PRODUCTION OF ARACHIDONIC ACID FROM *CANDIDA KRUSEI*

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Report submitted in partial fulfillment of the requirements
for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical and Natural Resources Engineering
UNIVERSITI MALAYSIA PAHANG

JANUARY 2012

SUPERVISOR'S DECLARATION

I hereby declare that I have checked this report and in my opinion this report is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology).

Signature:
Name of Supervisor:
Position:
Date:

STUDENT'S DECLARATION

I declare that the work in this project is my own except for quotations and summaries which have been duly acknowledged. The project has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Special dedication to my parents
Mr Abdul Manan Kechik and Mrs Rohani Haji Salleh
For your endless love, support and care

In memory of Allahyarhamah Aisya Bahiyah Abdul Razak
May Allah bless her soul and place her amongst the dwellers of Jannah

ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful, thank you Allah for giving me health and strength to complete this Undergraduate Research Project without any obstacles. First, I offer my sincerest gratitude to my supervisor, Miss Noraziah Abu Yazid for her valuable guidance, patience and support from the very beginning until the completion of this project. Her efforts in helping me had become my inspiration to work harder and produce better outcome.

I also would like to extend my appreciation to Mrs Hafizah Ramli who helped me in using Gas Chromatography for analysis. My utmost gratitude also extended to all lecturers and staffs of Faculty of Chemical and Natural Resources Engineering who are willing to share their ideas in improving my project. I highly appreciate all those helps since I am still lack in experience and need to be guided.

Finally I would like to thank fellow friends especially Nur Ain Sukri for always helping me and families for all their care and support throughout the duration of Undergraduate Research Project.

ABSTRACT

The potential of arachidonic acid (AA) in pharmaceutical field had been discovered for a long time. AA is known as a fatty acid that can help information transfer of nerve systems, other than involves in growth and repair of skeletal muscle tissue. Microorganisms such as yeasts are one of the sources that can be commercialized as AA producer. By using microorganisms, the production process become less complicated compared to using animal source, and the 'Halal' standard can be fixed. The works done in this research investigate the optimum condition for AA production from yeast. In this research, the best culture condition that produced the highest yield of AA was determined by varying the pH, the carbon source and the agitation speed. The range of pH is 5 to 9. The carbon sources that were used are sucrose, starch and the mixture of sucrose and starch. Speed of incubator shaker was set at 50 rpm, 150 rpm and 250 rpm. Extraction of lipid was done by using two-step Bligh and Dyer (1959) method. Chloroform and methanol were added in specific ratio before centrifugation. The lower phase which contain lipid was collected. After evaporation, the lipid extract was dissolved in small volume of chloroform and methanol. The lipids obtained were analyzed in gas chromatography. The highest dry biomass concentration was produced at a culture at pH 7 and 250 rpm agitation rate by using sucrose while the highest yield of AA was produced at pH 7 and 150 rpm agitation rate by using sucrose as carbon source. AA production from yeast had shown advantages over other sources. Further studies on optimization of AA production should be directed at varying other significant parameters including nutrients needed and method of extraction. The findings in this study will be helpful for other research about AA in the future.

ABSTRAK

Asid arakidonik (AA) mempunyai potensi yang besar dalam bidang farmaseutikal dan ianya telah ditemui sejak dahulu lagi. AA dikenali sebagai satu asid lemak yang membantu pemindahan maklumat dalam sistem saraf, selain daripada terlibat dalam pertumbuhan dan pembaikan tisu otot rangka. Mikroorganisma yang boleh digunakan untuk penghasilan AA adalah yis. Dengan menggunakan mikroorganisma, proses penghasilannya menjadi kurang rumit berbanding jika menggunakan sumber haiwan, malah status halal dapat dijamin. Kerja-kerja penyelidikan ini dijalankan untuk menentukan keadaan optima untuk menghasilkan AA daripada yis dari spesis *Candida krusei*. Dalam penyelidikan ini, keadaan yang terbaik bagi kultur yang dapat menghasilkan AA tertinggi telah ditentukan dengan cara memvariasikan pH, sumber karbon dan kadar kelajuan pusingan per masa. Nilai sasaran bagi pH ialah antara 5 hingga 9. Sumber-sumber karbon yang digunakan ialah sukrosa, kanji dan campuran sukrosa bersama kanji. Kelajuan pusingan per masa ditetapkan pada 50 rpm, 150 rpm and 250 rpm. Pengekstrakan lipid dilakukan dengan menggunakan kaedah Bligh dan Dyer (1959). Kloroform dan metanol dicampurkan dalam kadaran yang khusus sebelum proses pemisahan menggunakan mesin centrifuge dilakukan. Fasa yang berada di bahagian bawah selepas pemisahan mengandungi lipid diambil untuk dianalisis menggunakan kromatografi gas. Jumlah biomas yang tertinggi dihasilkan pada keadaan pH 7 dan 250 rpm dengan menggunakan sukrosa manakala jumlah hasil AA yang tertinggi dihasilkan pada pH 7 dan 150 rpm dengan menggunakan sukrosa sebagai sumber karbon. Penghasilan AA daripada yis dapat memberikan banyak kebaikan berbanding sumber-sumber lain. Kajian yang lebih mendalam terhadap penghasilan AA secara optimum perlu dijalankan dengan memvariasikan parameter lain seperti nutrisi yang diperlukan dan kaedah pengekstrakan. Penemuan dalam kajian ini boleh menjadi panduan pada kajian-kajian lain terhadap AA pada masa akan datang.

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

The role of yeast in biotechnology had been discovered for a long time. Other than used in production of food and beverages, yeasts also play important role in pharmaceutical field. They are involved in production of medicines, dietary supplements and probiotics. As the importance of yeast in biotechnology is expanding, huge amounts of yeast are cultivated nowadays. Despite the fact that yeast can cause many diseases, researchers continue to investigate other angelic side of yeast that can be developed for humans' sake. One of a newly discovered application of yeast is found in production of fatty acid, such as linoleic acid, alpha-linolenic acid, and arachidonic acid (Yazawa et al., 2009).

Arachidonic acid (AA) is a polyunsaturated fatty acid (PUFA) which is a fundamental nutrient component for human beings. It is pale yellow in colour and has slight odour. AA is liable to dissolve in organic solvent but not in water. According to Leray (2011), AA was first isolated in 1940 from phospholipids from beef suprarenal glands by Shinowara et al. (1940) and its structure was elucidated three years later by Arens et al. (1943). The first total synthesis of AA was made by Osbond et al. in 1961. AA is vital to human growth and nutrition. It has extensive application in pharmaceuticals, cosmetics and chemical materials.

