PRODUCTION AND PURIFICATION OF OMEGA-3 FATTY ACIDS FROM *Pichia pastoris* USING TRANS-ESTERIFICATION AND MOLECULAR DISTILLATION TECHNIQUES

Le Thi Thuy Trinh

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2020

กระบวนการผลิตและทำให้บริสุทธิ์ของกรดไขมันโอเมก้า 3 จาก *Pichia pastoris* โดยใช้ปฏิกิริยาทรานเอสเทอริฟิเคชั่นและเทคนิคการกลั่นระดับโมเลกุล

นางสาวลี ที ทุย ทริน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2563

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ลี ที ทุย ทริน : กระบวนการผลิตและทำให้บริสุทธิ์ของกรดไขมัน โอเมก้า 3 จาก *Pichia pastoris* โดยใช้ปฏิกิริยาทรานเอสเทอริฟิเคชั่นและเทคนิคการกลั่นระดับโมเลกุล (PRODUCTION AND PURIFICATION OF OMEGA-3 FATTY ACIDS FROM *Pichia pastoris* USING TRANS-ESTERIFICATION AND MOLECULAR DISTILLATION TECHNIQUES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.อภิชาติ บุญทาวัน, 76 หน้า

ทำการศึกษาหาสูตรอาหารในการเลี้ยง *Pichia pastoris* ที่ถูกดัดแปลงพันธุกรรมให้สามารถผลิตกรดไขมันโอเมก้า 3 เพื่อให้ได้ผลิตภัณฑ์ชีวมวลสูงสุดโดยใช้ กลีเซอรอลเป็นแหล่งคาร์บอนร่วมกับเกลือแอมโมเนียม เป็นแหล่งไนโตรเจนโดยใช้โปรแกรมออกแบบ Box-Behnken โดยมี 3 ปัจจัย และ 3 ระดับ เพื่อหาระดับที่เหมาะสมของกลีเซอรอล $(NH_4)_2SO_4$, และ $(NH_4)_2HPO_4$ พบว่าองค์ประกอบที่เหมาะสมคือ กลีเซอรอล 40 กรัม ต่อลิตร $(NH_4)_2SO_4$ 3 กรัมต่อลิตร และ $(NH_4)_2HPO_4$ 7 กรัมต่อลิตร สูตรอาหารนี้ถูกนำไปใช้ในเครื่องปฏิกรณ์ชีวภาพขนาด 500 ลิตร โดยควบคุมปริมาณออกซิเจนละลายอยู่ที่ 30-40 % ในวันที่ 1-3 และหลังจากนั้นลดปริมาณออกซิเจนละลายลงเหลือ 8-10 % จากนั้นในวันที่ 7 ทำการเก็บชีวมวล โดยใช้การกรองระดับไมโครและการปั่นเหวี่ยง ซึ่งได้เซลล์แห้ง 4.367 กรัมต่อลิตร เซลล์ที่ได้ถูกนำเข้ากระบวนการทำแห้งแบบพ่นฝอย หรือ การทำแห้งแบบแช่เยือกแข็ง พบว่า การทำแห้งแบบแช่เยือกให้ผงเซลล์ยีสต์ที่มีคุณภาพสูง หลังจากนั้น ผงยีสต์แห้งถูกนำไปทำปฏิกิริยาทรานเอสเทอริฟิเคชั่นกับเมทานอล เพื่อเปลี่ยนไตรกลีเซอไรด์ให้อยู่ในรูปเมทิลเอสเตอร์ของกรดไขมัน (FAME) การทำปฏิกิริยาทรานเอสเทอริฟิเคชั่น ทำให้สามารถเก็บ FAME ได้ถึง 70% และสามารถข้ามขั้นตอนการสกัดด้วยสารละลายเพื่อหลีกเลี่ยงการเกิดปฏิกิริยาออกซิเดชั่นของกรดไขมันโอเมก้า 3 ในขั้นสุดท้ายได้ใช้การกลั่นแยกสารระดับโมเลกุล (การกลั่นแบบระยะทางสั้น) ในการแยกสาร พบว่า สิ่งเจือปนในตัวอย่างลดจาก 60.93% เหลือ 6.7% กรดอิโคซะเพนตะอีโนอิก (EPA) หายไปในกระบวนการกลั่น แต่กรดโดโคซะเฮกซะอีโนอิก (DHA) เพิ่มจาก 0.012% เป็น 0.0135% ในอนาคตอาจจะนำเอทานอลมาใช้ทดแทนเมทานอลในปฏิกิริยาทรานเอสเทอริฟิเคชั่นเพื่อลดความเป็นพิษ นอกจากนั้นปฏิกิริยากลีเซอโรไลซิสยังเป็นอีกทางเลือกหนึ่งที่สามารถนำมาประยุกต์ใช้ในการเปลี่ยน FAME เป็นไตรกลีเซอไรด์

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา_____

LE THI THUY TRINH : PRODUCTION AND PURIFICATION OF OMEGA-3 FATTY ACIDS FROM *Pichia pastoris* USING TRANS-ESTERIFICATION AND MOLECULAR DISTILLATION TECHNIQUES. THESIS ADVISOR : ASSOC. PROF. APICHAT BOONTAWAN, Ph.D., 76 PP.

OMEGA-3 FATTY ACIDS/*PICHIA PASTORIS*/FERMENTATION/TRANS-ESTERIFICATION/MOLECULAR DISTILLATION

The compositions of the medium for recombinant *Pichia pastoris* producing omega-3 fatty acids were determined for maximum biomass production using crude glycerol as a carbon source and ammonium salts as nitrogen sources. The optimization of the medium was done using Box-Behnken, a design software with 3 factors, 3 levels, and 1 response for an optimal level of crude glycerol, $(NH_4)_2SO_4$, and $(NH_4)_2HPO_4$. The optimum composition was 40 g/L crude glycerol, 3 g/L $(NH_4)_2SO_4$, and 7 g/L $(NH_4)_2HPO_4$. This medium was used in a 500L bioreactor with DO 30-40% until day 3 and then reduced DO to 8-10% until day 7. The biomass at day 7 was 4.367 g/L DCW. Using microfiltration and centrifugation, the cells were obtained and drying by spray dry or freeze dry. The freeze dry method was chosen in this study since it gave high quality of dry *P. pastoris* cells. Direct trans-esterification was done with methanol to transfer triglycerides to FAME. The advantages of the direct trans-esterification are high yield up to 70% of FAME can be obtained, no oil extraction step is needed, and oxidation of omega-3 fatty acids can be avoided. Finally, the molecular distillation (short part distillation, SPD) was done. The impurity of the sample before running the SPD was reduced from 60.93% to 6.7 %. The eicosapentaenoic acid (EPA) was lost

after running the SPD, but the docosahexaenoic acid (DHA) increased from 0.012% to 0.0135%. In the future work, ethanol can be used instead of methanol for trans-esterification that is can be safe for human health. Another way, the glycerolysis can be applied to transfer FAME to triglycerides for human diet.

