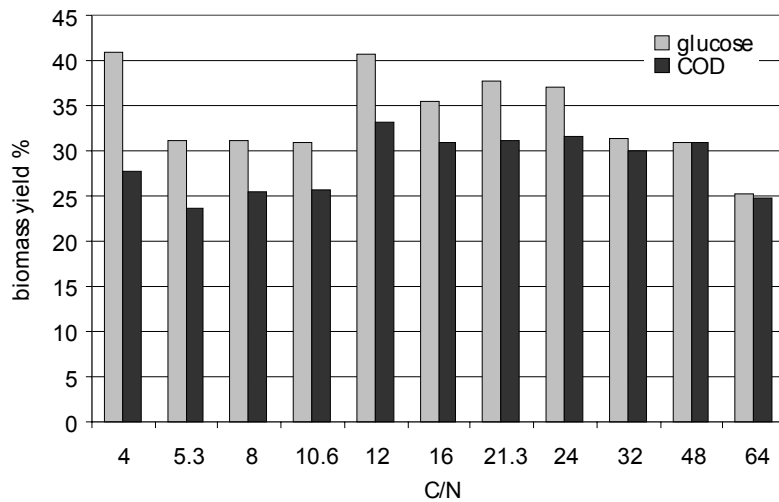


Fig. 2.3 Biomass yield to utilized glucose and COD for different C/N

Based on the biomass yield and substrate-cost-efficiency, the medium composed of 2% glucose and 0.5% yeast extract (with addition of 0.1% KH_2PO_4), with C/N of 16, was selected as the optimal medium for *Pythium irregulare* growth.

2.3.3 Lipid Content in the Biomass

In microbial cells, lipids exist as an important component of membrane and energy storage material in the form of oil.

Similar to the biomass yield results, the interaction of glucose and yeast extract significantly affected lipid content in biomass ($P=0.0001$). As seen in the previous section, the biomass yield and substrate utilization rate were affected primarily by glucose concentration, which could be divided into two groups, namely, low concentration (1% and 2%) and high concentration (3% and 4%). To avoid the possible misconception caused by pooling the same individual C/N from the different groups, the oil content in biomass vs C/N relationship is shown for the two separate groups (Fig. 2.4 (a) and (b)).

At the low concentration, where C/N ranged from 4 to 32, significantly high lipid content with 29.1% was achieved at C/N of 32. At the high concentration, where the C/N ranged from 12 to 64, the lipid content continuously increased to 27% in the subrange of C/N 21.3 to 64. Lipid accumulation could be triggered by nitrogen limitation (Kendrick, 1992). In this case, nitrogen limitation for *Pythium irregulare* may have occurred at the C/N of 32. The highest lipid content in biomass was obtained in the medium composed of 2% glucose and 0.25% yeast extract, corresponding to a C/N of 32.

Since the optimal C/N for lipid accumulation was out of the range of optimal C/N for biomass synthesis, a balance between biomass yield and lipid content in biomass was sought to achieve a high amount of total lipids. Lipid yield per volume broth, which was defined as the production of biomass per fermentation broth volume, served as an index. The highest lipid yield per volume broth (1.51 g/L) was obtained on the medium

composed of 4% glucose and 0.5% yeast extract, corresponding to a C/N of 32, followed by the medium composed of 2% glucose and 0.25% yeast extract, corresponding to a C/N of 32, of which the lipid yield was 1.43 g/L. If substrate-cost-efficiency was considered, another index, lipid yield to substrate would then apply. Considering that yeast extract is the predominately-cost component, the medium composed of 2% glucose and 0.25% yeast extract (C/N 32) achieved the highest yield of 0.286 g lipid/g yeast extract, which was much more efficient than any other medium combinations.

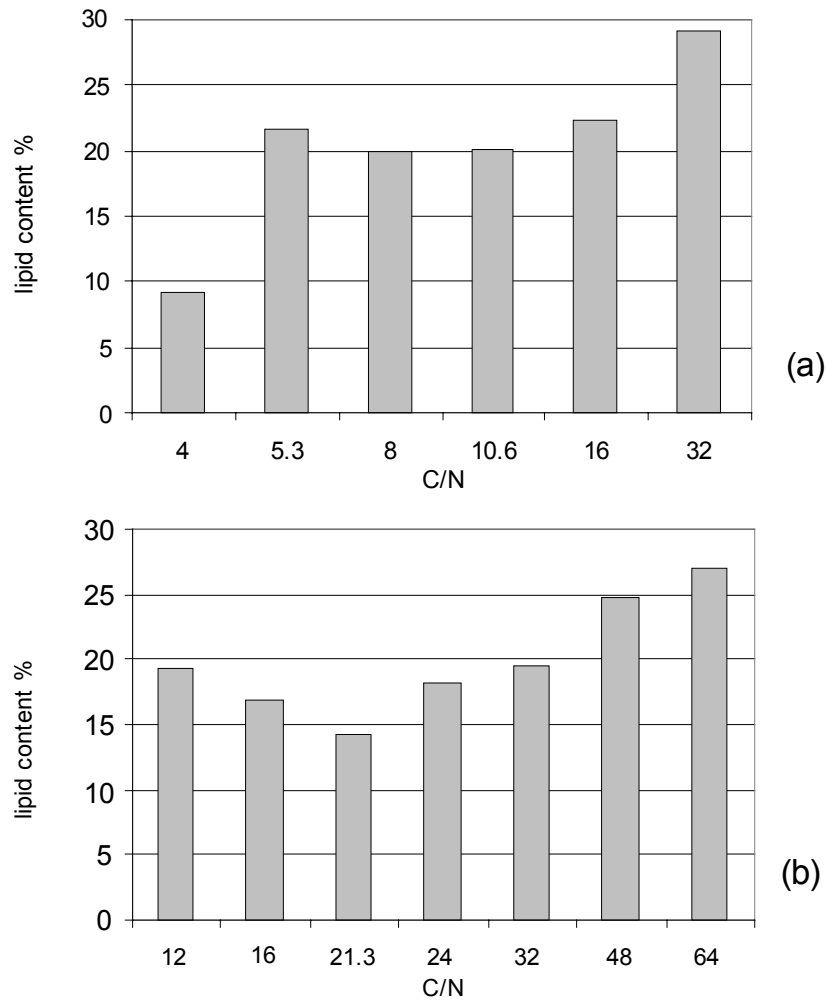


Fig. 2.4 Lipid content in biomass by C/N for (a) 1% and 2% glucose levels (b) 3% and 4% glucose levels

2.3.4 Fatty Acid Analysis

Fatty acids need to be converted into fatty acids methyl esters (FAME) for GC analysis. Fig. 2.5 shows an example of the FAME samples that were ready for GC analysis.



Fig. 2.5 FAME samples ready for GC analysis

The main fatty acids detected in *Pythium irregulare* lipids included myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), oleic acid (C18:1n9c), linoleic acid (C18:2n6c), γ -linolenic acid (GLA, C18:3n6), arachidonic acid (AA, C20:4n6) and eicosapentaenoic acid (EPA, C20:5n3), which was similar to O'Brien's report (1993). Stearic acid (C18:0), cis-11-eicosenoic acid (C20:1) and cis-8,11,14-eicosenoic acid (C20:3) were also detected, but the amount were relatively low. Fig. 2.6 shows a sample chromatogram showing these components. The main types of fatty acids remained the

same, but the yields of individual fatty acid varied depending on the growth media composition. The yields of these fatty acids are shown at Table 2.3.

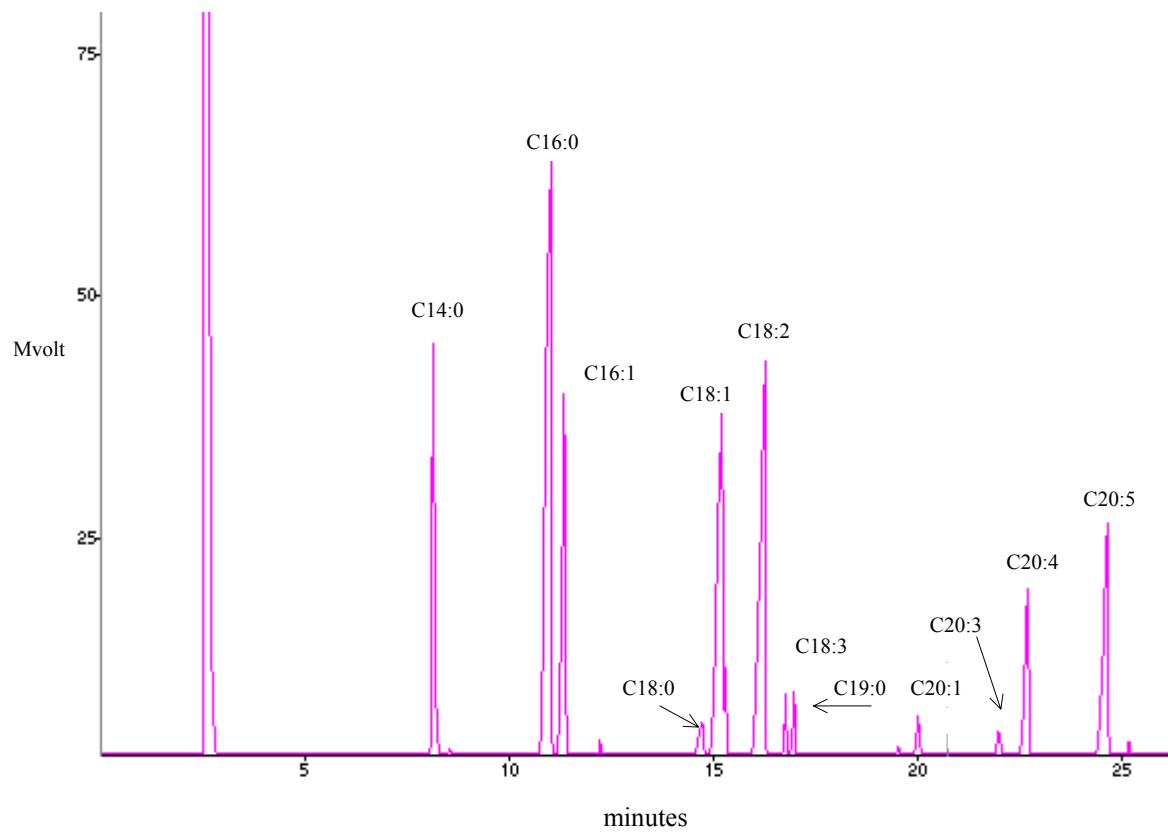


Fig. 2.6 Gas Chromatogram of a sample FAME

Table 2.3 Main Fatty Acid to Biomass Ratios for Medium Combinations Listed on Table 2.1 (mg/g)

Media No.	C14:0	C16:0	C16:1	C18:1	C18:2	GLA	AA	EPA
1	12.4	31.0	12.0	22.0	22.8	3.3	15.0	19.4
2	8.1	16.7	6.4	11.3	15.6	5.7	11.7	15.0
3	9.5	19.9	7.8	13.2	19.9	6.1	12.0	16.5
4	4.7	11.1	3.7	7.0	10.9	2.9	7.6	10.9
5	17.5	45.4	17.2	33.6	26.9	3.6	14.7	20.0
6	14.0	37.4	13.8	25.6	24.8	2.6	15.3	17.4
7	12.0	28.1	10.7	20.8	20.0	3.5	12.6	16.7
8	10.8	24.2	8.4	17.6	19.6	2.8	12.5	18.3
9	10.7	28.0	11.0	23.8	18.4	2.2	10.8	13.6
10	9.4	25.0	9.1	19.7	13.6	2.3	7.5	11.3
11	12.0	32.3	11.7	22.9	19.3	2.8	11.0	14.4
12	12.4	34.0	11.8	23.2	21.1	2.9	11.9	18.3
13	14.3	38.0	14.0	33.1	21.9	3.1	13.7	17.2
14	12.7	35.8	12.6	27.2	18.8	3.0	10.4	13.7
15	8.7	26.5	9.9	19.1	15.5	2.1	9.1	9.0
16	6.6	18.1	6.6	13.1	10.1	1.4	5.4	7.1

EPA and AA were the target PUFAs in this research, where the aim was to maximize these PUFA yields. The fatty acid yields per volume broth were related with the biomass yields. Same as the effect on biomass, the effect of interaction of glucose and yeast extract was significant for both EPA and AA yields per volume broth ($P < 0.0001$, Table 2.4).