AA is prominent to human at every stage. It mainly helps information transfer of nerve systems and can aid development of infants (Bigogno et al., 2002; Higashiyama et al., 2002). Thus, AA is very important in baby formula. Bigogno et al. (2002) also stated that AA is transferred directly from mother to infant by breast-feeding. This showed that AA is significant to pregnant women. AA also is necessary for old folks because it involves in control of memory (Higashiyama et al., 2002). Besides that, AA is capable of protection of gastric mucosa, treatment of skin psoriasis, reduction of fatty liver, killing of tumor cells and improvement of cirrhotic patients.

It is important to make sure that there is suitable amount of AA in our body to avoid various kinds of health effects. Any overtake or deficiency of AA may lead to risk in our health condition. Normally human gets AA through their diets that are usually found in red meat, egg yolk and organ meat. Besides that, AA was taken as supplement, which is famous among bodybuilder and athletes. AA also was produced into processed food, for example milk that is specialized for pregnant women.

Researchers are looking for the best producers of AA which can be consumed by everyone without any limitations. AA can be produced from fish and swine, however the process is more complicated as the removal of cholesterol, odours and tastes is quite difficult. Besides, marine resources are unstable due to unlimited fishing season and geographic locations (Yongmanitchai & Ward, 1989). Swine, the major producer of AA, are prohibited in certain religion and vegetarians. Nowadays higher plant such as algae was very popular among AA researcher. Besides that, microorganisms such as fungi were widely used in production of AA. In this research, works were done to extract AA from yeast as one of the promising producer of AA.

1.2 PROBLEM STATEMENT

Arachidonic Acid (AA) is presently isolated from numerous sources, in example animal adrenal gland and liver, and from sardine (Bajpai et al., 1991). Generally, porcine liver is used as the main source of AA (Yu et al., 2003). However, in a certain religion, any source from swine is forbidden or non-'Halal'. With the rising consumer demand for a 'Halal' integrated lifestyle, it is a need to find alternatives in producing products that can fit 'Halal' standards. Besides that, it is difficult to remove cholesterol and some objectionable tastes and odours from animal sources and fish oil concentrates (Jareonkitmongkol et al., 1993). Marine resources are also unstable due to limited fishing seasons and geographic locations (Yongmanitchai & Ward, 1989). Based on the potential need for AA in human, a few researches for new efficient source have been conducted. Some of microorganisms especially from fungi species turned out to have potential to accumulate lipid in their bodies equivalent to about 50 % of dry biomass (Higashiyama et al., 2002). Therefore, it is important to study the effectiveness of microorganism as a source of AA.

1.3 OBJECTIVE

The main objective of this research is to study the optimum condition for arachidonic acid (AA) production from *Candida krusei*. The measureable objectives are:

i. To determine the effect of pH for yeast culture
ii. To determine the effect of different types of carbon source needed by yeast in AA production
iii. To determine the effect of agitation rate that is suitable for culture of yeast in AA production

1.4 SCOPE OF STUDY

Arachidonic acid (AA) can be produced from various types of sources. In this study, AA was produced from yeast. The yeasts were cultured in production medium in which condition was optimized by varying the pH range, the carbon source and the agitation speed. Lipid from the yeast was extracted by methanol and chloroform as a solvent. The analysis of the lipids will be done by using gas chromatography (GC). The mobile phase or carrier gas comprised of nitrogen at 0.4 kg/ cm^2. The stationary phase consists of capillary column (50 m by 0.32 mm). Flame ionization detector was used as detector. Each sample was run for 40 minutes.

1.5 RATIONALE AND SIGNIFICANCE

Arachidonic acid (AA) production from yeast can reduce the production cost and it could fit the 'Halal' standard. Production of AA from yeast is also easier compared to other sources as yeast can be obtained easily. Besides that, the process is less complicated compared to animal source, which contain cholesterol and objectionable tastes and odors that need to be removed first. Yeast also can grow at higher speed compared to microalgae.

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CHAPTER 2

LITERATURE REVIEW

2.1 MICROORGANISM

Microorganisms are small living things which can be found everywhere. They exist in the air, food and also our body. Microorganisms play an essential role in ecosystem and human health. Some of them can cause diseases while some are used to prevent and cure diseases (Black, 2005). There are several types of microorganisms such as fungi, protozoa, bacteria and viruses. Fungi can be divided into two groups which are moulds and yeasts.

2.1.1 Yeast

Yeasts are eukaryotic microorganisms which come from family fungi. They are single-cell organisms which contain no chlorophyll and reproduce by budding. Yeasts are widely used in production of bread, beverages and vinegar. Certain yeast has several properties that make it as an important tool in expression of foreign proteins (Romanos et al., 1992). According to Devi et al. (2006), yeast is capable of producing high amount of oils and fats since late nineteenth century. Yazawa et al. (2009) stated that yeast *Saccharomyces cerevisiae* did not produce polyunsaturated fatty acid (PUFA) with more than two double bonds, but can produce saturated and monounsaturated fatty acids.

2.1.2 *Candida krusei*

Candida krusei is a budding yeast which is classified in fungi species. It is classified in Saccharomycetaceae family. It is widely used in chocolate production. According to Liu et al. (2004), it also involved in production of glycerol as it has the ability to ferment glucose into glycerol. *Candida krusei* is resistant to fluconazole and is the cause of fungemia in human (Abbas et al., 2000). Figure 2.1 illustrates the yeast *Candida krusei*.

Figure 2.1: *Candida krusei*

2.1.3 Optimum Condition for Yeast Growth

A culture medium provides all of the nutrients and the energy source required by the organism. Culture media can come in liquid, semisolid or solid forms. The liquid medium that lack of solidifying agent is called a broth medium whereas the solid or semisolid medium is prepared by adding a solidifying agent like agar into the broths. According to the US Pharmacopeia, agar can be defined as a hydrophilic colloid extracted from certain seaweeds of the Rhodophyceae class. It is insoluble in cold water but soluble in boiling water. Because of these properties, microorganisms can be cultivated at the temperature of 37.5^{o}C without fear of the medium liquefying. Semisolid medium contains a concentration of less than 1% agar (Cappuccino and Sherman, 1998). Semi-solid media can also be used

in fermentation studies, in determining bacterial motility, and in promoting anaerobic growth. A completely solid medium requires an agar concentration of about 1.5 % to 1.8 %. Solid media are usually used for the surface growth of microorganisms in order to observe colony appearance, for pure culture isolations, for store of cultures, and to observe specific biochemical reactions. Solid medium is usually used as agar plate, agar slants and agar deep tubes.

Agar plate is the agar that poured into a petri dish. Compared to broth that contains high concentration of microorganism, petri dishes allow observation, easy access (microorganism can be counted easily), and isolation, but are prone to environmental contamination and desiccation. Petri dish has to be inverted during the incubation to prevent any condensed moisture from falling on the surface of the agar so as to acquire a clear observation on microbes grown (Lester and Birkett, 1999). Yeast may be grown on a semi-solid agar medium and also in liquid media, in which it grows best.