School of Biotechnology

Academic Year 2020

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis advisor Assoc. Prof. Dr. Apichat Boontawan and Co – advisor Assoc. Prof. Dr. Mariena Ketudat-Cairns for their helpful comments and support of this thesis. I am most grateful for their teaching in thorough grounding in the principles of the research methodologies and also many other methodologies in life.

Besides that, I would like thank all my lab members and Dr. Chotika Gosalawit for help in the preparation of my research, the discussion and the suggestion in everything. Moreover, I would like to thank my committee: for their comments, questions and insightful.

Finally, I would like to add personal thank to my parents for who supports me throughout in my life.

Le Thi Thuy Trinh

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LIST OF ABBREVIATIONS

LC-PUFAs	=	long chain polyunsaturated fatty acid
EPA	=	eicosapenataenoic acid
DHA	=	docosahexaenoic acid
DNA	=	Deoxyribonucleic acid
P. pastoris	=	Pichia pastoris
LA	=	linoleic acid
ALA	=	α-linolenic acid
EFAs	=	essential fatty acid
STA	=	stearodonic acid
ETA	=	eicosantetraenoic acid
ARA	=	arachidonic acid
DPA	=	docosapentaenoic acid
GLA	=	γ-linolenic acid
SDA	=	stearidonic acid
D6-Des	=	delta-6 desaturase
D6-Elo	=	delta-6 elongase
DGLA	=	di – homo – γ - linoleic acid
$C_6H_6O_6$	=	glucose
FAEEs	=	fatty acid ethyl esters
GAP	=	glyceraldehyde-3-phosphate dehydrogenase
ICP-OES	=	inductively coupled plasma optical emission spectrometry

LIST OF ABBREVIATIONS (continued)

HPLC	=	high performance liquid chromatography
DCW	=	dry cells weight
DO	=	dissolve oxygen
MF	=	microfiltration
^{0}C	=	degree Celsius
OD	=	optical density
µg	=	microgram
SPD	=	short path distillation
TAG	=	Triacylglycerol

CHAPTER I

INTRODUCTION

1.1 Significance of study

Omega-3 long chain fatty acid are increasingly of commercial production for human health. In the past few decades, many clinical studies have been conducted on the health benefits of omega-3 LC-PUFAs. The most important of omega-3 long chain fatty acids are eicosapentaenoic acid (EPA; C20:5n-3) and docosapentaenoic acid (DHA; C22:6n-3). EPA can reduce heart attacks, strokes, and cardiovascular deaths (Xie et al., 2015). DHA is a structural component of the human brain, cerebral cortex, skin, and retina (Connor and Neuringer, 1988). Omega-3 LC-PUFAs are necessary for human, and have been highly recommended for human diet. Normally, omega-3 LC-PUFAs can be acquired from a variety of sources such as fish oil, plant oil, and krill oil. However, the primary source of omega-3 in the human diet is often derived from fish oil. The amount of omega-3 in fish is usually limited, and is difficult to collect. Furthermore, the use of fish oil is limited due to problems associated with its typical fishy smell, unpleasant taste, and poor oxidative stability.

Pichia pastoris, a yeast that is an established protein expression host mainly applied for the production of biopharmaceuticals and industrial enzymes. It has easily been applied to produce recombinant proteins such as an omega-3 fatty acids biosynthesis pathway proteins. Therefore, they have been considered as a promising alternative recombinant omega-3 fatty acid *P. pastoris* for fish oil as source of these

fatty acids. Cultivation of *P. pastoris* have many advantages in term of easy controlling, being uni-cellular, and can use of cheap raw materials such as cassava, molasses, crude glycerol, etc. In addition, scientists have found that the *P. pastoris* can be used for the production of omega 3 fatty acids containing docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) (Chotika, 2013). In this study, the recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6 was used to produce omega-3 fatty acid (Chotika, 2013). However, any products after fermentation can be collected from the downstream processing.

1.2 Objectives

Production and purification of omega-3 fatty acid from *Pichia pastoris* for human health:

- To identify optimum medium and fermentation conditions
- To compare two drying methods (spray dry and freeze dry) for the best method for *P. pastoris* preparation
- To apply the conditions of the trans-esterification such as reaction temperature, reaction times, ethanol/oil ratio, and catalyst concentration
- To purify omega-3 fatty acids by using molecular distillation

1.3 Research hypothesis

P. pastoris contains high omega-3 fatty acids in cells. High biomass can be collected by optimum condition of production. Purification of Omega-3 fatty acids collected by optimum condition methyl ester can be obtained by direct transesterification and molecular distillation.

1.4 Scope and limitations

The main focus of this project is to design an efficient process to extract omega-3 fatty acids from *P. pastoris*. This study can determine the optimal production and purification conditions of omega-3 fatty acids from *P. pastoris* such as fermentation condition, drying cells, direct trans-esterification, and molecular distillation. The fermentation conditions of *P. pastoris* can be investigated and supported high yield omega-3 fatty acids. As presented above, the omega-3 methyl ester yield is affected by the trans-esterification factors including temperature, quality of catalyst, and a shaker of rotation speeds. Therefore, the methodology can be applied to obtain high quality omega-3 fatty acid.

1.5 Expected results

This research can obtain the optimum conditions of medium, examined the optimum of fermentation to support yeast which can be produced high omega-3 fatty acids. In addition, we hope that our research can gather the scientific data from factors control parameters of the trans-esterification process. The results of molecular distillation can success to help purification processing for omega-3 fatty acids.

CHAPTER II

LITERATURE REVIEW

Before proceeding to the materials and methods of this study, it is necessary to summarize the knowledge that makes the study more clearly. Below is a literature review for this study.

2.1 Omega-3 fatty acids

2.1.1 Definition and the importance of omega-3 fatty acids

Omega-3 LC-PUFAs are necessary fatty acids for the human diet, including eicosapentaenoic acid (EPA, C20:5n-3), and docosahexaenoic acid (DHA, C22:6n-3) figure 2.1. DHA (given name 22:6n-3) is a structural component of the human brain, cerebral cortex, skin, and retina. It can be synthesized from α-linolenic acid ALA, or obtained directly from maternal milk (breast milk), fish oil, or algae oil (Sun et al., 2018). High levels of DHA intake can reduce the risk of depression, bipolar disorders, and mood disorders. Furthermore, DHA can prevent autism and behavior disorders through a selective and representative revision of different papers ranging from pure observational studies to randomized control trials. It is also good for pregnant women and lactation. During perinatal development through placental transfer and maternal milk, DHA for pregnant women will determine the status of the newborn and consequently effect on post-natal development of the brain and visual functions (Sun et al., 2018). Therefore, DHA is really important, and is widely used for human health.