Table 2.4 Statistical Effects of Glucose, Yeast Extract and Their Interaction on EPA and AA Yields

EPA					
Source	Degree of freedom	Type III SS	Mean Square	F Value	Pr > F
Glucose	3	23485.10	7828.37	181.34	<0.0001
Yeast extract	3	2320.12	773.39	17.91	<0.0001
Interaction	9	11995.80	1332.87	30.87	<0.0001
AA					
Glucose	3	12972.96	4324.32	147.37	<0.0001
Yeast extract	3	575.15	191.72	6.53	0.0014
Interaction	9	5601.76	622.42	21.21	<0.0001

To investigate the C/N effect on the EPA and AA yields to biomass, the 16 medium combinations were separated as low glucose concentrations and high glucose concentrations, as done previously for oil content in biomass. The EPA and AA yields vs C/N for low and high glucose concentrations are presented in Fig. 2.7 (a) and (b), respectively. For low glucose concentrations, where C/N ranged from 4 to 32, the EPA and AA yields increased with increasing C/N, the trend was similar to that of lipid content in biomass (see Fig. 2.4 (a)). However, for high glucose concentrations, where C/N ranged from 12 to 64, the trend was less clear than that of low glucose concentrations. The highest yields of the EPA and AA were 20 mg/g and 14.7 mg/g, respectively, which was achieved in the medium composed of 2% glucose and 0.25% yeast extract with a C/N of 32. The results are in the similar range of Cheng's results, where similar glucose-yeast extract media were used by *Pythium irregulare*. This medium also produced the highest oil content (29.1%) in biomass. The EPA and AA yields per volume in this medium were 98 mg/L and 72 mg/L, respectively. Though these

two values were not the highest in all the medium combinations, if EPA and AA yield per unit yeast extract was applied as done for the lipid in Section 2.2.4, this medium had much higher substrate-cost-efficiency than any other media. Therefore, it was adopted as the optimal medium for the EPA and AA production in the following studies (Chapter 3 and 4).

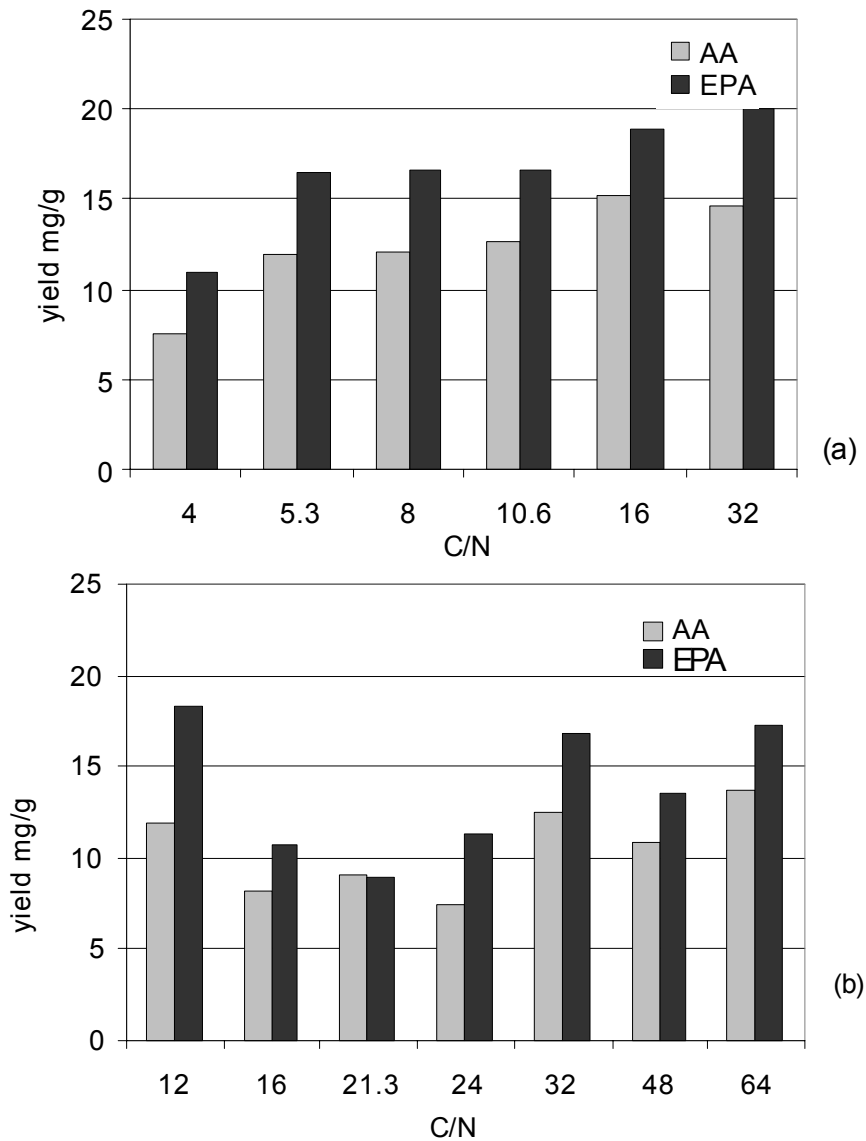


Fig. 2.7 AA and EPA yields to biomass by C/N for (a) 1% and 2% glucose levels (b) 3% and 4% glucose levels

The ratio of EPA to AA in most media (14 of 16 media) were located in a narrow range of 1.3 to 1.5, which was not affected greatly by the quantity of media nutrients or C/N. Shimizu (1988) indicated that the enzyme system in certain fungi catalyzing the conversion of AA to EPA was activated by cold temperatures.

For the other main fatty acids, their yields to biomass were proportional to oil content in biomass. Palmitic acid (C16:0) had the highest yield per unit biomass among all the individual fatty acids in all the medium combination. Oleic acid (C18:1n9c) and linoleic acid (C18:2n6c) yields were also high. Interestingly, at 1% glucose level, the oleic acid had higher yield than linoleic acid, but at rest of the three glucose levels, linoleic acid had greater yield. More interestingly, at each glucose level, the oleic acid/linoleic acid ratio decreased with increasing yeast extract levels, which may indicate that nitrogen has a potential to inhibit the desaturation of fatty acid.

Compared with yields of oleic acid and linoleic acid, the yield of another important C₁₈ fatty acid, γ -linolenic acid (GLA, C18:3n6), was low. In most media, the ratio of γ -linolenic acid to linoleic acid was around 15%. However, significantly higher ratios were observed at 1% glucose level with yeast extract addition of 0.5%, 0.75% and 1%, where the ratios were 37%, 31% and 27%, respectively. Again, the effect of increasing nitrogen levels decreased GLA levels for the fixed 1% glucose level.

CHAPTER 3

TEMPERATURE OPTIMIZATION AND KINETIC MODELING

3.1 Introduction

Temperature is considered as an important factor for polyunsaturated fatty acid (PUFA) production. Research has shown that low temperatures may stimulate PUFAs production, especially EPA synthesis (Stinson, 1991; O'Brien, 1993; Cheng, 1999). To achieve efficient PUFA production, a culture technique called “temperature shifting” was applied in these studies. With this technique, the microorganism was incubated at optimal growth temperature (normally 25°C for fungi) for a certain period until the nutrients were exhausted and the culture was then incubated at a lower temperature for a defined period. However, some discrepancies with the effectiveness at this technique exist. Opposite results were seen in Gandhi's (1991) report. Shimizu (1988) indicated that at low temperatures, the enzyme system in certain fungi catalyzing the conversion of AA to EPA would be activated. As a result, low temperature incubation may increase EPA production on the expense of decreasing AA production.

In most PUFA microbial fermentations, studies were mainly focused on the final target PUFA production, while the biomass, substrate utilization, lipid and fatty acids changes with time were rarely reported. From an engineering perspective, these kinetic relationships are essential for fermentation process control and optimization. Furthermore, this information gives greater insight at the metabolic activities of the microorganism and could be applied to fermentation models that could be helpful to the process utilizing other substrates as feedstock.

In the previous chapter, the media was optimized for EPA and AA production. The objective of the second study was to investigate the biomass, substrate utilization, lipids and individual PUFA profiles during the fermentation process at different temperatures. The results were then applied to determine an optimized incubation temperature mode (with or without “temperature shifting”) for target PUFA production. From the growth kinetic data, microbial growth models were applied to determine the appropriate kinetic parameters.

3.2 Materials and Methods

Unless specified, the materials and methods were the same for this study as indicated in Section 2.1.

The glucose-yeast extract media were composed of 2% glucose, 0.25% yeast extract and 0.1% KH_2PO_4 (w/v), adjusted initially to pH of 6.5.

Temperature setting was as follows: Three constant incubation temperatures were set at 13 °C, 19 °C and 25 °C in shaker. Duplicate samples were taken daily. For the 25 °C incubation temperature, six cultures were shifted to 13 °C after a defined incubation period (3 days, 4 days or 5 days) until the 7th day to harvest to investigate temperature shifting effect.

3.3 Results and Discussion

3.3.1 Substrate Utilization and Growth Curves at Different Temperatures

Mechanisms for growth of filamentous fungi are very different from those of unicellular microorganisms. All cells in the multi-cellular structures may contribute to the growth process by extension and branching of hyphae (see Fig. 2.2. (b)). As a consequence, the growth curves of filamentous fungi are different from those of

unicellular microorganisms. The most obvious phenomenon is that the filamentous fungi do not have exponential phase, but a so-called “rapid phase”.

For the three experimental temperatures, *Pythium irregulare* showed typical growth characteristics of filamentous fungi. Growth curves for the three temperatures are presented in Fig.3.1 (a). There was no significant difference for the maximum biomass production among the three temperatures, which were 8.84 g/L, 8.56 g/L and 8.28 g/L, achieved at the 4th, 6th and 8th days for 25 °C, 19 °C and 13 °C, respectively.