Culture media generally contain a source of carbon, nitrogen and vitamins. Glucose is the most widely utilizable carbon source, and hence is the most commonly used in growth media. Fructose and mannose are the next most commonly utilized sugars by yeast and are found in media from natural sources. Sucrose also may be used in some media. Some species can use other more complex carbohydrates such as starch, glycerol and maltose, due to special enzymes they release. This glucose can be respired (used to provide energy) in a similar way to respiration in most other organisms. Research from Bajpai et al, (1991) showed that growth of fungi *Mortierella alpina* was very good by using glycerol and linseed oil. However, the growth was found to be very poor with lactose, starch and sucrose as carbon source and moderate with glucose, fructose and maltose.

Yeast is an organism which can be found everywhere, whether in water, air or land, depending on their species. *Candida krusei*, an ascomycetes, is an environmental yeast that is usually present in milk products, beer and bird feces. This yeast is known for its use in chocolate production. *C. krusei* also is an osmophilic yeast that can ferment glucose into glycerol (Liu et al., 2004). To culture this microorganism, 10% of pre-cultured seed was

transferred into fermentation medium and then followed by 5-day incubation at 35 °C and 150 rpm (Chen et al., 2006).

2.2 ARACHIDONIC ACID

Arachidonic acid (AA), $C_{20}H_{32}O_2$, is a carboxylic acid which contains 20 carbon chains. Figure 2.2 illustrates the structure of AA.

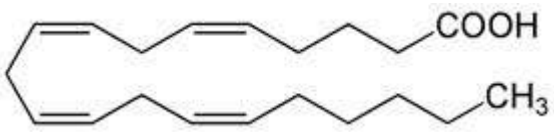

Figure 2.2: Arachidonic acid structure

AA is one of a common unsaturated fatty acid. Unsaturated refers to the presence of one or more double bonds between carbons. The double bonds results in introduction of a kink in the molecule shape, which makes it difficult to pack together. The intermolecular interactions are weaker, and hence the melting points are much lower than saturated fatty acid. Table 2.1 shows basic properties of AA.

Table 2.1: Arachidonic acid properties

Molecular weight	304.47 g/mol
Density	0.922 g/mL
Melting point	-49 °C
Boiling point	170 °C
Specific gravity	0.922

(Source: www.sciencelab.com)

2.2.1 Sources of Arachidonic Acid

Arachidonic acid (AA) can be obtained from various sources. AA, widespread in the animal kingdom, can be isolated from lipids extracted from pig adrenal gland or pig liver and sardines as well, however the yield of AA is only 0.2 % or lower, which is very difficult to be industrialized (Yuan et al., 2002). Furthermore, swine is forbidden for certain religion and vegetarian as well. In addition, cholesterol and some objectionable tastes and odours are still difficult to remove from fish oil concentrates and animal sources (Jareonkitmongkol et al., 1993). According to Yongmanitchai and Ward (1989), marine resources are unstable due to limited fishing seasons and geographic locations. AA also can be produced from microorganisms. Nevertheless, just a few of microbial oils have been commercialized due to high production cost as compared with the low cost of conventional edible oil extracted from natural resources (Higashiyama et al., 2002).

The interest in arachidonic acid (AA) and other polyunsaturated fatty acids (PUFAs) inspired the search for new sources of these PUFAs. Several microalgae were shown to contain high proportions of long-chain PUFAs (LC-PUFA) among their fatty acid (Bigogno et al., 2002). According to Araki et al. (1990) in *Gracilaria sp*., the proportion of AA can be as high as 60 % of total fatty acids, however, the dry weight content does not exceed 0.2 %. Araki (1990) and Cohen (1990) produced AA from *Porphyridium cruentum* and found out that the yield of AA was high only when the growth is slowed (Cohen, 1990). Bigogno et al. (2002) found that the fresh-water green microalgae *Parietochloris incisa* is the only AA-rich vegetal organism. The sources of AA were summarized in the Table 2.2.

Table 2.2: Sources for AA production

Authors	Sources
Bigogno et al. (2002); Cheng- Wu et al. (2002)	Algae *Parietochloris incisa*
Cohen (1990); Araki et al. (1990)	Algae *Porphyridium cruentum*
Hartmann et al. (1986)	Moss *Leptobryum pyriforme*
Bajpai et al. (1991); Streekstra (1997); Park et al. (1999); Eroshin et al. (2000); Yuan et al. (2002); Yu et al. (2003); Zhu et al. (2005)	Fungi *Mortierella alpina*
Bajpai et al. (1991); Cheng et al. (1999);	Fungi *Mortierella elongata*
Cheng et al. (1999)	Fungi *Pythium irregulare*

2.2.2 Application of Arachidonic Acid

Arachidonic acid (AA) has various applications in many fields. AA is a major constituent and plays the role of maintaining membrane fluidity in biological cells (Higashiyama et al., 2002). Owing to its unique biological properties, AA has been used in medicine, pharmacology, cosmetics, food industry, agriculture, and other fields (Eroshin et al., 2000). AA is an essential dietary component for human beings and a precursor of many important eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes (Yuan et al., 2002) with important function in circulatory as well as central nervous systems (Innis, 1991). Ahern et al. (1983) stated that AA serves as a starting material for the biosynthesis of the prostaglandin PGE2.

Higashiyama et al. (2002) also stressed on the various physiological functions of AA, for example protection of gastric mucosa, treatment of skin psoriasis, reduction of fatty liver, killing of tumor cells, and improvement of lipid metabolism of cirrhotic patients. Recently, a new function of AA was reported. Anandamide and sn-2 arachidonylglycerol

may function as a natural ligand for the cannabinoid receptor, which is expressed in areas of the central nervous system that contribute to the control of memory, cognition, movement and pain perception (Higashiyama et al., 2002)

Besides that, AA can aid the development of infants. AA is necessary for the visual acuity and better cognitive development of infants after birth (Koletzko et al., 1996). Various health authorities recommend AA in baby formula (Bigogno et al., 2002). Koletzko et al. (1989) reported that AA is a component of human milk and therefore potentially valuable ingredient in various formulation of artificial baby food. Bigogno et al. (2002) mentioned that AA is also transferred directly from mother to infant during the last intrauterine trimester and after birth by breast feeding. Therefore, the mother also requires AA in her diet. Since AA is widely used in many fields, it is strongly desired to develop AA abundantly, safely, and economically. The applications of AA were summarized in Table 2.3.

Table 2.3: Applications of AA

Authors	Applications
Higashiyama et al. (2002)	• Maintain membrane fluidity in biological cells • Protection of gastric mucosa • Treatment of skin psoriasis • Reduction of fatty liver • Killing of tumor cells • Improvement of lipid metabolism of cirrhotic patients
Yuan et al. (2002)	Precursor of many important eicosanoids
Ahern et al. (1983)	Starting material for biosynthesis of prostaglandin
Koletzko et al. (1996)	• Aid development of infants • Component of human milk

CHAPTER 3

METHODOLOGY

3.1 GENERAL PROCEDURE

The general procedure of the whole research was summarized in the Figure 3.1.