EPA is a carboxylic acid with a 20-carbon chain and five cis double bonds; the first double bond is located at the third carbon from the omega end. EPA can reduce heart attacks, strokes, and cardiovascular deaths (Xie et al., 2015).

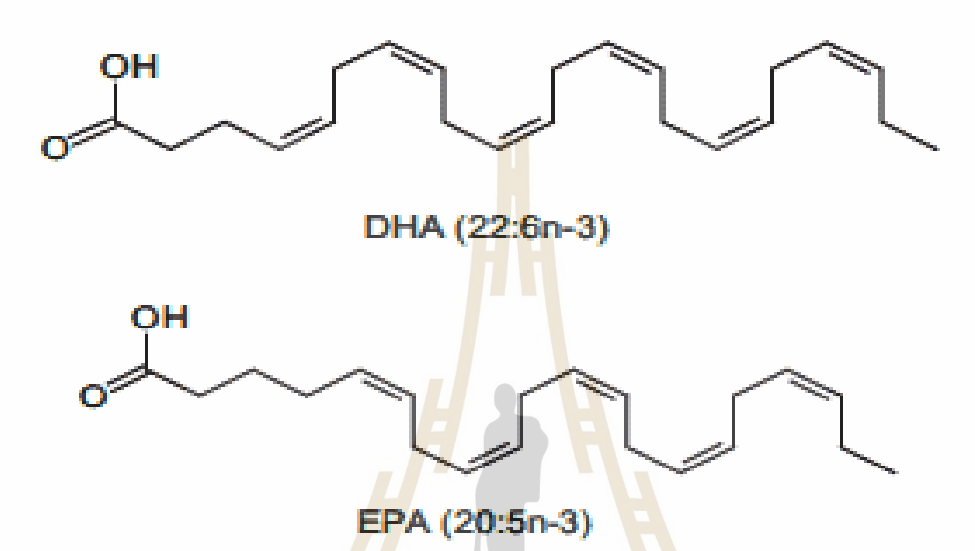


Figure 2.1 Chemical structure of DHA and EPA

2.1.2 Omega-3 fatty acids biosynthesis pathway

Previous studies have suggested biosynthesis of these fatty acids. Linoleic acid (LA) and α-linoleic acid (ALA) cannot be synthesized in mammalian tissues, and hence are regarded as essential dietary components. Higher plants are the major source of these essential fatty acids (EFAs). Fatty acid biosynthesis in plants takes place in plastids. The metabolic pathway of omega-3 LC-PUFAs synthesis from dietary α-linoleic acid (ALA) is shown in Figure 2.2. Stearodonic acid (STA) is the first metabolite synthesized from α-linoleic acid (ALA), which subsenquently leads to the synthesis of eicosantetraenoic acid (ETA), eicosapentaenoic acid (EPA),

docosapentaenoic acid (DPA), and DHA. The conversion requires desaturases (Δ6, Δ5, and Δ4), an elongase of the microsomal system, and oxidation in the peroxisomes for chain shortening. Besides, the pathway of subject omega-6 PUFAs synthesis is from linoleic acid (LA) where arachidonic acid (ARA) is the major end product.

The PUFAs pathway of lower eukaryotes (fungi, algae and protozoa) contains Δ9 desaturase (Δ9), Δ12, and Δ15. Lower eukaryotes also have Δ8 desaturase (Δ8) which is an alternative pathway for generating of EPA (Eicosapentanoic acid) (C20:4n-3). Another enzyme, Δ4 can add a double bond at the forth carbon atom of DPA (Docosapentaenoic acid) (22:5n-3) to produce DHA (Venegas-Calerón et al., 2010). The expression of Δ4 has been an alternative pathway for the generation of DHA in vertebrate (Li et al., 2010). Some fungi can add double bond by the activity of Δ17 desaturase (Δ17) for the generation of both ETA and EPA as shown in Figure 2.2.

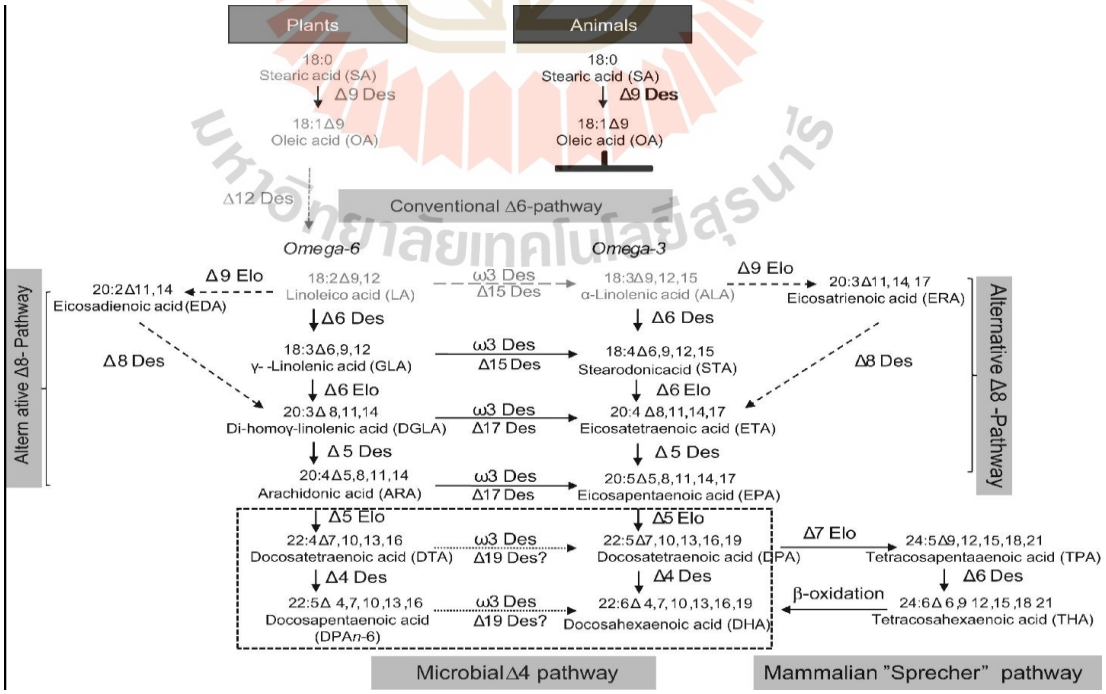


Figure 2.2 PUFAs biosynthesis pathway (Sayanova and Napier, 2011)

2.2 Sources of oil

Rich sources in omega-3 fatty acids include fishes, microalgae, yeasts, and some oil plants. However, the commercial production of omega-3 fatty acids uses fishes, microalgae, and yeast, for the human diet. The following sections compare advantages and disadvantages of oil sources.