Filamentous fungal mycelium has potential to form pellets in submerged culture. For pellet-formed fungal growth, Pirt (1966) developed a model to describe the growth, which is also known as the “Cube-root model”, as follows:

$$X_B^{1/3} = X_{B0}^{1/3} + \frac{k * (36\pi\rho)^{1/3} * t}{3}$$

Where

X_B : biomass concentration

X_{B0} : initial biomass concentration

k : derivation of microbial pellet radius over incubation time (dr/dt)

ρ : density of the microbial pellet

t : incubation time

The Pirt model could be simplified as follows by substituting with K , which is related to growth rate.

$$X_B^{1/3} = X_{B0}^{1/3} + K * t$$

where

$$K = \frac{k * (36\pi\rho)^{1/3}}{3}$$

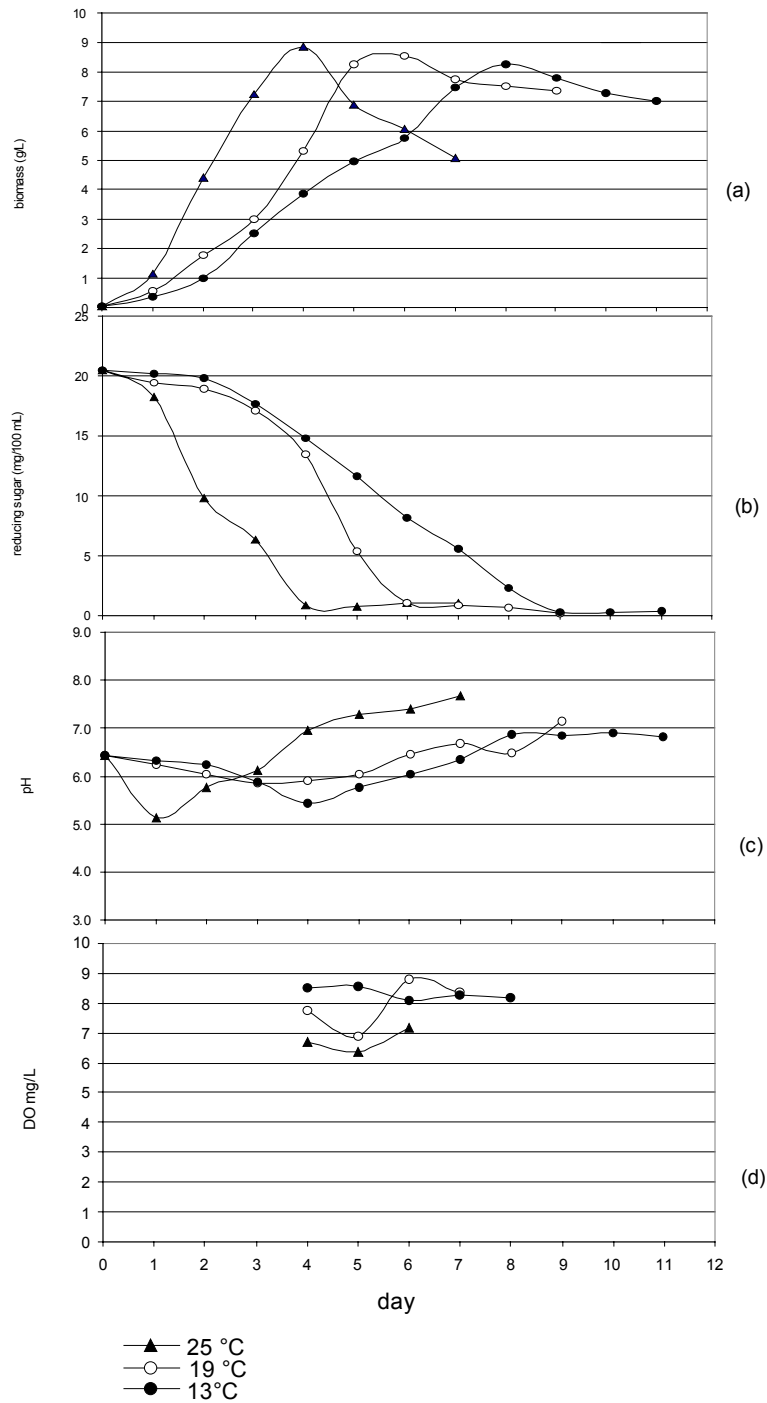


Fig. 3.1 (a) Growth curves (b) Reducing sugar in broth (c) Broth pH (d) Broth DO for *Pythium irregulare* grown on 2% glucose and 0.25% yeast extract media for the three experimental temperatures

In this research, a disk-shaped mycelium flocculus was formed instead of a pellet in the orbital shaker. There was no direct growth model for a disk-shaped mycelium floc. However, the simplified Pirt Model was applied to the active growth data for the three temperatures and corresponding linear regressions were obtained as shown in Fig. 3.2.

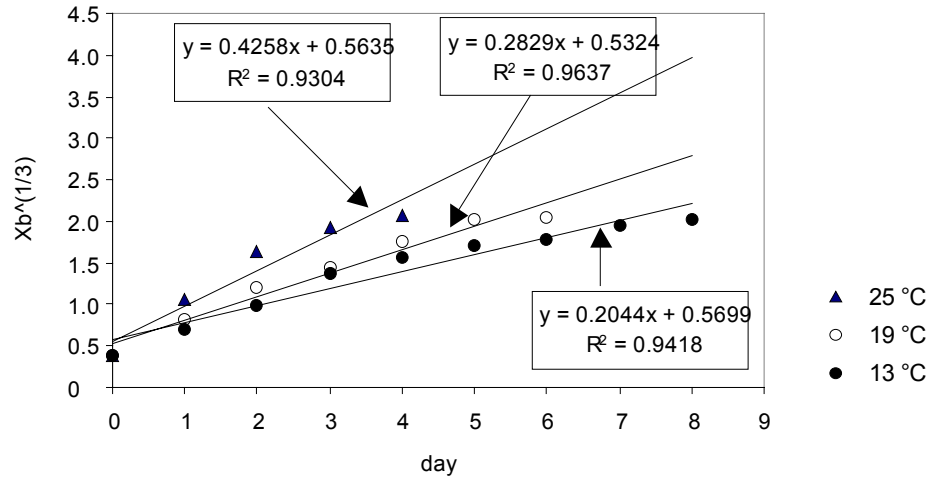


Fig. 3.2 Linear regressions of Pirt Model for *P. irregulare* active growth in glucose-yeast extract media at the three temperatures

As a result, three functions with reasonable accuracy were obtained as follows (in the unit of g/L):

$$25\text{ }^{\circ}\text{C:} \quad X_B^{1/3} = 0.4258t + 0.5635, R^2 = 0.9304, t=1 \text{ to } 4 \text{ days}$$

$$19\text{ }^{\circ}\text{C:} \quad X_B^{1/3} = 0.2829t + 0.5324, R^2 = 0.9637, t=1 \text{ to } 6 \text{ days}$$

$$13\text{ }^{\circ}\text{C:} \quad X_B^{1/3} = 0.2044t + 0.5699, R^2 = 0.9418, t=1 \text{ to } 8 \text{ days}$$

K ($\text{g}^{1/3}/(\text{L}^{1/3} \cdot \text{day})$) was a parameter whose value relies on microbial growth rate.

Since all culture conditions except incubation temperature were the same, the difference among the three K parameters was considered to be completely due to the difference in

temperature. For the experimental temperature ranges, a higher temperature resulted in a higher K value.

The influence of temperature on growth rate of a microorganism was similar to that observed for the activity of an enzyme. For the temperature between the minimum and maximum growth temperature, the relationship could be expressed as the Arrhenius function (Nielsen and Villadsen, 1994):

$$u = A * \exp\left(-\frac{E_g}{RT}\right)$$

Where

u : growth rate

A: constant

E_g : the activation energy of the growth process (J/mole)

R: the Arrhenius Constant (J/kg.mole)

T: the absolute temperature (K)

Because K was a parameter describing growth rate, it was substituted for u in the above function. Then the Arrhenius function was converted to the following:

$$\ln K = \ln A - \frac{E_g}{R} * \frac{1}{T}$$

The $\ln(K)$ over $1000/T$ is plotted in Fig. 3.3. A linear regression from the three points was obtained. The Arrhenius parameters, A and E_g , for *Pythium irregulare* active growth in this glucose-yeast extract media were then obtained with the values of 2.24×10^8 and 44.5 KJ/mole, respectively.

Temperature affected not only the growth rate, but also other metabolic activities, such as substrate consumption and pH changes. At higher temperatures, the substrate

consumption rates were higher. Reducing sugars retained in the broth were monitored during the fermentation period (Fig.3.1 (b)). The changes in reducing sugar levels matched the biomass trends at the corresponding temperature. Carbon source exhaustion appeared when the maximum biomass production was achieved, which occurred on the 4th, 6th and 9th days for the temperatures of 25 °C, 19 °C and 13 °C, respectively. At these points, about 95% of the glucose was utilized.

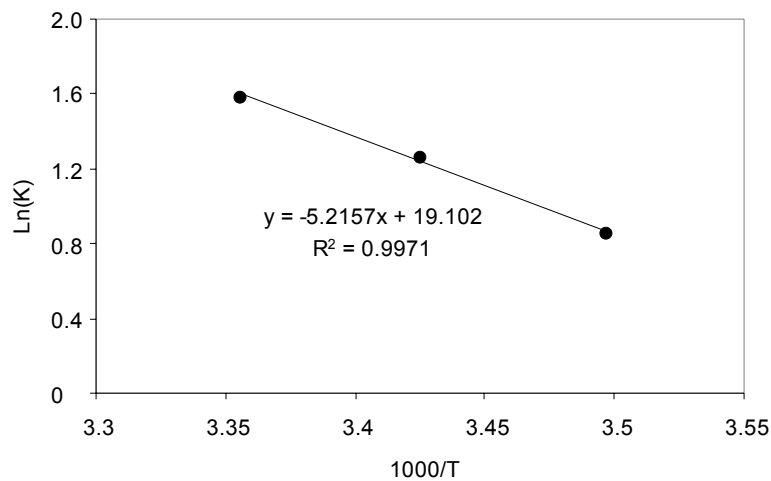


Fig. 3.3 The influence of temperature on parameter K from Pirt Model for *P. irregulare* active growth in glucose-yeast extract media

The pH changes were typical (Fig. 3.1 (c)) for fungal cultures where pH decreased during the early growth phases due to ammonia consumption from the media as well as due to carbonic acid formation resulting from the aerobic carbon source consumption. Because of the difference of metabolic rates with temperature, the pH bottom point appeared earlier at higher temperatures. Then the pH increased owing to biomass decay and the disappearance of the carbon source from the media.

Dissolved oxygen (DO) in the broth was monitored in the stable growth phase as shown in Fig. 3.1 (d), where higher temperatures resulted in higher DO values. The DO value for the room temperature air was 7.52 mg/L. It was assumed that oxygen limitation was negligible at the surface of mycelium flocculus during fermentation for all the three temperatures. However, oxygen limitation could occur inside the flocculus when it got certain size.

3.3.2 Lipid and Fatty Acid Profiles

Existing as a component of the cell membrane and as an energy-storage substrate, the fungal lipids are a mixture of primary and second metabolites. Profiles of lipid content in biomass and lipid yield per volume broth for the three temperatures are shown in Fig. 3.3. Lipid content in biomass increased in the active growth period for the three temperatures (Fig. 3.4 (a)). For temperatures of 25 °C and 19 °C, lipid content continued to increase until one day after the biomass peak, then kept relatively stable around 32% and 17%, respectively. For 13 °C, the maximum lipid content occurred when the biomass peaked and remained stable for 2 days around 17%. Unlike other reported literature (Stinson, 1991; O'Brien, 1993; Cheng, 1999), the lower temperature in this study did not enhance lipid content in biomass. Instead, at 25°C, the lipid content in the biomass was much higher than the other two lower temperatures.

The stationary phase is fairly short for filamentous fungi. The biomass dropped quickly after biomass peak possibly due to oxygen deficiency inside the flocculus and thus occurrence of biomass decay. Even though the lipid content in biomass increased or remained stable after the maximum production of biomass occurred, the trends of lipid yield per volume broth were similar to these of biomass for all the three temperatures (Fig.

3.4 (b)). The maximum lipid yield per broth volume occurred when the biomass was maximized for all the three temperatures. The lipid yield reached 2.28 g/L, 1.26 g/L and 1.42 g/L at 25 °C, 19 °C and 13 °C, respectively. The lipid yield per volume broth harvested from 25 °C was much higher than those from the other two lower temperatures.