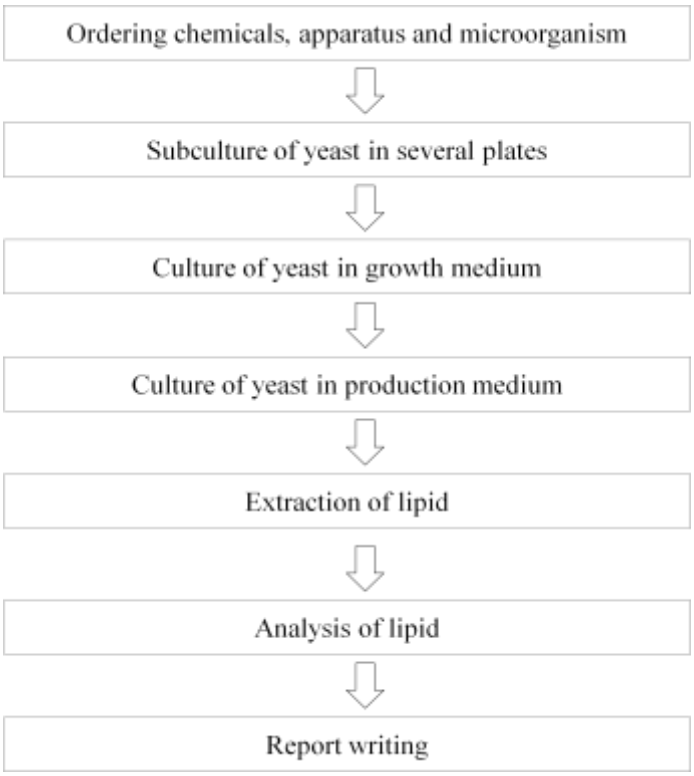

Figure 3.1: General procedure of the research

3.2 MATERIALS

The chemicals that were used in this study are of analytical grades, and they are summarized in the Table 3.1.

Table 3.1: List of chemicals

Chemical	Supplier	Principle usage
Methanol	Sigma-Aldrich	Extraction
Chloroform	ChemAR	Extraction
Ethanol	R&M Chemicals	Sterilization
Arachidonic acid	Sigma-Aldrich	Analysis (Standard)

3.3 EQUIPMENTS

Several equipments were used during this research. Those equipments were incubator, incubator shaker, laminar flow hood, autoclave, centrifuge, and gas chromatography. The equipments that were used in this study are summarized in the Table 3.2.

Table 3.2: List of equipments

Equipment	Brand/Origin	Principle Usage
Incubator	Memmert/ Germany	Heating, ventilation, humidification
Laminar flow hood	ESCO/ USA	Air filtration
Autoclave	Hirayama/ Japan	Sterilization
Incubator shaker	Infors AG/ Switzerland	Heating, agitation
Centrifuge	Eppendorf/ Germany	Separation
Gas chromatography	Agilent/ USA	Analysis

3.3.1 Incubator

Incubator was used to provide the desired temperature, ventilation and humidity for the growth of culture. The temperature and humidity levels were set for healthy growth of the cells after placing the culture in the incubator. Within a few days, the cells multiplied in number. The incubator is heated electrically or through water jet circulating over the outer phase of the incubator box. Hence suitable heat was passed to the samples in the laboratory incubator. Microbiological incubator (Memmert Model BE600) which is originated from Switzerland was used in this study and is illustrated in Figure 3.2.

Figure 3.2: Microbiological incubator model BE600

3.3.2 Incubator Shaker

Incubator shaker is equipped with shaking function which is needed for growing culture in liquid form. It offers quiet, maintenance-free shaking under a constant and reproducible temperature environment. Agitation speeds affect aeration and mixing of the culture. The greater the aeration the greater the oxygen transfer rate will be, and hence, increasing the cell growth rate. Figure 3.3 shows incubator shaker (Ecotron 100000, Infors) from Switzerland.

Figure 3.3: Incubator shaker model Ecotron 100000

3.3.3 Autoclave

In culturing the fungi, it is important to prevent any contamination. Autoclave was used to sterilized media and equipment required for growing microorganisms. Basic chemistry principle is used in autoclave, that is when pressure increase, the temperature also increase proportionally. Air was expelled from the autoclave and the pressure rose to 15 psi. With attained temperature 121 ℃, all organisms were killed in 15 minutes. Figure 3.4 illustrates the autoclave (PH PMD 287, Hirayama) from Japan.

Figure 3.4: Autoclave model PH PMD 287

3.3.4 Laminar Flow Hood

Laminar flow hoods are provided with an atmosphere of filter to prevent the pure culture of microorganism from contamination during inoculation or transfer, at the same time, protect the researcher against infectious agents. In the beginning of the experiment, the ultraviolet light button of the flow hood is on for about 20 to 30 minutes to sterilize the inner part of it. After the cover of the flow hoods is taken away, the air flow button has to be on immediately. Then, researchers have to swab the surface of the inner hood with alcohol to prevent any contamination to the specimen. Operations, which are done in the hoods, are carried out in the vicinity of a Bunsen burner flame to decontaminate surfaces that are briefly exposed. Figure 3.5 shows the laminar flow hood model AHC- 4A1.

Figure 3.5: Laminar flow hood model AHC-4A1

3.3.5 Centrifuge

A centrifuge is a device that separates particles from suspensions according to their size, shape and density. In a solution, particles whose density is higher than that of the solvent sink, and particles that are lighter than it float to the top. The material to be

centrifuged was distributed into centrifuge tubes which attached in a symmetric manner to a rotating block called the rotor. When the centrifuge tubes are spun, the centrifugal action creates an induced gravitational field in an outward direction relative to the axis of rotation and this drives the precipitated matter towards the bottom of the tube. Figure 3.6 illustrated the centrifuge (5810 R, Eppendorf) which is from Germany.

Figure 3.6: Centrifuge 5810 R

3.3.6 Gas Chromatography

The gas chromatograph separates the volatile components of a very small sample and determines the amount of each component present. Mobile phase and stationary phase were required in running chromatography. The mobile phase or carrier gas comprised of nitrogen. The stationary phase consists of capillary column (50 m by 0.32 mm). The components in the sample can be separated because of interaction between the compound and the stationary phase. The stronger the interaction, the shorter time it will take to be spent in mobile phase, and the longer time it will move through the column. Hence, the retention time will be longer. The component in the sample will exit the column and flow past the detector. The detector shows different response with different component. Flame ionization detector is the most widely used detectors for organic sample. Figure 3.7 shows gas chromatography Agilent Technologies 6890 Series from USA.