2.2.1 Fish oil

Fish oil is obtained from the tissues of various kinds of fish such as salmons, herrings, mackerels, anchovies, and sardines. Although omega-3 fatty acids are collected by fish, it does not synthesize it (Falk-Petersen et al., 1998). They obtain omega-3 fatty acids from the algae or plankton in their diets (ibid.). The main composition of fish is omega-3 including eicosapentaenoic (EPA), and docosahexaenoic acid (DHA). Other types of fish such as tunas also contain omega-3 fatty acids, but it has a small amount.

However, the use of fish oil is limited due to problems associated with its typical fishy smell, unpleasant taste, and poor oxidative stability. The amounts of fish are also limited. Moreover, fish oil supply is difficult to predict, especially in the long term, due to the decline in fish stocks in many of the world's oceans. Thus, other sources such as microalgae, yeast, invertebrates are therefore considered as a promising alternative for fish oil.

2.2.2 Microalgae oil

Microalgae, a kind of photosynthesis, are typically found in fresh water, and marine systems. Depending on types of species, their sizes can range from a few micrometers to a few hundred micrometers. Omega-3 long chain PUFAs can be

synthesized from microalgae. Algae oil has more advantages, compared to fish oil such as the consistency of the composition, sensory properties, and ease of production. Plus, algae oil does not possess odors like fish oil which is a reason for limiting its use. Moreover, the production of algae biomass is easier, and more environmentally friendly which gives higher yields than fish oil production (Ratledge and Cohen, 2008).

However, the algae cultivation system requires a big area, and needs sunlight to grow resulting in a long cultivation time. Moreover, the production process can be easily contaminated when the culture is in an open pond.

2.2.3 Microbial oil

Omega-3 fatty acids can be synthesized by some kind of yeast such as *P. pastoris*. This yeast is an established protein expression host which has been mainly applied for the production of biopharmaceuticals and industrial enzyme (Ahmad et al., 2014). Therein, *P. pastoris* mutant can produce a high level of polyunsaturated fatty acids (PUFAs), which are typically synthesized by strings of reactions catalyzed by desaturase and elongase enzymes. For example in Kang et al, 2008, the Elo-like enzyme was involved in multiple reactions leading to the production of PUFAs, and that the *TaElo*, *Tad5*, and *Tad4* genes were capable of functioning together to produce DPA and DHA by using GLA and SDA (Kang et al., 2008).

Another hand, *P. pastoris* is easy to grow, and uses glucose carbon source from cassava roots (hydrolysis by enzymes) which is the decrease in price of final products. The *P. pastoris* exhibits several advantages compared to other oil sources such as high levels of recombinant protein production in a protein free medium, safety for human, ability to purify secreted protein from growth medium without harvesting

yeast cells, with low costs the cost for purify secreted protein from growth medium without harvesting the yeast cells, and elimination of endotoxin and bacterial contamination (Chotika, 2013). *P. pastoris* expressed all of the Δ9, Δ12, and Δ15 desaturase activity (Chotika, 2013). Moreover, it can be easy to construct the recombinant *P. pastoris* containing a high level of PUFAs. That results in the insertion of Δ6, E6 and Δ5 could generate EPA and DHA in *P. pastoris* cells (Chotika, 2013).

2.3 Metabolism engineering of an oleaginous yeast

The natural producers of omega-3 fatty acid are mainly marine bacteria, fungi, and microalgae. Nevertheless, this study focuses on yeasts that the sources can be a promising alternative for fish oil. They follow aerobic desaturase and elongase pathway. The enzymatic activities encoded by all these genes can be divided into two classes: fatty acid desaturation, and chain elongation. The desaturation, is generally catalyzed by a front-end desaturase, introduces a double bond between the pre-existing double bond and the carboxyl end of a fatty acid substrate or a methyl-end desaturase that inserts a double bond between the pre-existing double bond and the methyl-end (Hashimoto et al., 2008).

Figure 2.3 shows the process of producing fatty acid, fatty alcohols, alkanes, etc. The production pathway of omega 3 fatty acids has two parts: the conventional D6-pathway which utilizes a delta-6 desaturase (D6-Des), and delta-6 elongase (D6-Elo) which converts LA and ALA, produces DGLA and ETA, respectively. Thanks to the elongation desaturation, the fatty acetyl-CoA converted into to a long chain (>20) (SCD1, stearoyl-CoA desaturase).