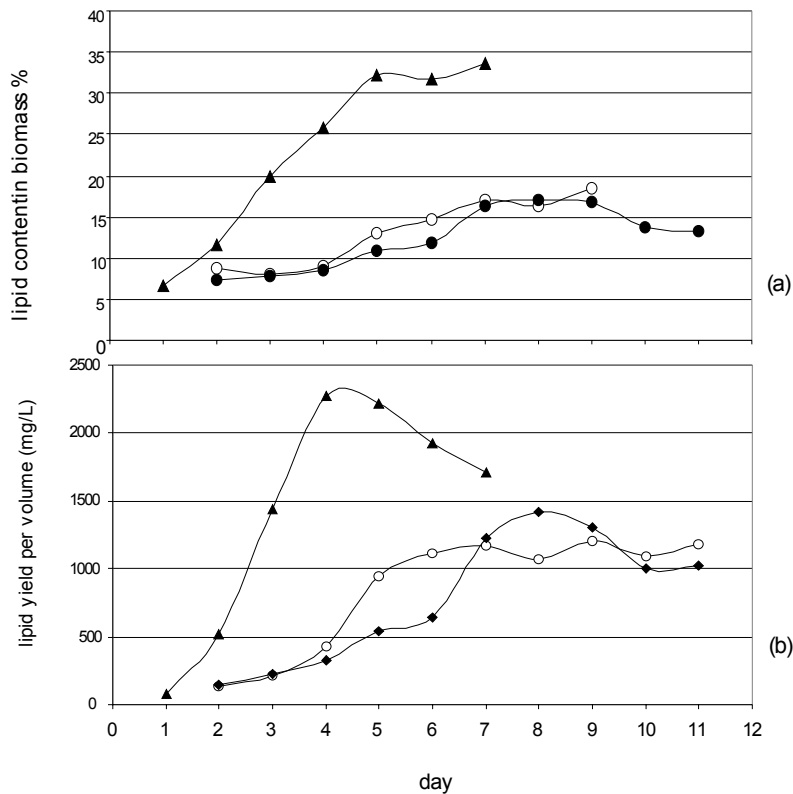


Fig. 3.4 (a) *P. irregulare* lipid content in biomass (b) Lipid yield per volume broth over time for the three experimental temperatures

Similar to the results stated in Section 2.3.3, the fatty acids detected in the fungal lipids included myristic acid (C14:0), myristoleic acid (C14:1), palmitic acid (C16:0), palmitoleic acid (C16:1), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), γ -linolenic acid (C18:3), arachidic acid (C20:0), cis-11-eicosenoic acid (C20:1), cis-11,14-eicosenoic acid (C20:2), cis-8,11,14-eicosenoic acid (C20:3), arachidonic acid (C20:4) and cis-5,8,11,14,17-eicosapentaenoic acid (C20:5).

The main fatty acids were C14:0, C16:0, C16:1, C18:1, C18:2, C20:4 and C20:5 at all the three temperatures. The profiles of these main fatty acid yields per volume broth under the three temperatures during incubation are shown in Fig. 3.5 (a), (b) and (c).

The trends of these individual fatty acid profiles at the three temperatures were nearly the same. At 25 °C, a plateau was formed during the 4 to 6 day period, where the lipid yields were also the highest. On the 4th day, maximum EPA yield was achieved at 124 mg/L and the AA yield was 75 mg/L. The maximum yield of AA (81 mg/L) was achieved on the 6th day, while EPA yield decreased to 109 mg/L. Both maximum EPA and AA yields appeared on the 4th and 5th days for 19 and 13 °C, respectively. However, even much more significant than that of lipid yields, all the main individual fatty acid yields were much lower than the corresponding yields at 25 °C. The EPA and AA yields for 19 °C were only 21 mg/L and 11 mg/L, respectively. At 13 °C, the values were 22 and 8 mg/L for EPA and AA, respectively.

For all the three temperatures, all the individual fatty acids dramatically dropped to near zero after peaks or peak plateau. The mechanism is not clear, but may be due to the release of fatty acids from cell to broth, where the fatty acids were not determined in this research; or the conversion of neutral fatty acids to free fatty acids, which could not be determined by the method used in this research. However, this phenomenon was important for setting the cutoff point of fermentation process. As a recommendation, the cutoff point for EPA and AA production for 13 °C, 19 °C and 25 °C were 5 days, 4 days and 4 days, respectively.

The results indicated that lower temperature had no advantage over the optimal growth temperature (25 °C) for EPA and AA production. Either from the point of

application or consideration of process maintenance cost, the incubation temperature of 25 °C was much more efficient. The possible reason for this result might be related to the lipid synthesis mechanism in the cell at different temperatures.

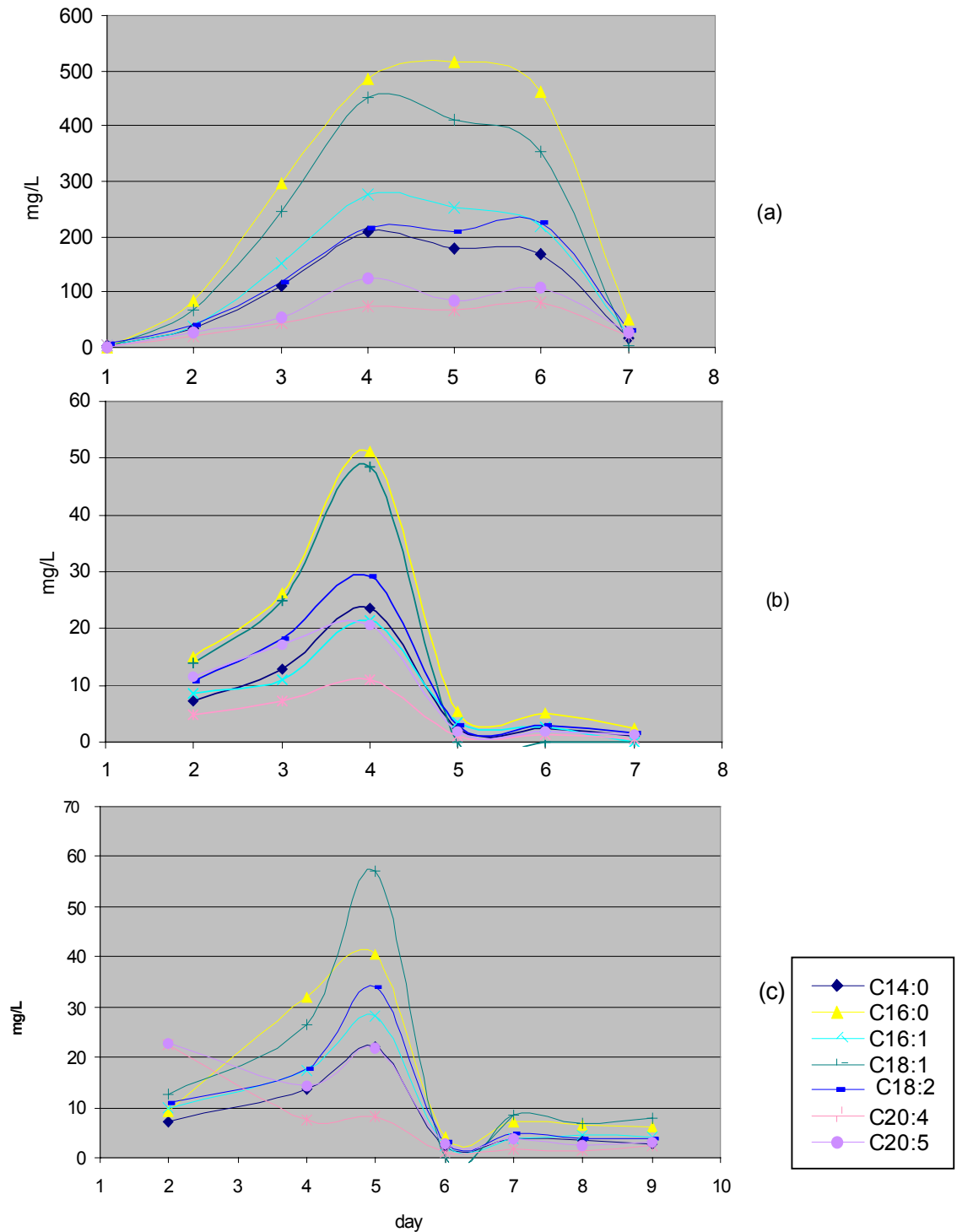


Fig. 3.5 Profiles of main fatty acid presented in *P. irregulare* lipids grown at (a) 25 °C (b) 19 °C (c) 13 °C

Though a lower temperature did not increase the fatty acid yields, the average EPA/AA ratio during incubation had increased by lowering the temperature. The ratios were 1.34, 1.88 and 2.05 at the temperatures of 25 °C, 19 °C and 13 °C, respectively. This may be due to the activation of enzymes at low temperature, which convert AA to EPA (Shimizu, 1988).

3.3.3 Effects of Temperature Shifting on Biomass and Lipid Yields

To test if the “temperature shifting” technique could enhance the EPA and AA yields, three strategies were carried out. Cultures were incubated at 25 °C for 3, 4 or 5 days, and then the incubation temperature was lowered to 13 °C until the cultures were harvested on the 7th day. The results were compared for samples incubated at 25 °C for 4 days and those incubated at 13 °C for 7 days. The biomass and lipid yields in these cultures are shown in Fig.3.6 (a) and the corresponding EPA and AA yields are shown in Fig. 3.6 (b).

Biomass yield decreased as the incubation period at 25 °C increased from 3 to 5 days, which may be due to biomass decay. However, the lipid yield per volume increased as the incubation period at 25 °C increased. The three-day incubation at 25 °C did not improve the increase in lipid yield, because nitrogen exhaustion had not yet occurred when the temperature was lowered. The lipid content in biomass was 34% and lipid yield per volume reached 253 mg/L when the culture was incubated at 25 °C for 5 days and then reduced to 13 °C for 2 days (denoted as 25-5 in the graph). This lipid yield was about 10% higher than that of lipids obtained at 25 °C for 4 days.

Similar to the lipid yield, both EPA and AA yield enhancement was only achieved when the fungus had a 5-day incubation at 25 °C followed by 2-day incubation at 13 °C.

The EPA yield reached 147 mg/L with a 19% increase and the AA yield was 97 mg/L with a 30% increase.

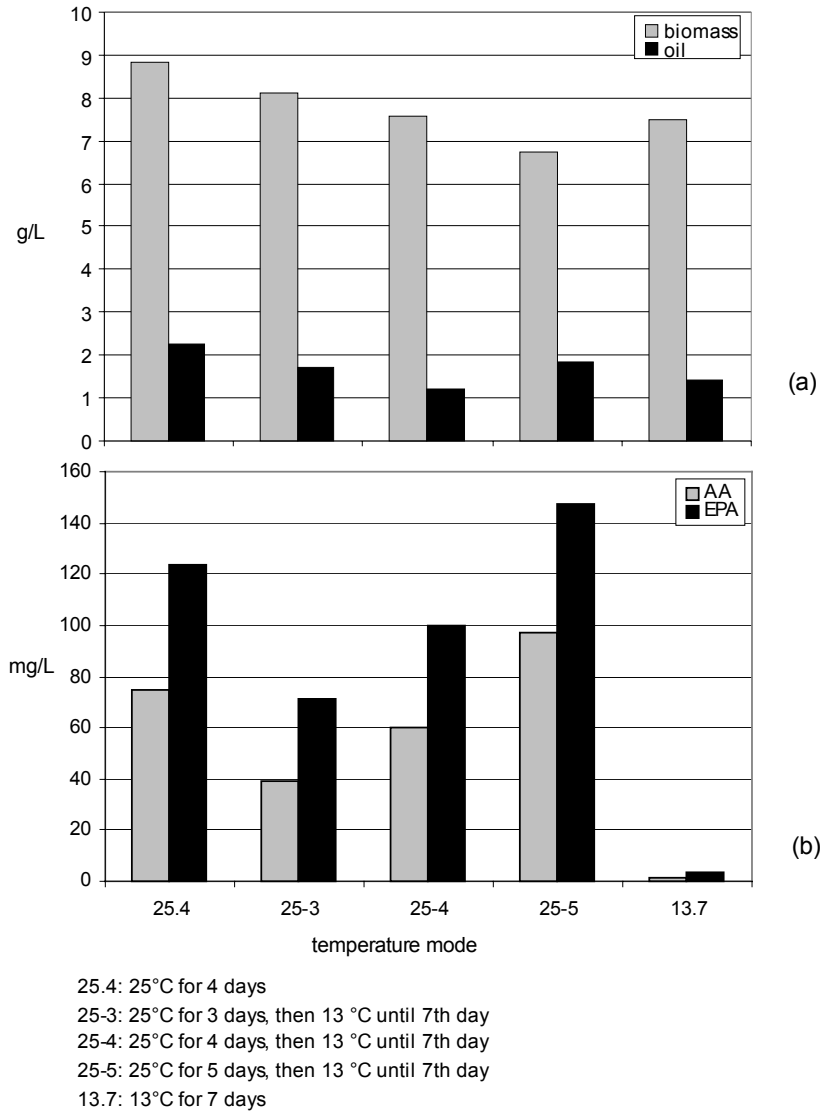


Fig. 3.6 Effects of temperature shifting on (a) *P. irregulare* biomass and oil yields (b) EPA and AA yields

In summary, when temperature was not shifted, the optimal lipid, EPA and AA production occurred at the incubation temperature of 25 °C; when the temperature shifting technique was implemented, greater yields occurred for cultures incubated for 5 days at 25 °C followed by 2 days at 13 °C.