Figure 3.7: Gas chromatography Agilent Technologies 6890 Series

3.4 MICROORGANISM

A plate of yeast *Candida krusei* was obtained from Kuliyyah of Science (Biotechnology), International Islamic University of Malaysia, Kuantan. The yeast obtained need to be subcultured in order to increase the number of yeast growth. Potato dextrose agar was mixed with 500 ml distilled water in a Schott bottle, and was autoclaved for 15 minutes. Under sterile condition, the media was cooled down before being poured into six petri dish. The agar was allowed to be solidified. Using inoculums loop, a loopful of the yeast culture was obtained and was spread on the agar by streaking method. The agar plates were sealed before being incubated at 36 ℃ for 4 to 5 days in inverted position. After incubation, they were stored at 4 ℃ and were subcultured for every two weeks to provide more nutrients. Figure 3.8 illustrates *Candida krusei* culture in petri dish.

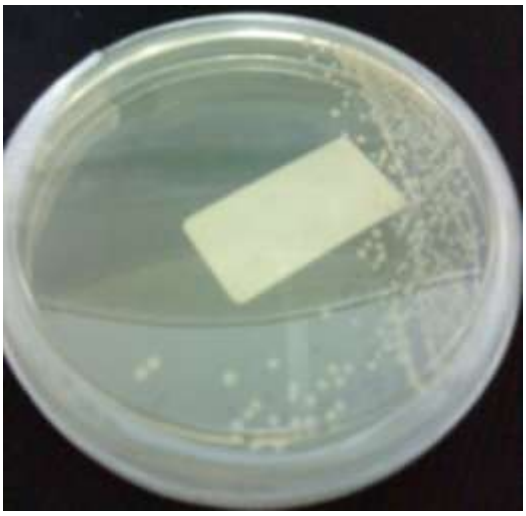

Figure 3.8: *Candida krusei* culture

3.5 MEDIA PREPARATION

3.5.1 Preparation of Growth Media

In preparing the growth media, 4 g of glucose and 2 g of yeast extract were added to 200 ml of distilled water and mixed homogenously. The mixture was divided into two different conical flasks and was autoclaved. After cooled, three loopful of yeast from the culture plate were transferred into the flasks containing growth media. The flasks were then placed in incubator shaker for 24 hr at 36 °C with 180 rpm agitation.

3.5.2 Preparation of Production Media

The production media is the main role for the production of our desired product. The condition for production media was varied in order to study the optimum condition. The composition of production media consists of (g/l): carbon source, 30; yeast extract, 5.0; KNO_3, 1.0; KH_2PO_4, 2.4; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_20$, 0.1; $FeCl_3.6H_2O$, 0.015;

$ZnSO_4.7H_2O$, 0.0075; $CuSO_4.5H_2O$, 0.0005 (Bajpai et al., 1991). The carbon sources used were sucrose, starch and mixture of sucrose and starch. The pH was varied at 5, 7 and 9, and was adjusted using 0.5 M hydrochloric acid (HCl) and 0.5 M sodium hydroxide (NaOH). After being autoclaved, the media was inserted with 10 % of growth media and incubated for 48 hr at 36 ℃. The agitation speed was set to 50, 150 and 250 rpm.

3.6 EXTRACTION OF LIPID

Lipid was extracted by the two-step Bligh and Dyer (1959) method. First, ultrasonication bath was used to disrupt the cell. 1 ml of cell suspension was taken and was put into a centrifuge tube containing 3.75 ml of a mixture of chloroform: methanol (1:2). The mixture was vortex for 5-10 min. After that, 1.25 ml of chloroform was added with 1 min of mixing, and 1 ml of distilled water was added at following step with 1 minute of mixing. Then, the sample was centrifuged for 15 min at 8000 rpm. Subsequently, the lower phase was collected in another tube. About 1.88 ml of chloroform was added to the non-lipid residue and was vortex before centrifuged. The lower phase was mixed with the upper phase from the first centrifugation. The lipid extract was evaporated until it was about to dry, and dissolved in a small volume with a ratio of chloroform: methanol (2:1). The samples were filtered using 0.2 μm Nylon filter and placed in vials for further analysis. The procedure was summarized in the Figure 3.9.

Figure 3.9: Flowchart for lipid extraction procedure

3.7 SAMPLE ANALYSIS

Arachidonic acid (AA) extract was analyzed by using gas chromatography which was equipped with flame ionization detector and a 50 m by 0.32 mm capillary column. The carrier gas was nitrogen (N_2), operated at pressure of 0.4 kg/cm^2. The temperature program of the column was: injector 250 °C, initial 10 min at 180 °C, heating 3 °C/min to 240 °C and holding for 20 min at 240 °C (Chiao et al., 2001). AA was identified by comparing the retention time with the commercialized AA as a standard purchased from Sigma-Aldrich, Malaysia.

CHAPTER 4

RESULT AND DISCUSSION

4.1 SUBCULTURE OF YEAST

Yeast need to be subcultured in order to increase the number of yeast growth. At the beginning of experiment, due to limitation of incubator in the laboratory, the agar cultures were placed at 27 °C condition. Within a few days, there was no growth of yeast in all plates. This might be due to unsuitable temperature for the yeast growth or presence of contamination during the streaking procedure. After the problem solved, the agar culture were able to be grown in incubator at 36 °C. Yeast colony was observed at each plate and can be seen at Figure 4.1. The strains were then stored at 4 °C.

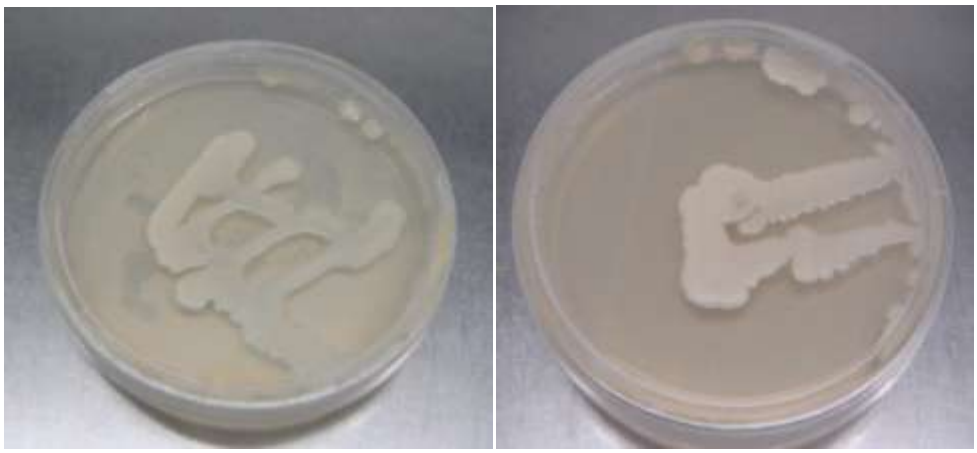

Figure 4.1: Yeast culture on agar plates

4.2 CULTURE OF YEAST IN GROWTH MEDIUM

Yeasts were first cultured in growth medium before being inoculated in the production medium. Growth medium supports the growth of yeast in liquid form. The conditions of all culture were kept constant. Cotton plugs were used as closures in all flasks to allow air to enter the culture and also to avoid contamination of the culture. After 24 hours of incubation in incubator shaker, wall growth can be observed, indicating the growth of yeasts in the medium. As soon as being removed from the shaker, the cell produced can be seen settling at the bottom of the flasks. Figure 4.2 illustrates the culture of yeast in growth medium.