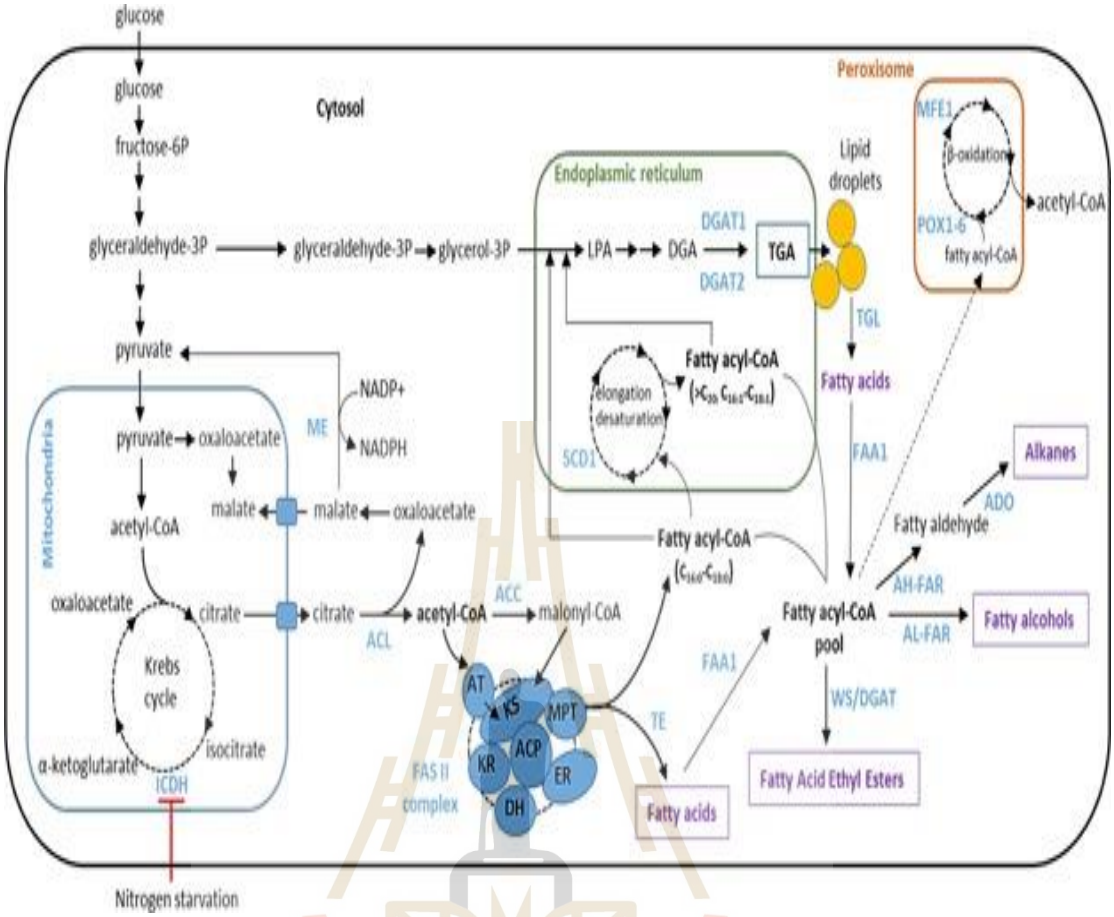


Figure 2.3 Biochemistry of lipid (TAGs) accumulation in oleaginous yeast and engineering pathways for the production of oleochemicals (Hashimoto et al., 2008). ICDH, isocitrate dehydrogenase; ME, malic enzyme; ACL, ATP citrate lyase; ACC1, acetyl-CoA carboxylase; TE, thioesterase; FAA1, fatty acyl-CoA synthetase; SCD, stearoyl-CoA desaturase; DGAT1/DGAT2, diacylglycerol acyltransferase; TGL, triacylglycerol lipase; WS/DGAT, wax ester synthase/acyl-CoA diacylglycerol acyltransferase; AH-FAR, aldehyde-forming fatty acyl-CoA reductase; AL-FAR, alcohol-forming fatty acyl-CoA reductase; ADO: aldehyde deformilating oxygenase; POX1-6, peroxisomal acyl-CoA oxidases, MFE1, hydroxyacyl-CoA dehydrogenase (Adrio, 2017).

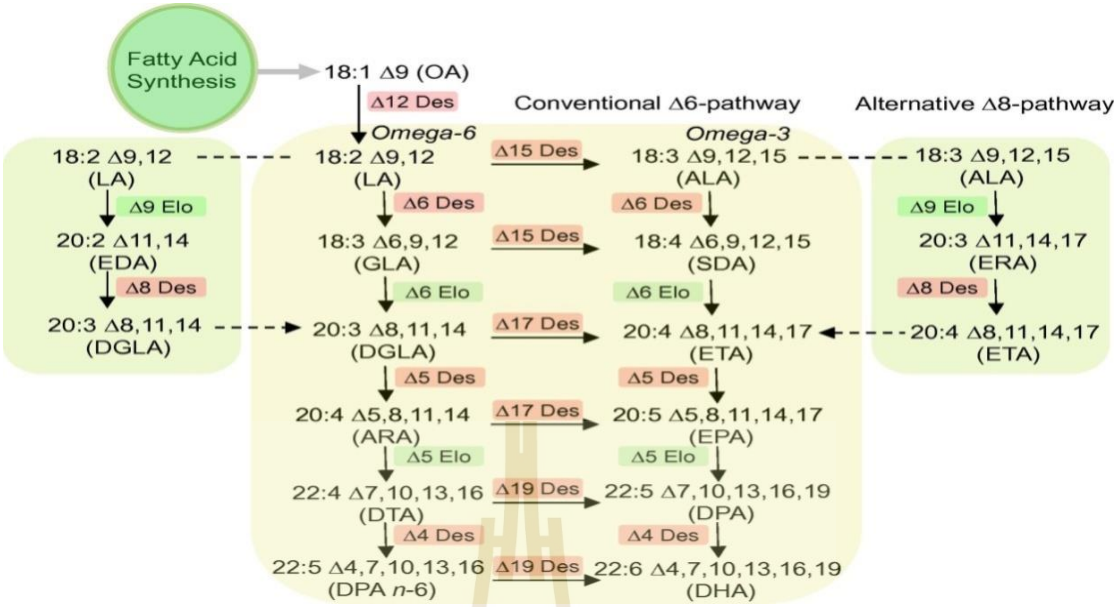


Figure 2.4 Schematic diagram of the aerobic pathway for EPA and DHA synthesis. The conventional D6-pathway utilizes a delta-6 desaturase)D6-Des(and delta-6 elongase (D6-Elo) convert LA and ALA, producing DGLA and ETA, respectively. The alternative D8-pathway utilizes a delta-9 elongase and delta-8 desaturase to convert LA and ALA, producing DGLA and ETA, respectively. Both pathways need a delta-5 desaturase (D5-Des), delta-5 elongase (D5-Elo) and delta-4 desaturase (D4-Des) to synthesize DHA. Conversion of 18 carbon, 20 carbon or 22 carbon omega-6 fatty acids to omega-3 fatty acids is catalyzed by the delta-15 desaturase (D15-Des), delta-17 desaturase (D17-Des) and a hypothetical delta-19 desaturase (D19-Des), respectively. Fatty acids are OA, oleic acid; LA, linoleic acid; GLA, c-linolenic acid; DGLA, di-homo-c-linolenic acid; AA, arachidonic acid; ALA, a-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid and DHA, docosahexaenoic acid (Kamisaka et al., 2016).

From Figure 2.4, it can be seen that there are two distinct and converging pathways existing in the omega-3-LCPUFA-synthesizing microorganisms: the conventional D6-pathway, and the alternative D8-pathway. In the conventional D6-pathway, the biosynthesis of x3-LCPUFAs begins with the D6-desaturation of LA (n-6) and ALA (n-3), which is catalyzed by D6-desaturase, resulting in the synthesis of c-linolenic acid (GLA, 18:3D6,9,12) and stearidonic acid (SDA, 18:4D6,9,12,15), respectively. These D6-desaturated fatty acids are then elongated by a D6-elongase to yield di-homo-c-linolenic acid (DGLA, 20:3D8,11,14) and eicosatetraenoic acid (ETA, 20:4D8,11,14,17), respectively. A D5-desaturase is required for further conversion of DGLA and ETA to form ARA (n-6) and EPA (n-3).

In the alternative D8-pathway, the biosynthesis of x3-LCPUFAs is initiated by the D9-elongation of the same substrates as D6-pathway (LA and ALA), which is catalyzed by a specific D9-elongase, generating eicosadienoic acid (EPD, 20:2D11,14) and eicosatrienoic acid (EPA, 20:3D11,14,17).

2.4 Carbon source

For cultivation of microorganism, carbon source is an important compound of medium for cells grow up. Depending on kind of microorganism, carbon source consumption ability will be different. For yeast, it cannot digest starch but fungi can use as good. In general, popular carbon sources are such as glucose, cassava starch, molasses, crude glycerol, etc.