CHAPTER 4

RICE BRAN UTILIZATION AS FERMENTATION FEEDSTOCK

4.1 Introduction

As discussed in Section 1.4, rice bran has the potential to serve as a substrate for fungal submerged fermentation for production of polyunsaturated fatty acids (PUFAs). The optimal glucose concentration for *Pythium irregulare* growth on glucose-yeast extract media determined in Chapter 2 was 2% glucose, with a C/N ranged from 12 to 24, while the optimal C/N for EPA and AA production was 32.

The estimated C/N (regardless of the utilization ratio of carbon and nitrogen sources) in rice bran was about 10. Theoretically, rice bran may serve well as an adequate substrate for *Pythium irregulare* growth in submerged fermentation if the initial rice bran concentration is set to about 5% (w/v) to achieve an approximate 2% usable carbon source.

The objective of this study was to investigate the feasibility of utilizing rice bran as a fermentation substrate for *Pythium irregulare* to produce EPA and AA based on the information reported in the previous chapters. Biomass yield, COD removal rate in the byproduct composed medium, lipid content, and target PUFA yields during the fermentation were then determined. The eventual goal for the research was to define a feasible fermentation process to utilize rice bran to produce EPA and AA by *Pythium irregulare*.

4.2 Materials and Methods

Unless specified, the materials and methods were the same as given in Sections 2.2 and 3.2.

Rough rice (*Cypress* variety) was obtained from Crowley Rice Research Station (LA) and rice bran was then obtained from the pilot-scale Sataki Rice Mill located in the Biological and Agricultural Engineering Department, LSU Agricultural Center. Rice bran was stabilized by heating in a microwave for 4 minutes until the internal temperature reached 110-115 °C to denature the enzymes which cause rice bran oil hydrolysis.

Stabilized rice bran was added to tap water to obtain approximately 5 % (w/v). The pH of the solution was about 6.5. 50 ml of the mixture was then placed in a 250 ml culture flask and autoclaved at 121 °C for 15 min. 50 ml rice bran medium was inoculated with 5 ml seed culture, then incubated at 25 °C. Triplicate samples were taken daily. After a 5-day incubation period at 25 °C, six flasks of cultures were shifted to 13 °C incubating for 3 or 4 days (three of each) and the samples were taken on the last day of incubation to investigate temperature shifting effect.

The cultures were vacuum filtered with Whatman No.1 filter paper. The total residue on the top of filter, which was the mixture of fungal mycelium and rice bran residue, was considered as total biomass. The biomass was then freeze-dried until its weight was constant.

4.3 Results and Discussion

4.3.1 Biomass, Reducing Sugar, COD and pH Profiles During Incubation

A sample of total biomass, which was the mixture of mycelium and rice bran particle residue, taken from a five days culture without filtering, is shown in Fig. 4.1.

Some insoluble rice bran particles still could be seen in the biomass as they embraced the fungal mycelium tightly.



Fig. 4.1 Harvested biomass on a plate: mixture of mycelium and rice bran residue

The total biomass relationship with incubation time is shown in Fig. 4.2 (a). Since rice bran hydrolysis and the fungal growth occurred simultaneously, the real biomass (pure mycelium) changes were not accurately represented by the total biomass data shown in this graph. However, the real fungal biomass at certain time should be higher than the “biomass” difference between that point of that certain day and the bottom point (the bottom point appeared at the day 1 as seen in the graph). For instance, the “biomass” difference between the 1st day and the 2nd day was 5.3 g/L, the real biomass yield on the 2nd day should be more than 5.3 g/L. This was because the rice bran hydrolysis by the microorganism was irreversible.

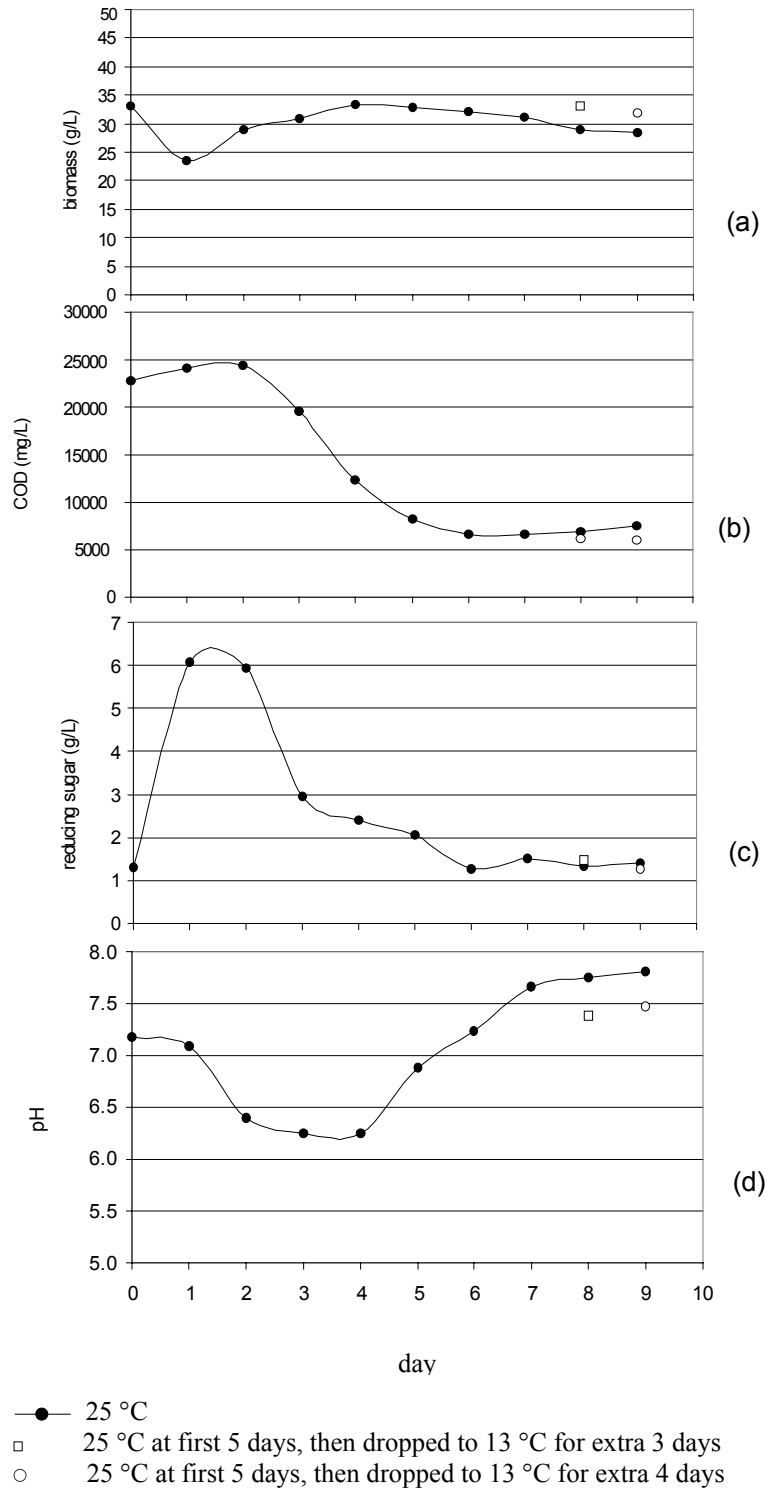


Fig. 4.2 (a) Total biomass (b) Soluble COD in broth (c) Reducing sugar in broth (d) Broth pH profile during the incubation period

The initial rice bran concentration in the media was 50 g/L. Some of its components were possibly hydrolyzed during the autoclave step. Excluding the soluble components and some of tiny particles in rice bran that passed through the Whatman No. 1 filter, the initial total biomass at day 0 was about 33 g/L. On the following day, new fungal mycelium formed, but the rate of mycelium synthesis was expected to be less than the hydrolysis rate of the rice bran's insoluble components due to the high enzyme activity of newly produced fungal enzymes. As a result, the total biomass dropped to 23 g/L after the first day of incubation. Then the biomass began to increase until the 4th day when it reached 33 g/L. The data suggested that the fungal biomass on the 4th day was higher than 9.83 g/L by the difference from the 1st day. Near the stationary growth phase, high enzyme concentrations were expected in the media causing the substrate hydrolysis rate to be potentially higher than the rate of mycelium synthesis. From observation of the samples, the fungal biomass continued to increase until the 6th or 7th day, followed by biomass decay, which occurred after the 8th day. The culture with temperature shifting (5 days incubation at 25°C followed by 3 or 4 days at 13 °C,) had significantly higher biomass yield compared to the corresponding cultures without temperature shifting (day 8 and day 9), which implied that biomass decay was delayed at lower temperatures.

Fungal growth was slower in the rice bran media compared with that of glucose-yeast extract media (Chapter 3), which may be accounted for by some of the nutrients in the rice bran not being utilized by the microorganism directly. These nutrients would be utilized only after they were hydrolyzed by the enzymes produced by the fungus. To investigate substrate hydrolysis and utilization, both chemical oxygen demand (COD) and soluble reducing sugars in the broth were measured during the incubation period

(Fig. 4.2 (b) and (c)). One point that should be mentioned was that the soluble COD in the broth included some tiny particles that could pass through the filter, causing the initial COD to be nearly 50% of the total substrates (50 g/L). The biomass change could be explained by the following: after one-day incubation, the microorganism produced certain enzymes to hydrolyze the substrate. As a result, the reducing sugars dramatically increased from 1.3 g/L to 6.1 g/L and the COD also increased until the 2nd day. Along with the growth, soluble nutrients were taken up by the fungus and the reducing sugars and COD continued to decrease until decay occurred on the 7th or 8th day. During that period, substrate hydrolysis continued, but it was predominated by substrate consumption. The original rice bran medium and biomass-free broth from a five-day culture are shown in Fig. 4.3. The original medium was rather turbid, but after a five-day-incubation, the broth was much clearer, which indicated that most of the particles in the medium were hydrolyzed or removed.