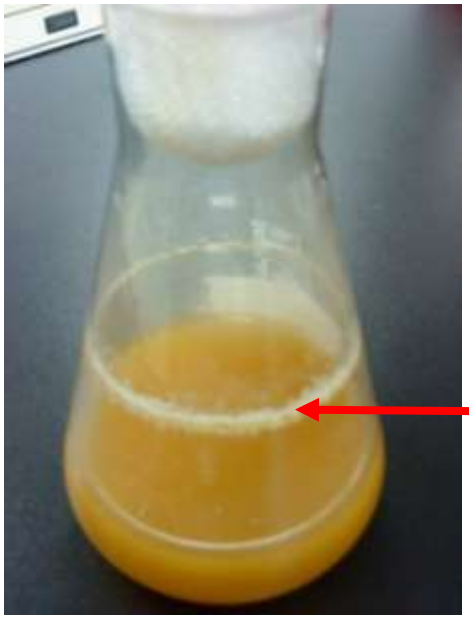

Figure 4.2: Yeast culture in growth medium

4.3 CULTURE OF YEAST IN PRODUCTION MEDIUM

Production medium provides nutrients for the production of desirable products. Different view of cultures can be observed since the conditions of the production medium were varied. Based from the observation, a few wall growth was formed when slow agitation rate is used, which is at 50 rpm. At pH 5, the colour of the culture is lighter compared to culture at pH 9, as can be seen from Figure 4.3. We can conclude that when acidity increase, the colour become lighter.

(a) (b)

Figure 4.3 : *Candida krusei* culture in different conditions of production medium (a) condition: pH 9, 50 rpm, sucrose+starch ; (b) condition: pH 5, 50 rpm, sucrose+starch

The colours also tend to be lighter when starch was used. This is probably because of the starch itself is in white powder form and it affected the colour of the culture when used in high amount. When agitation rate was set at 150 rpm, more wall growth can be observed compared to at 50 rpm. This shows that growth of yeast is greater at higher agitation rate. Figure 4.4 shows the view of the cultures.

(a) (b)

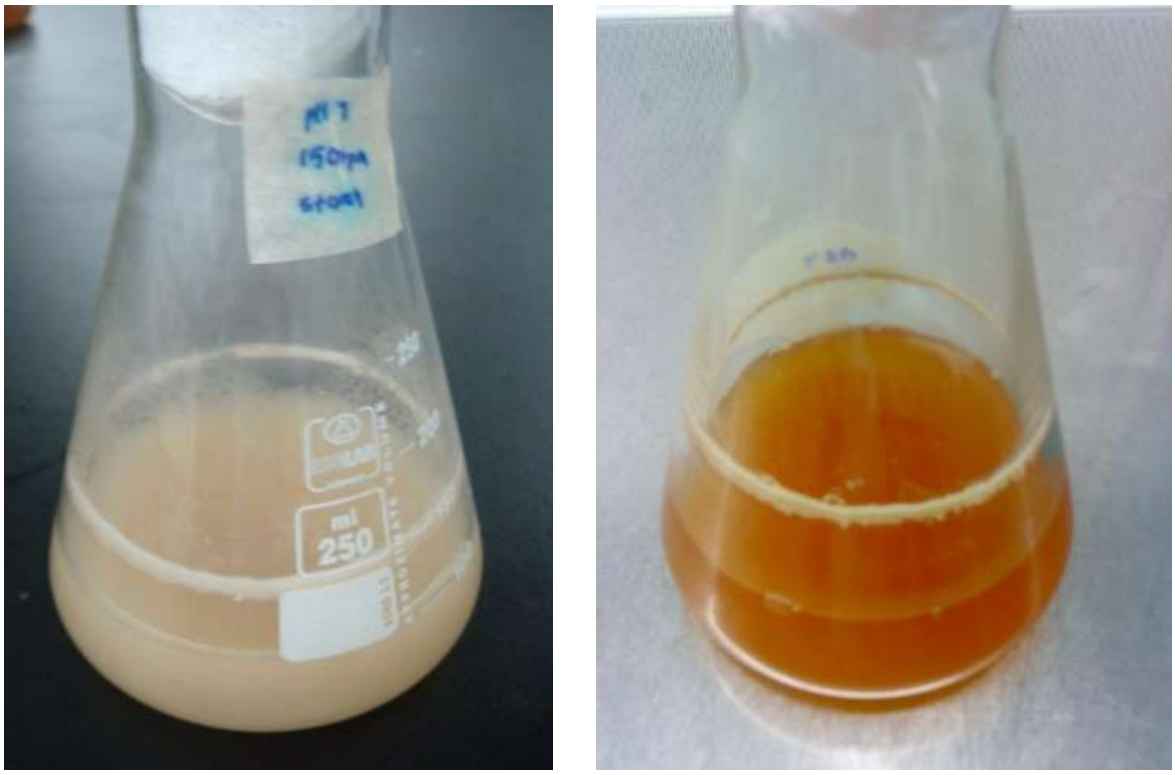

Figure 4.4 : *Candida krusei* culture in different conditions of production medium (a) condition: pH 7, 150 rpm, starch; (b) condition: pH 7, 150 rpm, sucrose

4.4 ANALYSIS RESULT

Arachidonic acid (AA) standard was diluted to three different concentrations. The area obtained at retention time around 38.73 min from the result of gas chromatography was recorded and calibration curve need to be plotted. However, only one of the standards showed peak with 38 min of retention time. Hence, another method was used to determine the concentration of samples, which is by interpolation. The result for every design of experiment was calculated and tabulated in Table 4.1.

Table 4.1: Biomass and arachidonic acid (AA) yield obtained from experimental design

Run	pH	Carbon source	Speed (rpm)	Dry biomass (g/ml)	AA yield (g/ml)
1	9	starch	250	0.0561	0.1493
2	7	sucrose	150	0.1289	5.0916
3	9	starch+sucrose	250	0.0211	0.0800
4	5	starch+sucrose	50	0.0169	0.0000
5	5	starch	250	0.1010	1.5350
6	7	starch+sucrose	150	0.0977	0.0000
7	9	starch+sucrose	50	0.0194	0.0232
8	5	starch+sucrose	250	0.0808	1.3092
9	7	sucrose	250	0.1473	0.0014
10	5	starch	50	0.0204	0.8094
11	9	sucrose	150	0.1175	1.0709
12	5	sucrose	150	0.1234	0.2886
13	7	starch	150	0.0837	0.2055
14	7	sucrose	50	0.0831	0.1993
15	9	starch	50	0.0210	0.0448

4.4.1 Effects of pH

The highest dry biomass concentration was obtained at pH 7. Barath et al. (2010) studied the effect of pH for growth of cell of *Candida krusei* and concluded that the optimum pH was 7. Suitable pH is necessary for growth of yeast as the amount of acid and base can affect the growing process. Different microorganisms species require different pH value. The highest AA content also was obtained at pH 7. Biomass produced was higher at pH 5 compared to pH 9. At high pH value the minerals in yeast become exhausted and it can be in emergency alkalosis (Vincent, 2011). Hence high pH value is not suitable for growth of yeast. However, at pH 5 the yield of AA was the lowest among other pH value. This result shows that lower pH value is not suitable for AA production when using sucrose at 150 rpm. Figure 4.5 shows the biomass obtained for each pH value.