2.4.1 Glucose

Glucose (monosaccharide) is a simple type of sugar with molecular formula $C_6H_{12}O_6$ that is an important energy source in living things. Almost microorganism can metabolize simple sugar such as glucose for living. Glucose source using for commercial is prepared using starch such as cassava root, rice, corn, etc. The commercial amylase enzyme has been used hydrolysis starch to glucose. Based on the starch, amount of amylase and type of amylase will be different.

2.4.2 Molasses

Molasses is a viscous by-product from refining sugarcane into sugar. All most sugar in molasses is glucose, sucrose, fructose and some impurities (Ghorbani et al., 2011). Normally, microorganism prefer to use glucose more than fructose. For sucrose, start of sucrose consumption and microorganism consumption rate was slower and depend on initial sugar concentration (Ghorbani et al., 2011).

2.4.3 Crude glycerol

Crude glycerol is main by-product from biodiesel production. Therefore, decreasing the waste crude glycerol, scientist have been used crude glycerol like carbon source for microorganism. Previous studies, a lot of microorganism can use crude glycerol as well including yeast. In general, crude glycerol contains glycerol, salts, methanol or ethanol, water, soap and other impurities (Luo et al., 2017). Methanol is a toxic to most microorganism but P. pastoris can utilize it (Anastácio et al., 2014). Depending on the oil and catalyst for biodiesel reaction, the impurities have free fatty acid, monoacylglycerols and biodiesel, salts, etc (Anastácio et al., 2014). Based on the recombinant protein and plasmid, *P. pastoris* can use a different amount of crude

glycerol for high products. Furthermore, the other nutrients were added to support getting more product (Table 2.1).

Table 2.1 Consumption of crude glycerol, nitrogen sources of *P. pastoris*

Microbial	C source	N source	Biomass	Products	References
P. pastoris X-33	1 % or 2 % (w/v) crude glycerol	1 % yeast extract, 2% peptone	OD = 9	α-amylase	(Anastácio et al., 2014)
P. pastoris (pGAPZ)	70 g/L crude glycerol (80% glycerol)	5.17 g/L ammonium sulfate	OD = 18, 146 g/L, 60 hours	phytase	(Tang et al., 2009)
P. pastoris E17	12.6 g/L glycerol	1 g/L yeast extract 2 g/L peptone YNB 1.34 g/L 4.10^{-5} g/L biotin	11 g/L at 50 hours	RHuEPO	(Eda Çelik et al., 2008)
P. pastoris X-33	40 g/L crude glycerol (84% glycerol w/w)	12.4 $(NH_4)_2HPO_4$ 2.00 citric acid;	15g/L at 20 hours, adjusted to pH 7 with KOH;	recombinant lipase B from *Candida antarctica*	(Robert et al., 2017)
P. pastoris	40 g/L glycerol	12.6 g/L $(NH_4)_2HPO_4$	14g/L at 25 hours	L-lactic acid	(Lima et al., 2016)
Pichia pastoris strain TBS2Man4-pPICZaA-X33	4-5% (w/v) with glycerol 80%	BSM medium and maintained by 17% (w/v) NH_4OH	15g/L for flask 22 g/L for fermentor 5L	thermostable b-mannanase	(Luo et al., 2018)
Pichia pastoris strain pGAPZ:Δ6&Δ5:E6.	40 g/L crude glycerol	3 g/L $(NH_4)_2SO_4$, and 7 g/L $(NH_4)_2HPO_4$	4.367 g/L 500L fermenter	Omega-3 fatty acid	In this study

Table 2.2 Consumption of crude glycerol, nitrogen sources of *P. pastoris* (Continued)

Microbial	C source	N source	Biomass	Products	References
Pichia pastoris SMD 1168	Glycerol 13 g/L	yeast extract, 10 g/L; casein peptone, 20 g/L; yeast nitrogen base with ammonium sulfate, 13.4 g/L; biotin, 4.10^{-4} g/L in 100 mM and casamino acids, 20 g/L	24 g/L	anti LDL (-) single-chain variable fragment	(Arias et al., 2019)

2.5 Fermentation

The growth of yeast or other microorganisms in batch culture can be modeled with four different phases as follow in Figure 2.5

2.5.1 Lag phase

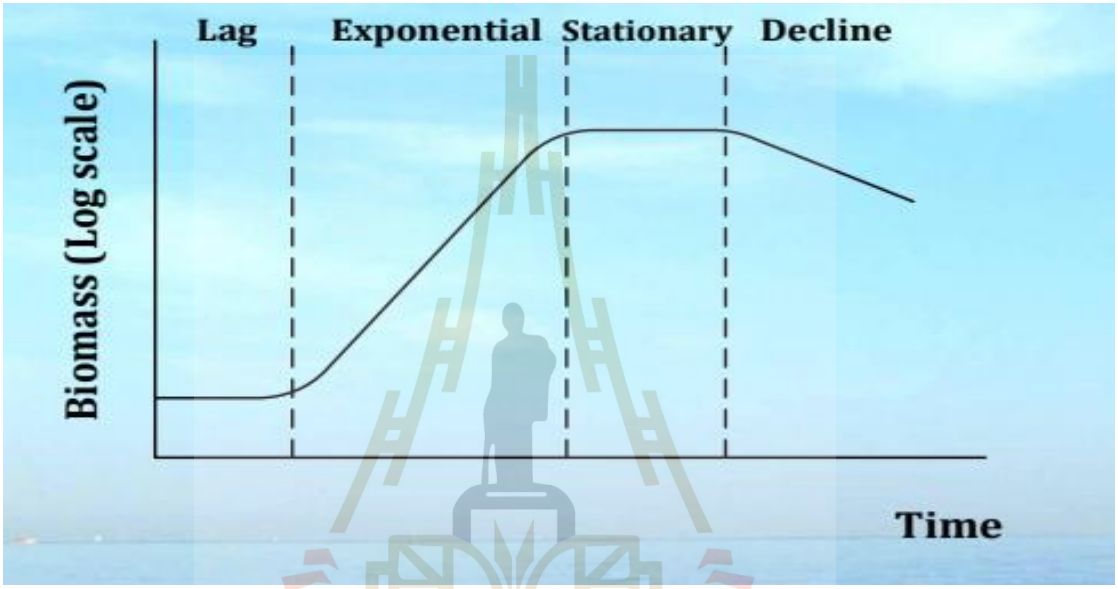


Figure 2.5 Growth curve of yeast or other microorganism

The rate of growth is essentially zero. Because yeast is inoculated into new medium, the cells cannot reproduce new cells in a new medium. Thus, cells use the lag phase to adapt to their new environment, new enzymes or structural components may be synthesized. Depending on microorganism, yeast was needed around 1 day for this lag phase.