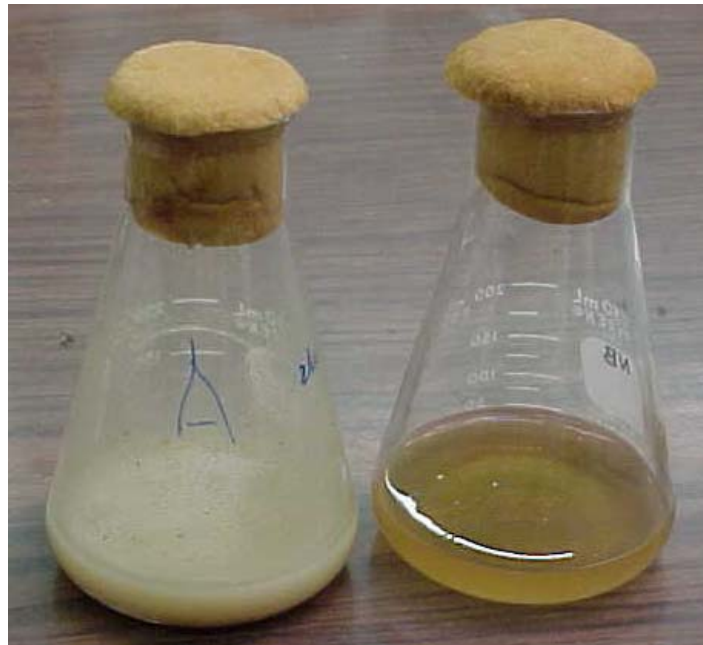


Fig. 4.3 Rice bran medium before (left) and after (right) 5-day fermentation

The pH changes in the rice bran media (Fig.4.2 (d)) were similar to that of glucose-yeast extract media. The pH decreased during the early growth phases due to ammonia consumption from the media as well as carbonic acid formation resulting from the aerobic carbon source consumption. Then the pH started to increase owing to biomass decay and the disappearance of carbon source in the media.

The effects of temperature shifting on growth and nutrient consumption were also typical. When the incubation temperature shifted from 25 °C to 13 °C, the metabolic activities were slowed and biomass decay was delayed. Therefore, biomass concentration in the temperature-shifted culture was higher and COD in the broth and pH were lower than corresponding cultures without temperature shifting.

4.3.2 Lipid and Fatty Acid Profiles During the Incubation

Rice bran used in this study contained 18.8% lipids, some of which could be destroyed in the medium heat-sterilization step. After sterilization, the collected rice bran residue from the medium, which was considered as biomass at day 0, only contained 62% amount of original lipids from raw rice bran. Possibly, during sterilization, a process similar to hot-water oil extraction occurred, which led to a 38% loss in biomass lipids. The percentage of lipids in the mixture of mycelium and rice bran residue during incubation is presented in Fig. 4.4 (a). The percentage continued to increase in the active growth phase, then formed a plateau in the 6th to 8th day. On the 6th day, the percentage was as high as 28.6%, which was about 10% higher than that of raw rice bran. To illustrate this more directly, the lipid yield per volume is shown in Fig. 4.4 (b). The highest lipid yield was obtained on the 6th day at 9.21 g/L, with a 57% increase from the 0th day. This increase was completely attributed to fungal lipid synthesis. Lipid yield in

the rice bran medium prior to sterilization was 9.4 g/L. After fungal bioconversion, 98% of the lipids were recovered. More importantly, the fatty acids in the lipid fraction might change with addition of those of greater value attribute to ability of *Pythium irregulare* to synthesize some high-value PUFAs, such as EPA and AA.

Unlike the results in glucose-yeast extract media (Section 3.3.3), neither the lipid content in biomass nor lipid yield per volume of the temperature-shifted samples were as high as the peak values obtained from the constant-temperature samples.

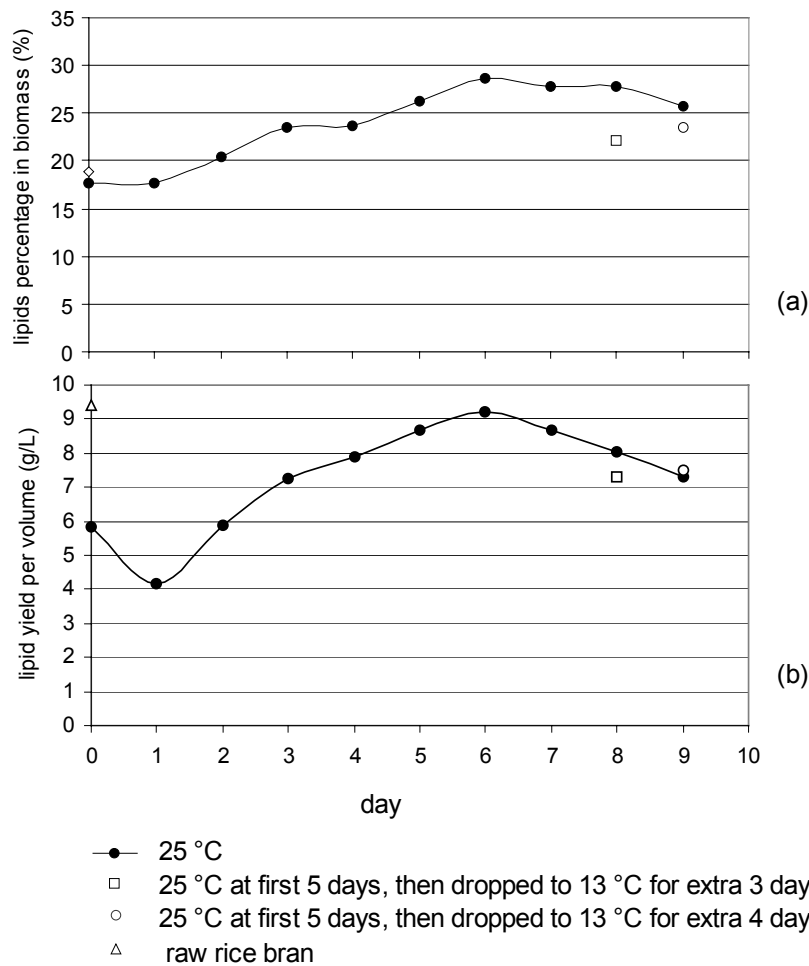


Fig. 4.4 (a) Lipid content in total biomass (fungal biomass and rice bran)
 (b) Lipid yield per volume during the incubation period

Fatty acids in the experimental rice bran and the rice bran residue collected in the media after sterilization (considered as biomass at day 0) were determined. The types of main fatty acids remained the same in rice bran and the residue, and their percentages were changed slightly (Table 4.1). However, the biomass loss was significant, only 33 g/L rice bran residue was obtained after sterilization while originally 50 g rice bran was added to 1 L medium. The individual fatty acid yields in the biomass of day 0 were about 40% lower than that of rice bran. EPA and AA were not detected in rice bran and the biomass.

Table 4.1 Fatty Acid Percentages in Rice Bran and The Rice Bran Residue Collected from Media after Sterilization (day 0)

fatty acid	C14:0	C16:0	C16:1	C18:0	C18:1n9c	C18:2n6c	C18:3n6
rice bran	0.05	2.39	0.08	0.12	7.11	5.55	0.24
residue	0.04	2.23	0.07	0.12	6.85	5.25	0.23

The time profiles of main individual fatty acid yield per broth volume during the bioconversion process are presented in Fig.4.5 (a)-(k). On the first day of incubation, all the fatty acids that were initially contained in rice bran were degraded possibly due to substrate hydrolysis; their yields dropped significantly. With fungal growth, new fatty acids were synthesized and the yields increased. EPA and AA were detected from the 1st day of incubation, which was attributed to fungal lipid synthesis.

The peaks of C₁₆ and C₁₈ fatty acids appeared on the 3rd or 4th day. Their yields were higher than the yields in the initial media, which indicated more of these fatty acids were being produced by the fungus. C₁₈ fatty acids are precursors of C₂₀ fatty acids, which may explain why C₂₀ fatty acids peaked later than C₁₈ fatty acids. The peaks of

C20:0, C20:1 and C20:4 appeared on the 6th day while the peak of C20:5 appeared on the 7th day. This delay possibly due to the desaturation process. Maximum EPA yield was 207 mg/L, which was achieved after 7 days incubation, where the AA yield was 70 mg/L. Maximum AA yield was 82 mg/L, which was achieved after 6 days incubation, where the EPA yield was 99 mg/L.

The EPA yield was significantly greater than that obtained in the optimal glucose-yeast extract media (2% glucose and 0.25% yeast extract) from Chapter 3, while the AA yield was about the same. This indicated that rice bran media is competitive with glucose-yeast extract media for EPA and AA production, even without considering the cost-efficiency by using this low-cost byproduct as a potential feedstock.

Similar to the results in the glucose-yeast extract media, after the EPA yield peaked, the yields of all the individual fatty acids suddenly dropped dramatically to very low levels, with some even becoming undetectable. However, the lipid yield did not show such a dramatic decrease (see Fig. 4.4). One possible reason might be these fatty acids were hydrolyzed to free fatty acids, which could not be detected by the FAME method applied in this research. This information is important for setting a cutoff point for the fermentation process to optimize target fatty acid production. As a recommendation, a 7-day-incubation at 25°C was suitable for EPA and AA production, where the bioconversion percentages from rice bran to EPA and AA were 0.42% and 0.14%, respectively. The individual fatty acid amounts in 50 g of rice bran, the corresponding peak amount during fermentation and the amount after 7 days incubation from biomass collected from 1 L of rice bran media, are shown in Table 4.2. The bioconversion

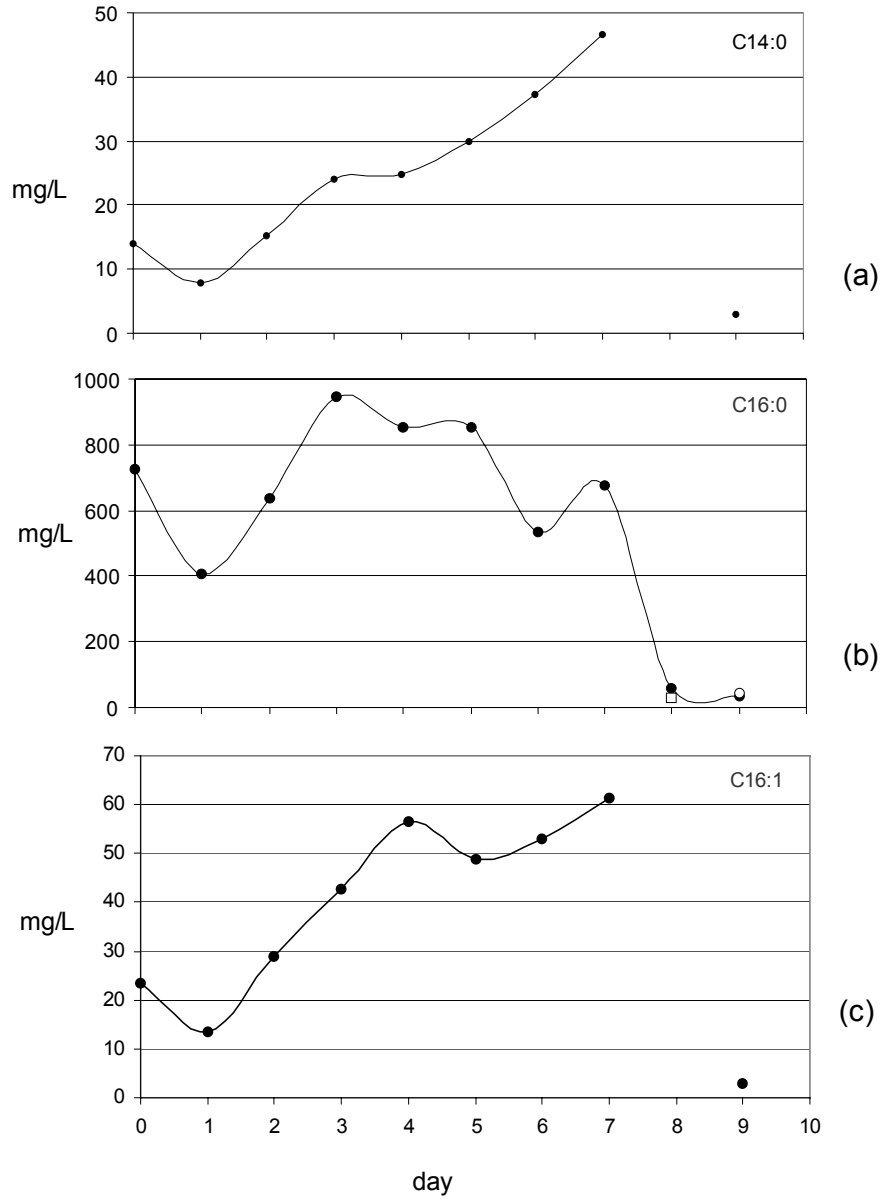
percentage, which is defined as fatty acid yield (g/L) divided by rice bran yield in media (50g/L), is shown for EPA and AA.