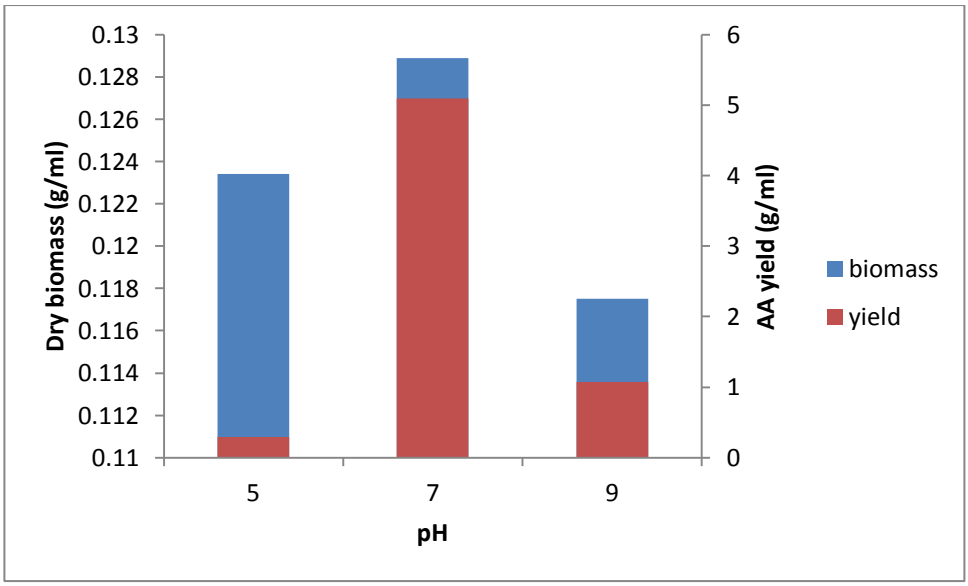

Figure 4.5: Effect of pH on biomass and yield of AA at 150 rpm using sucrose

4.4.2 Effect of Carbon Source

Figure 4.6 shows the effect of carbon source on biomass and yield. From the graph we can see that the highest value of biomass (0.1289 g/ml) was obtained when sucrose was used as carbon source. This result deflects from the findings by Yuan et al. (2002) in which biomass of fungi *Mortierella alpina* was higher in starch compared to sucrose. The highest yield of AA was obtained when using sucrose (5.0916 g/ml). There was no AA yield when Yuan et al. (2002) used sucrose and low yield produced when starch was used. Different carbon sources at 30 g/l were used. Most of the culture that used sucrose produced high biomass. According to Bajpai et al. (2010), both sucrose and starch show poor fungal *Mortierella alpina* growth compared to other sources such as glucose, glycerol and maltose. The mixture of starch and sucrose was found to be not suitable production of AA as zero AA yields was obtained. The mixing of those two substrates may result in reaction that unable AA to be produced.

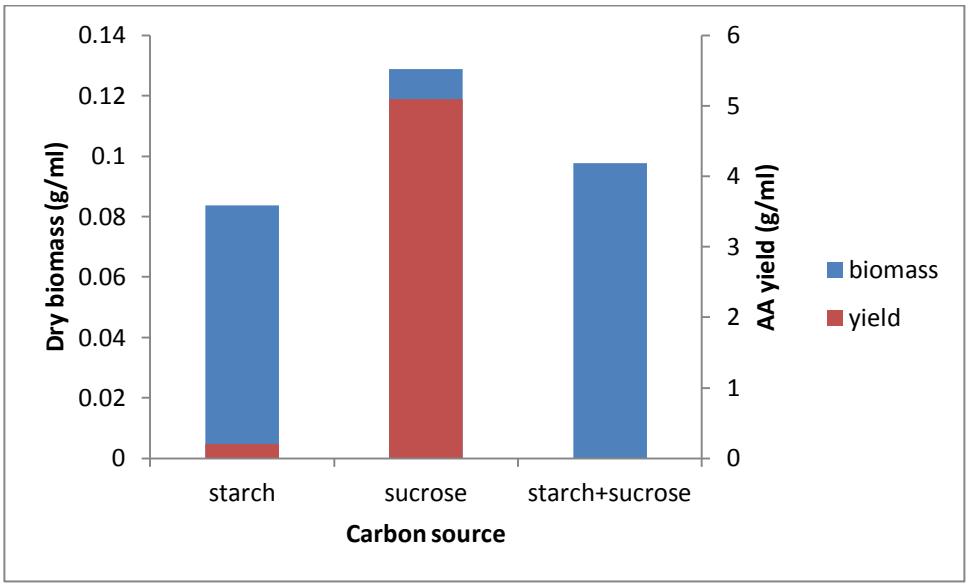

Figure 4.6: Effect of carbon source on yield of AA at pH 7 and 150 rpm

4.4.3 Effect of Agitation Rate

The highest biomass concentration was obtained at 250 rpm. Agitation of culture allows more contact with air and thus increasing the cell produced. Liu et al. (2006) used 200 rpm of agitation rate for culture of *Candida krusei* in their study. The best yield was obtained at 150 rpm. Most researchers used high agitation rate of orbital shaker in production of AA, showing that higher speed is preferable. The yeast growth was the highest at 250 rpm with pH 7 by using sucrose (0.1473 g/ml). However the AA yield was very low, which is 0.0014 g/ml. High speed of agitation may damage the cell and cause the enzyme that is needed in AA production to be disrupted. Hence the yield of AA became very low. As been reported by Palma et al. (1995), excessive agitation resulted in low enzyme xylanase activity for *Penicillium janthinellum.* Figure 4.7 shows the effect of agitation rate on biomass and yield production.