2.5.2 Exponential phase

After lag phase, cells can grow really fast, cells exponentially multiply. Nutrients in the broth later depleted or inhibitory products accumulate, growth slows down and the cells enter the stationary phase.

2.5.3 Stationary phase

In this phase, cells cannot growth well due to nutrients source, inhibit product and waste itself. There is no further growth and some cultures exhibit a death phase as the cells lost viability. The growth curve becomes plateau.

2.5.4 Dead phase

The growth enters death phase in which the active mass reduce as the cell population dies away.

Growth rate modeling

Growth rate of yeast or microorganism depend on concentration of cells. It can describe as follow:

$$\frac{dx}{dt} = \mu x$$

Integration of equation

$$\int_{x_0}^{x_t} \frac{dx}{x} = \int_{0}^{t} \mu dt$$

$$\ln(x_t) - \ln(x_0) = \mu(t - 0)$$

$$\ln\left(\frac{x}{x_0}\right) = \mu t$$

x = concentration of cells at time

x_0 = initial concentration

μ = specific grpowth rate

t = time

For exponential phase the specific growth rate is maximum as follow:

$$\ln\left(\frac{x}{x_0}\right) = \mu_{max} t$$

Doubling time (t_d) is solve by assume that cells increase double. Thus, $x = 2x_0$

$$\ln\left(\frac{2x_0}{x_0}\right) = \mu_{max} t_d$$

$$\ln(2) = \mu_{max} t_d$$

$$t_d = \frac{\ln(2)}{\mu_{max}}$$

$$t_d = \frac{0.693}{\mu_{max}}$$

2.6 Cells harvesting

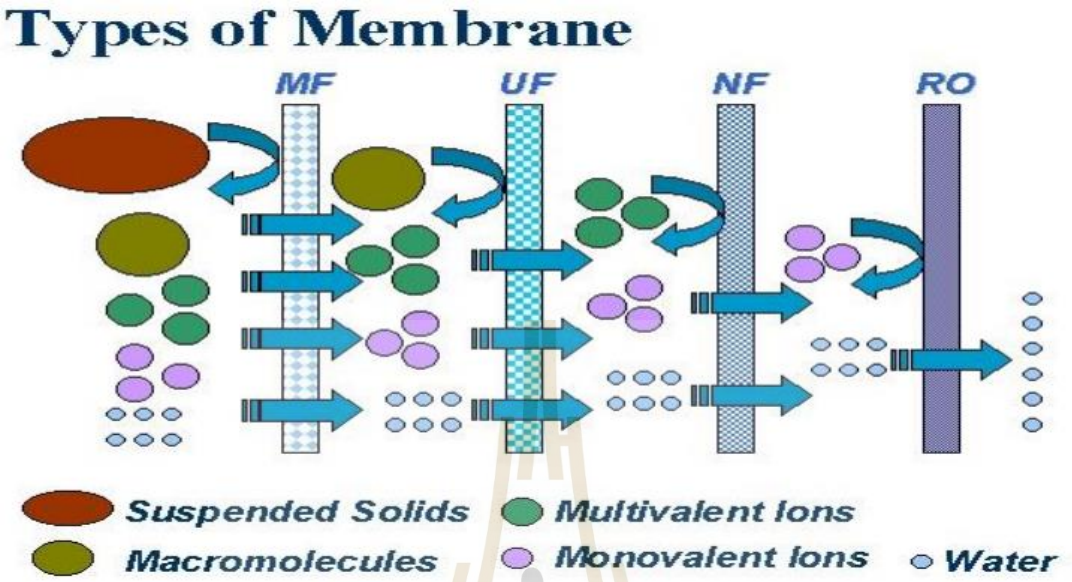


Figure 2.6 Comparison of membrane techniques

After fermentation, yeast cells should be separated from broth by using membrane technology and centrifugal. Using membrane technology is almost reduced water in broth that helping for next step centrifugation.

2.6.1 Membrane technology

Membrane separation process have many kind of membrane that depend on the pore of membrane such as microfiltration, ultrafiltration, nanofiltration, and reverse osmosis, etc. Based on the separation mechanisms and size of the separated particles, the membrane can choose to get a main product that show in Figure 2.6.

Microfiltration is a membrane separation process whose objective is to remove bacterial cells from broth. The size range of the membrane is around 0.1μm. The filtration has two types: dead-end filtration and cross-flow filtration. For the dead-end filtration, the direction of the flow is from the perpendicular to the membrane. All

particles accumulate on the membrane surface, and the permeation rate reduces quickly. In order to maintain the permeation rate, the pressure must increase. At some point, there will be no permeate and the membrane must be changed. Regarding the cross-flow filtration, the flow is parallel to the membrane surface. The feed re-circulates across the surface, producing permeate and retentate streams. High liquid flow rate prevents the formation of cake layers, and constant flux is obtained. The membrane requires pumps, module designs, and piping. After the process finished, back-washing can be done to regain flux. The flux characteristic both cross flow and dead end is shown is Figure 2.8. Hence in this study, the microfiltration will use the cross-flow filtration.