Table 4.2 Individual Fatty Acid Amounts in 50 g Rice Bran and The Biomass Collected from 1 L of Rice Bran Media

Fatty acid	Per 50 g rice bran (mg/50 g)	Peak amount (mg/L) and (corresponding day)	In the 7 th day biomass (mg/L)	% bioconversion after 7 days
C14:0	27	47 (7)	47	
C16:0	1194	948 (3)	677	
C16:1	40	61 (7)	61	
C18:0	60	70 (4)	53	
C18:1	3556	2908 (3)	2199	
C18:2	2776	2234 (3)	1779	
C18:3	121	105 (4)	76	
C20:0	0	40 (3)	27	
C20:1	0	63 (6)	62	
C20:4	0	82 (6)	70	0.14
C20:5	0	208 (7)	208	0.42

The amounts of C16:0, C18:1, C18:2, which were predominated fatty acids in rice bran, decreased after the bioconversion process, probably due to the loss of 40% of individual fatty acids during media sterilization, where oil would be removed by hot-water extraction. A noticeable quality of oil was observed on the culture flask wall. Though the fungus synthesized some amount of these fatty acids during incubation, the yields had not recovered to the original level. These three fatty acids peaked at the 3rd day, and then the yields decreased. Same phenomena had been observed in C18:3. However, besides EPA and AA, the amounts of C14:0, C16:1, C18:0, C20:1 increased.

The incubation period for the temperature shifted culture was 8 or 9 days. The yields for all individual fatty acids were low and were much less than the peak production of corresponding fatty acids under constant temperature. Therefore, temperature shifting did not show any advantages of enhancing the target polyunsaturated fatty acids yield in this study.



● 25 °C
 □ 25 °C at first 5 days, then dropped to 13 °C for extra 3 days
 ○ 25 °C at first 5 days, then dropped to 13 °C for extra 4 days
 ** Some points are missing since they were undetectable

Fig. 4.5 (a)-(k) Individual fatty acid profiles

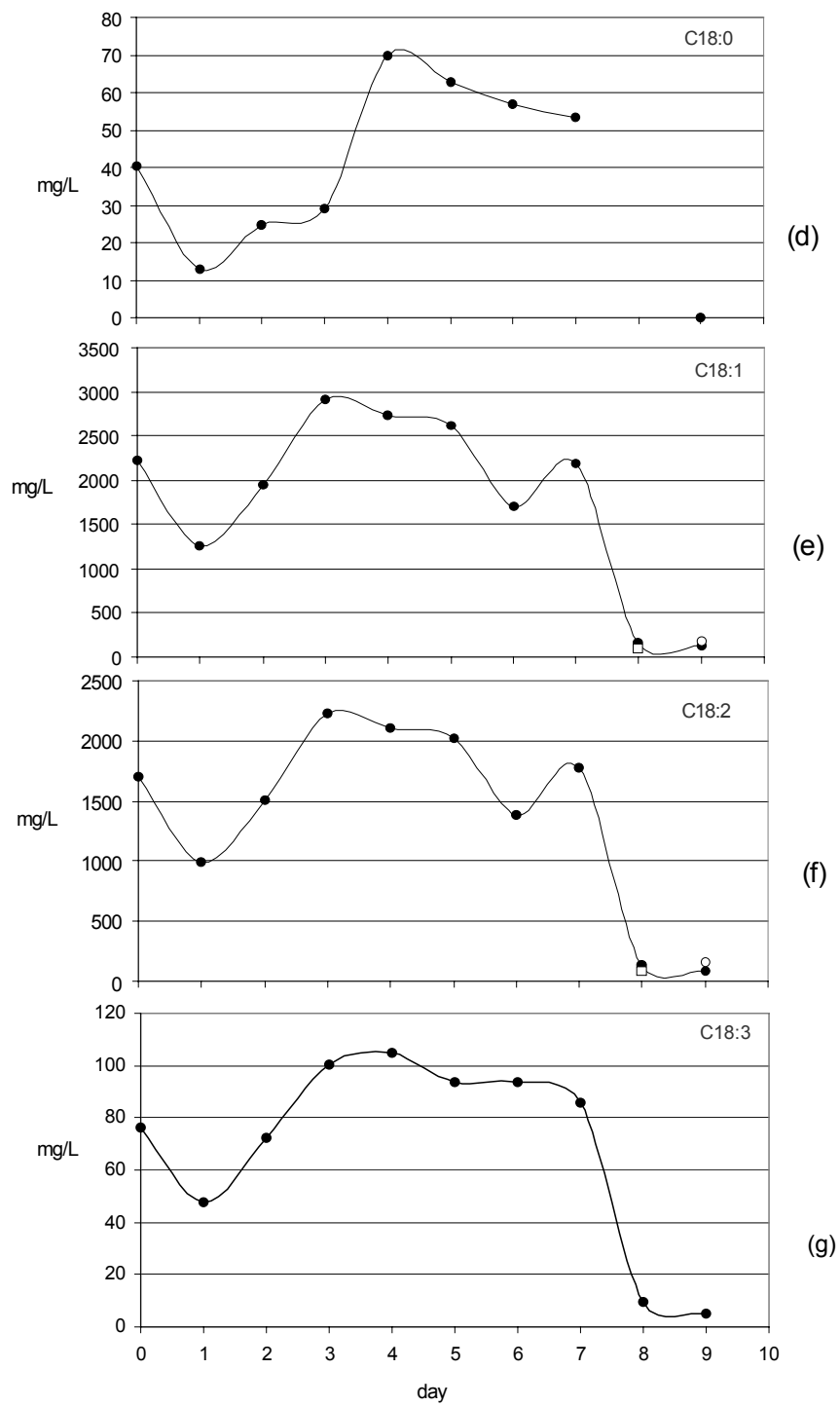


Fig. 4.5 (a)-(k) (continued)

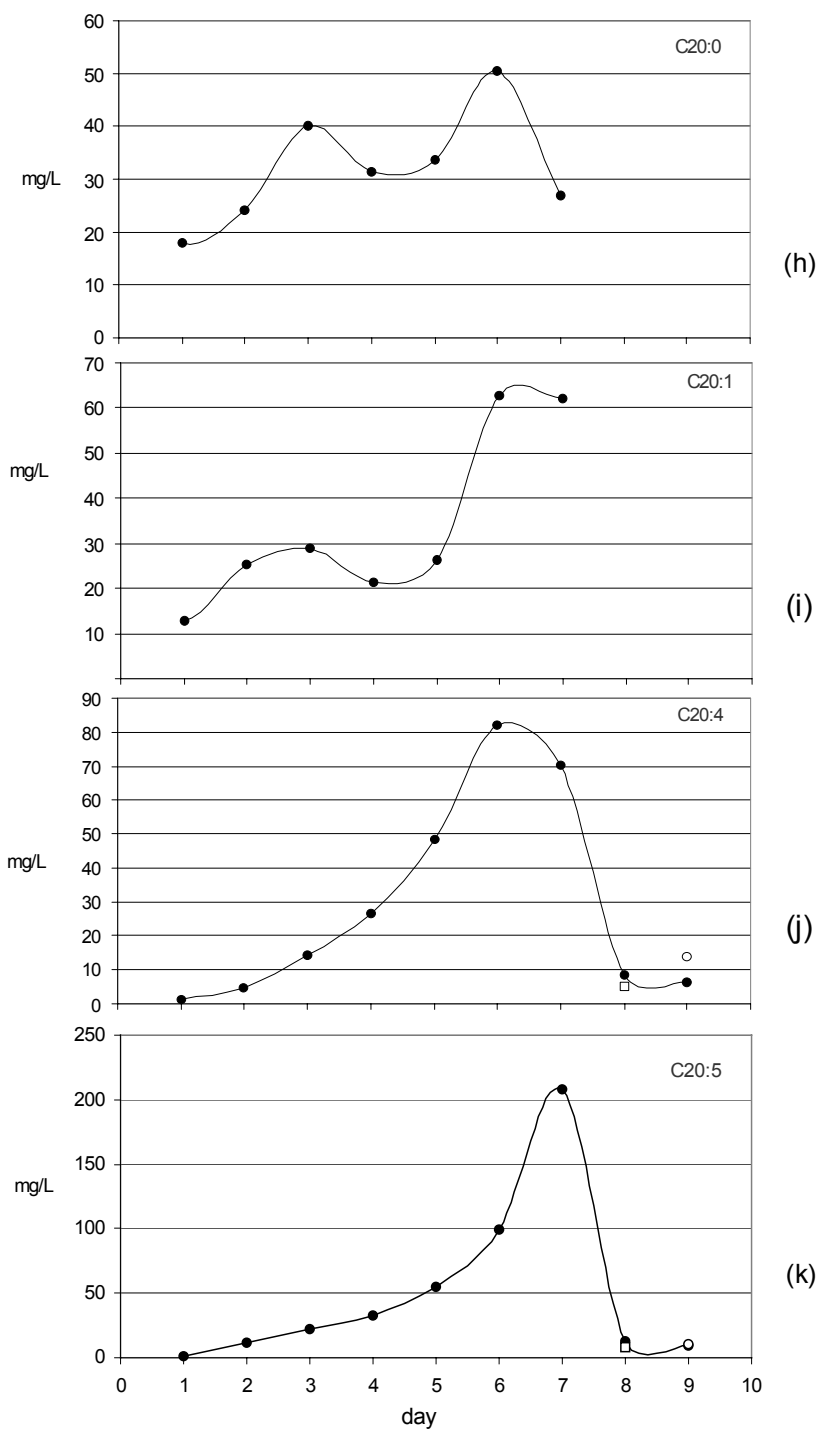


Fig. 4.5 (a)-(k) (continued)

CHAPTER 5

SUMMARY AND CONCLUSIONS

Growing interest in polyunsaturated fatty acids (PUFAs) was resulted in the development of fermentation technologies to produce microbial lipids high in PUFA content. The main objectives of this research were to investigate the nutrient requirements including media composition and C/N ratio for *Pythium irregulare* on glucose-yeast extract media for lipid production and then to optimize the media; to characterize and model the temperature effect using the optimized medium to optimize the temperature condition; from information obtained in the glucose-yeast extract media, studies utilization of rice bran as a potential substrate for fungal lipid production in submerged fermentation was investigated as a means of accomplishing rice bran value-added processing.

Among the four levels of glucose, i.e., 1%, 2%, 3% and 4%, the optimal glucose concentration was 2%. The optimal C/N for biomass ranged from 12 to 24 while that for lipid accumulation in biomass was 32, which indicated nitrogen limitation enhanced lipid accumulation. There was no overlap for C/N for biomass synthesis and lipid accumulation. To achieve high production of the target PUFAs, the balance among biomass, lipid content in biomass and individual PUFA yield were considered and the substrate quantity/cost in the medium was evaluated for production efficiency as well. The medium composed of 2% glucose and 0.25% yeast extract was considered the optimal medium for EPA and AA production in this study.

Among the three experimental incubation temperatures namely 13 °C, 19 °C and 25 °C, on the optimized glucose-yeast extract media, the kinetic data for biomass, substrate utilization, lipid yield and individual fatty acid yields over time were determined. Most of the literature does not consider kinetic data and neglect the PUFA decay process, which appears to be significant in this research. The lower temperatures did not show any advantage of PUFA production compared with room temperature (25 °C). Room temperature operation would make the fermentation process more economical.