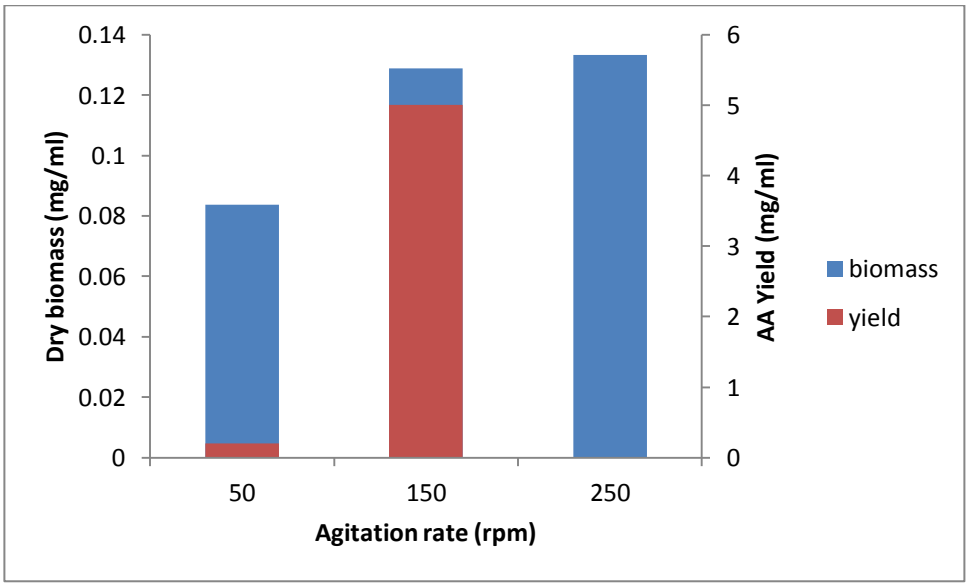

Figure 4.7: Effect of agitation rate on yield of AA at pH 7 using sucrose

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The optimum culture condition for arachidonic acid (AA) production from *Candida krusei* was found to be pH 7 by using sucrose at 150 rpm because the dry biomass and AA yield were the highest. The dry biomass concentration is 0.1289 g/ml while AA yield is 5.0916 g/ml. Whereby, pH 7 happens to be the most suitable pH for AA production. The best carbon source is sucrose compared to starch and mixture of sucrose and starch. Moderate agitation rate that is 150 rpm was favourable.

Arachidonic acid (AA) production from yeast shows advantages over other sources as the production process can be completed in shorter time. Furthermore, it can be consumed by every people as it fit the 'Halal' standard. Production of AA from yeast is also easier compared to other sources as yeast can be obtained easily. Besides that, the process is less complicated compared to animal source, which contain cholesterol and objectionable tastes and odors that need to be removed first.

The wide application of arachidonic acid (AA) can guarantee the potential market place. Its application in pharmaceutical field was discovered a long time ago and people are aware of its significance in life. AA is indeed prominent to infants, adults and old folks. AA can be taken in supplements and is contained in processed food.

5.2 RECOMMENDATION

During analysis process using gas chromatography, the equipment ran out of gas several times. This situation might affect the outcome and made the result undesirable. The vials that were used also may lead to error in analyzing. Those vials which had been used by others and were filled with various contents might not be washed well and can still appear as peak in current analysis. Hence it is important to ensure the cleanliness of the apparatus used.

Limitations of equipment in the laboratory makes the whole project takes longer time than expected. Equipments such as incubator, autoclave and gas chromatography were continuously used by other users. The unavailability of certain chemicals also had delayed the project. Better result might be obtained if the problems faced can be solved earlier.

Further studies on optimization of arachidonic acid (AA) production should be directed at varying other significant parameters including nutrients needed and method of extraction. There are other parameters that must be considered and should not be ignored. Production of a fatty acid is a difficult process and hence suitable method must be used. Long time duration is needed in order to succeed in producing AA.

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APPENDIX A

APPENDIX A1

TABLE OF DATA

Run	Mass of empty tube 1(g)	Mass of freeze- dried sample tube 1 (g)	Dry biomass tube 1	Mass of empty tube 2 (g)	Mass of freeze- dried sample tube 2 (g)	Dry biomass tube 2
1	9.9824	11.2909	1.3085	9.9708	11.1872	1.2164
2	9.7566	12.8072	3.0506	9.9628	12.7109	2.7481
3	9.9374	10.4016	0.4642	9.8847	10.3694	0.4847
4	9.8120	10.2019	0.3899	9.8946	10.2652	0.3706
5	10.0927	12.3844	2.2917	10.0452	12.2954	2.2917
6	9.8829	10.2727	0.3898	9.9108	11.9630	2.0522
7	9.8975	10.3501	0.4526	9.8923	10.3122	0.4199
8	10.0596	11.7955	1.7359	9.9470	11.8484	1.9014
9	9.8627	13.4619	3.5992	9.9106	12.9394	3.0288
10	9.8989	10.3492	0.4503	9.8759	10.3445	0.4686
11	9.9662	12.5449	2.5787	9.9015	12.6098	2.7083
12	10.0927	12.8072	2.7145	9.8734	12.7109	2.8375
13	9.8569	11.6061	1.7492	10.0404	12.0598	2.0194
14	9.9475	11.6615	1.7140	9.8722	11.8981	2.0259
15	9.8974	10.3421	0.4447	10.0025	10.5034	0.5009

APPENDIX A2

CALCULATION FOR DRY BIOMASS

Run 1

pH: 9

Carbon type: starch

Speed: 250rpm

Mass of sample from tube 1 = (11.2090- 9.9824) g

= 1.3085 g

Sample concentration = $\frac{1.3085\ g}{45ml}$ = 0.0291 g/ml

Mass of sample from tube 1= (11.1872- 9.9708) g

= 1.2164 g

Sample concentration = $\frac{1.2164g}{45ml}$ = 0.027 g/ml

Total biomass = (0.0291+ 0.027) g/ml

= 0.0561 g/ml

	Tube 1	Tube 2
Mass of tube (g)	9.9824	9.9708
Mass of tube + freeze-dried sample (g)	11.2909	11.1872
Mass of sample (g)	1.3085	1.2164
Sample concentration (g/ml)	0.0291	0.027
Total (g/ml)	0.0561	

APPENDIX B

APPENDIX B1

GAS CHROMATOGRAPHY

DATA FOR STANDARD

APPENDIX B2

GAS CHROMATOGRAPHY

DATA FOR SAMPLE

APPENDIX C

APPENDIX C1

CALCULATION FOR AA YIELD

Run	Area	Yield
1	1.10815	0.1493
2	36.99206	5.0916
3	0.59379	0.0800
4	-	0.0000
5	11.39401	11.3933
6	-	0.0000
7	0.17220	0.0232
8	9.71730	1.3092
9	0.01039	0.0014
10	6.00763	0.8094
11	7.94856	1.0709
12	2.14208	0.2886
13	1.52534	0.2055
14	1.47927	0.1993
15	0.33252	0.0448

Run 2

pH: 7

Carbon type: sucrose

Speed: 150 rpm

	Standard	Sample
Area	26.72036	36.99206
Yield (g/ml)	3.6	x

$$\frac{3.6}{26.72036} = \frac{X}{36.99206}$$

x= 5.0916 g/ml