This study showed the feasibility of rice bran utilization in submerged fermentation to produce lipids containing target PUFAs using *Pythium irregulare*. In the 5% (w/v) rice bran medium, which had about 2% carbohydrate with an estimated C/N of 10, after 7 days incubation, the lipid yield increased about 10% compared with the initial rice bran. The composition of fatty acid fraction was improved with the addition of EPA and AA synthesized by the fungus. Maximum EPA yield reached 207 mg/L and AA yield reached 70 mg/L after a 7 days incubation period, respectively. The EPA yield was even much higher than obtained in the optimized glucose-yeast extract media. By then, the bioconversion percentages from rice bran to EPA and AA were 0.41% and 0.14%, respectively. Other important fatty acids, including GLA and ALA, were also produced by the fungus. PUFA yields decreased dramatically after maximum EPA yield appeared, which made it essential for determining a cutoff point in the fermentation process. Most of the components in the rice bran were utilized by the fungus, with an observed COD removal percentage of 73% in the byproduct media after 7 days incubation when the EPA peak occurred.

As recommendation, further research should focus on the nutrient adjustment on rice bran media. Rice bran serves well as main substrate for *Pythium irregulare* to produce EPA and AA and slight nutrient adjustment, such as supplement of some minerals, may increase the target PUFA yields. Further work should also be conducted to determine the dramatic drop in natural lipid fatty acids while the total lipid content remained stable. Free fatty acid determination would serve as the logical starting point to investigate potential lipid hydrolysis in the decay process.

REFERENCES

1. Aki T, Nagahata Y, Ishihara K, et al. 2001. Production of arachidonic acid by filamentous fungus *Mortierella alliacea* strain YN-15. *Journal of the American Oil Chemists Society*. 78 (6): 599-604.
2. APHA (American Public Health Association). 1995. Standard methods for the examination of water and wastewater. 253-255.
3. Bajpai P, Bajpai PK, Ward OP. 1991. Eicosapentaenoic acid (EPA) formation: comparative studies with *Mortierella* strains and production by *Mortierella elongate*. *Mycological Research*. 95 (11): 1294-1298.
4. Brown CM, Rose AH. 1969. Fatty acid composition of *Candida utilis* as affected by growth temperature and dissolved-oxygen tension. *Journal of Bacteriology*. 99: 371-376.
5. Cheng MH, Walker TH, Hulbert GJ, et al. 1999. Fungal production of eicosapentaenoic and arachidonic acids from industrial waste streams and crude soybean lipid. *Bioresource Technology*. 67 (2): 101-110.
6. Christie WW. 1982. A simple procedure for rapid transmethylolation of glycerolipids and cholesteryl esters. *Journal of Lipid Research*. 23 (7): 1072-1075.
7. Conti E, Stredansky M, Stredanska S, Zanetti F. 2001. Gamma-Linolenic acid production by solid-state fermentation of *Mucorales* strains on cereals. *Bioresource Technology*. 76 (3): 283-286.
8. Costa JAV, Alegre RM, Hasan SDM. 1998. Packing density and thermal conductivity determination for rice bran solid-state fermentation. *Biotechnology Techniques*. 12 (10): 747-750.
9. Feng KC, Liu BL, Tzeng YM. 2000. *Verticillium lecanii* spore production in solid-state and liquid-state fermentations. *Bioprocess Engineering*. 23 (1): 25-29.
10. Gandhi SR, Weete JD. 1991. Production of the polyunsaturated fatty acids by fungus *Pythium ultimum*. *Journal of General Microbiology*. 137: 1825-1830.
11. Gill Iqbal, Rao Valivety. 1997. Polyunsaturated fatty acids, part 1: occurrence, biological activities and applications. *Trends in Biotechnology*. 15(10), 401-409.
12. Higashiyama K, Yaguchi T, Akimoto K, et al. 1998. Enhancement of arachidonic acid production by *Mortierella alpina* 1S-4, *Journal of the American Oil Chemists Society*. 75(11): 1501-1505.
13. Holman RT, 1998. The slow discovery of the importance of omega 3 essential fatty

- acids in human health. *Journal of Nutrition*. 128 (2): 427S-433S.
14. Hong H, Datla N, Reed DW, et al. 2002. High-level production of gamma-linolenic acid in *Brassica juncea* using a Delta 6 desaturase from *Pythium irregulare*. *Plant Physiology*. 129 (1): 354-362.
 15. Jang HD, Lin YY, Yang SS. 2000. Polyunsaturated fatty acid production with *Mortierella alpina* by solid substrate fermentation. *Botanical Bulletin of Academia Sinica*. 41 (1): 41-48.
 16. Jen Nielsen, John Villadsen. 1994. *Bioreaction Engineering Principles*. 183.
 17. Kendrick A, Ratledge C. 1992. Lipid formation in the oleaginous mold *entomophthora-exitalis* grown in continuous culture - effects of growth-rate, temperature and dissolved-oxygen tension on polyunsaturated fatty-acids. *Applied Microbiology and Biotechnology*. 37 (1): 18-22.
 18. Lepage G, Roy C.C. 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *Journal of Lipid Research*. 25: 1391-1396.
 19. Li ZY, Lu YY, Yadwad VB, Ward OP. 1995. Process for production of arachidonic acid concentrate by a strain of *Mortierella alpina*. *Canadian Journal of Chemical Engineering*. 73(1): 135-139.
 20. Mandels M, Andreotti R, Roche C. 1976. Measurement of saccharifying cellulase. *Biotechnology and Bioengineering Symposium*. 6: 21-23.
 21. Milan Certik, Sakayu Shimizu. 1999. Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *Journal of Bioscience and Bioengineering*. 87(1): 1-14.
 22. Obrien DJ, Kurantz MJ, Kwoczak R. 1993. Production of eicosapentaenoic acid by the filamentous fungus *pythium irregulare*. *Applied Microbiology and Biotechnology*. 40 (2-3): 211-214.
 23. Orthofer FT. 1996. Rice bran oil: Healthy lipid source, *Food Technology*. 50 (12): 62-64.
 24. Pirt SJ. 1966. A theory of the mode of growth of fungi in form of pellets in submerged culture. *Proceeding of Royal Society*. 166 (1004): 369-373.
 25. Robert Kuehl. 2000. *Design of experiments: statistical principles of research design and analysis*. Second Edition. 181-189.

26. Shimizu S, Kawashima H, Akimoto K, et al. 1989. Conversion of linseed oil to an eicosapentaenoic acid-containing oil by *Mortierella alpina* 1s-4 at low-temperature. *Applied Microbiology and Biotechnology*. 32 (1): 1-4.
27. Shimizu S, Shinmen Y, Kawashima H, et al. 1988. Fungal mycelia as a novel source of eicosapentaenoic acid. *Biochemistry and Biophysics Research Communication*. 150 (1): 335-341.
28. Singh A, Ward OP. 1997. Production of high yields of arachidonic acid in a fed-batch system by *Mortierella alpina* ATCC 32222. *Applied Microbiology and Biotechnology*. 48 (1): 1-5
29. Stinson EE, Kwoczak R and Kurantz M. 1991. Effect of cultural conditions on production of eicosapentaenoic acid by *Pythium irregulare*. *Journal of industrial Microbiology*. 8 (3): 171-178.
30. Stredansky M, Conti E, Salaris A. 2000. Production of polyunsaturated fatty acids by *Pythium ultimum* in solid-state cultivation. *Enzyme and Microbial Technology*. 26 (2-4): 304-307.
31. Suutari M, Laakso S. 1994. Microbial fatty-acids and thermal adaptation. *Critical Reviews in Microbiology*. 20 (4): 285-328.
32. Yasuhisa Koike, Hongjie Cai, Kenichi Higashiyama, et al. 2001. Effect of consumed carbon to nitrogen ratio on mycelial morphology and arachidonic acids production in cultures of *Mortierella alpina*. *Journal of Bioscience and Bioengineering*. 91 (4): 382-389.
33. Ykema A, Verbree EC, Kater MM, et al. 1988. Optimization of lipid production in the oleaginous yeast *Apiotrichum curvatum* in whey permeate. *Applied Microbiology and Biotechnology*. 29 (2-3): 211-218.
34. Zhu H, He GQ. 1999. The nutrition requirement for submerged culture of *Flammulina velutipes* utilizing starch-processing wastewater. *Chinese Journal of Biotechnology*. 15(4): 512-516.

APPENDIX A

GC TEMPERATURE PROGRAMMING FOR FAME ANALYSIS

Temp
Inlet Pres
Detector
Time Prog.
PTV/OCI Temp

Oven Parameters

Max Temp: C

Equilib Time: Min

Isothermal Zone Temperatures

INJ1	Set	<input style="width: 40px;" type="text" value="250"/>	Max	<input style="width: 40px;" type="text" value="300"/>	C
DET1	Set	<input style="width: 40px;" type="text" value="260"/>	Max	<input style="width: 40px;" type="text" value="300"/>	C
Aux1:	Set	<input style="width: 40px;" type="text" value="25"/>	Max	<input style="width: 40px;" type="text" value="340"/>	C
Aux2:	Set	<input style="width: 40px;" type="text" value="25"/>	Max	<input style="width: 40px;" type="text" value="340"/>	C
Aux3:	Set	<input style="width: 40px;" type="text" value="25"/>	Max	<input style="width: 40px;" type="text" value="340"/>	C
Aux4:	Set	<input style="width: 40px;" type="text" value="25"/>	Max	<input style="width: 40px;" type="text" value="250"/>	C

Step	Rate [°C/min]	Temp [°C]	Wait (min)
Init		130	2.0
1	10.0	180	0.0
2	2.0	215	0.0
3	10.0	230	9.0
4			
5			

Total Program Time : 35 min

APPENDIX B

RELATIVE RESPONSE AREA FOR INDIVIDUAL FATTY ACID TO INTERNAL STANDARD (C19:0)

Fatty acid	RR _f
C14:0	0.969
C14:1	0.974
C16:0	0.941
C16:1	0.964
C18:0	0.937
C18:1	0.921
C18:2	0.965
C18:3	1.131
C20:0	0.980
C20:1	0.964
C20:2	1.059
C20:3	1.031
C20:4	1.104
C20:5	1.041

APPENDIX C

SAS CODE FOR THE EFFECTS OF GLUCOSE AND YEAST EXTRACT ON BIOMASS YIELD

```
Data one;
Do glucose=1 to 4;
Do YE=1 to 4;
Do rep=1 to 3;
input y@@;
output;
end;
end;
end;
drop rep;
cards;
2.802
3.304
3.368
2.718
2.75
2.756
3.03
2.958
3.184
3.926
4.036
4.056
4.84
4.79
5.102
5.632
5.848
5.172
6.00
6.41
6.036
6.634
6.802
6.718
4.736
5.726
5.204
7.37
7.326
6.744
7.124
7.39
7.458
7.51
8.56
8.12
4.332
5.158
```

```
4.71
6.38
7.786
8.41
8.792
7.756
10.602
9.486
10.094
9.646
;
proc print data=one;
run;
proc glm;
class glucose YE;
model y=glucose YE glucose*YE;
means glucose YE/tukey lsd;
lsmeans glucose*YE/stderr pdiff out=means;
run;
proc plot data=means;
plot lsmeans*YE=glucose;
run;
```

VITA

Hui Zhu was born in Yinxian, Zhejiang Province, People's Republic of China, in May, 1971. In 1989, he left for Hangzhou to begin his college education at Zhejiang University, where he obtained his bachelor of science and master of science degrees in food science in 1993 and 1996, respectively. During 1996-1999, he worked for the Biotechnology Institute of Zhejiang University as research associate and then as research assistant professor, where his research area was microbial fermentation.

In 2000, he came to the United States and joined the graduate program in the Department of Biological and Agricultural Engineering at Louisiana State University and worked on bioprocess engineering. He is expected to obtain his master's degree in biological engineering in December, 2002.

He is a member of Sigma Xi, Scientific Research Society and the Institute of Biological Engineering.