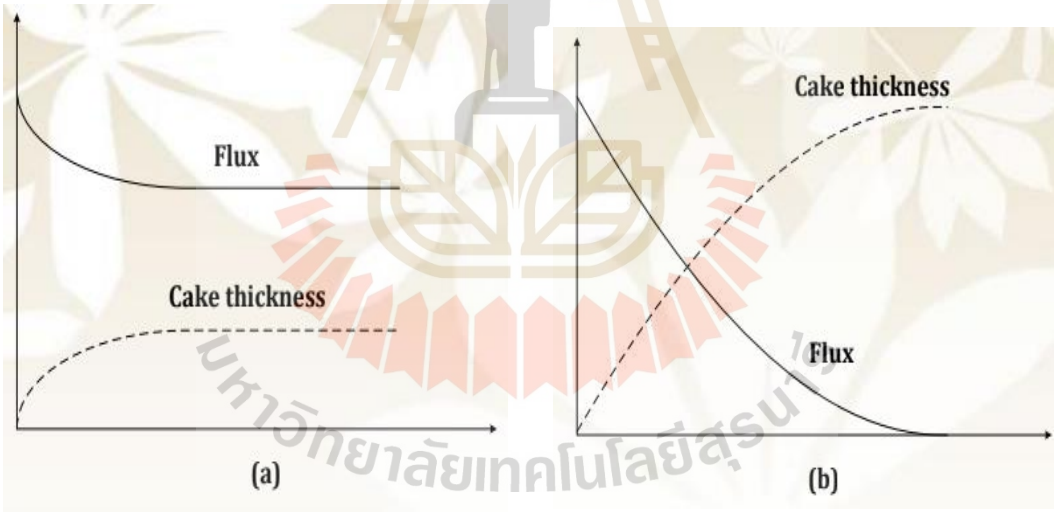


Figure 2.7 Types of filtration: cross flow and dead end

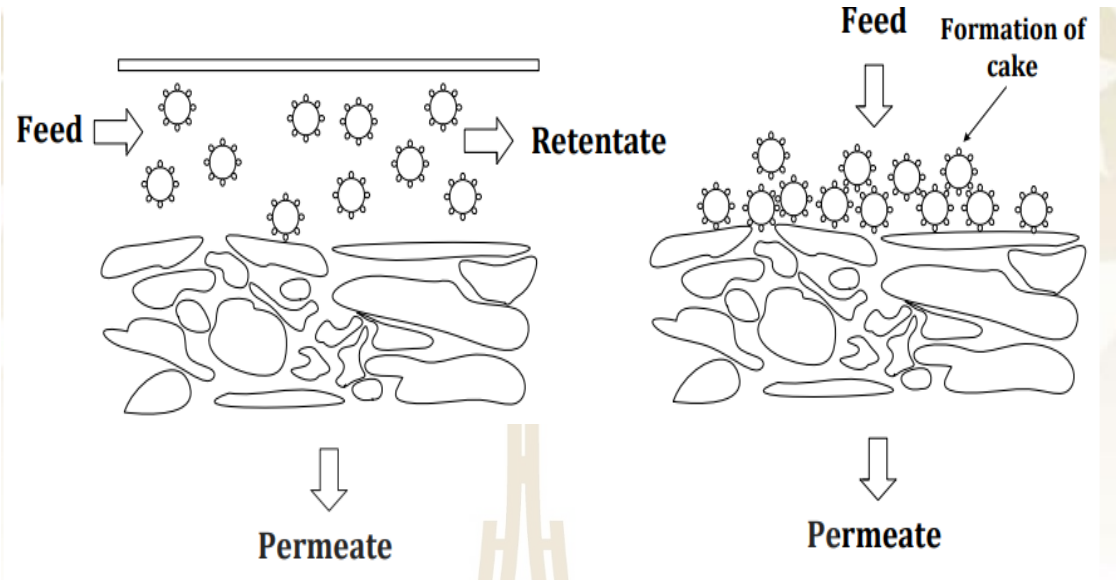


Figure 2.8 Flux characteristic of (a) cross flow and (b) dead end

2.6.2 Centrifugation

Thus, microfiltration has the disadvantage that cannot separate all of water from broth, the centrifugation should be used to obtain high concentrated cells. Depending on volume of broth, centrifugal have models for lab scale or commercial scale. In addition, a centrifuge is a device for separating particles on a solution based on their size, weight, density, viscosity of the medium and rotor speed.

2.7 Spray dry and freeze dry

Spray drying is a method of producing dry powder from a liquid or slurry by rapidly drying the liquid with a hot gas. Freeze drying is a low temperature dehydration process which involves freezing the product, lowering the pressure, then, removing the ice by sublimation. The shift is from a solid directly into a gas. Below is a table which shows the comparison between spray drying and freeze drying.

Table 2.3 Comparison between spray drying and freeze drying

Spray drying	**Freeze drying**
Quick method	Time-consuming method
Free-flowing powder	Compact bulk, grinding needed
Easy scale-up	Difficult scale-up
Suitable for solvents	Difficult with certain solvents
Limited recovery	Yield almost 100%
Temperature sensitive	Extremely temperature sensitive

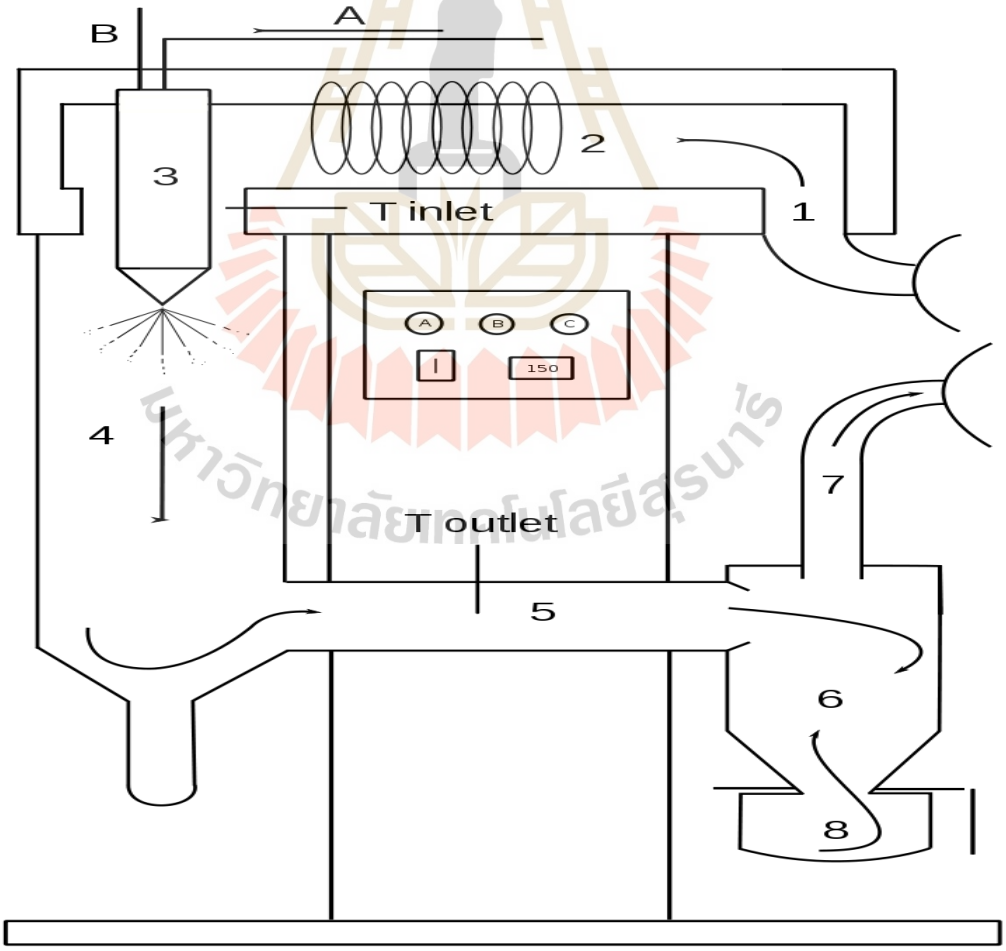


Figure 2.9 Spray dryer

Each method has disadvantages and advantages. Table 1 shows that spray drying can be easily applied to scale-up and collected yeast more quickly than freeze drying. Based on the purpose of this study, the better method will be chosen for dry yeast.

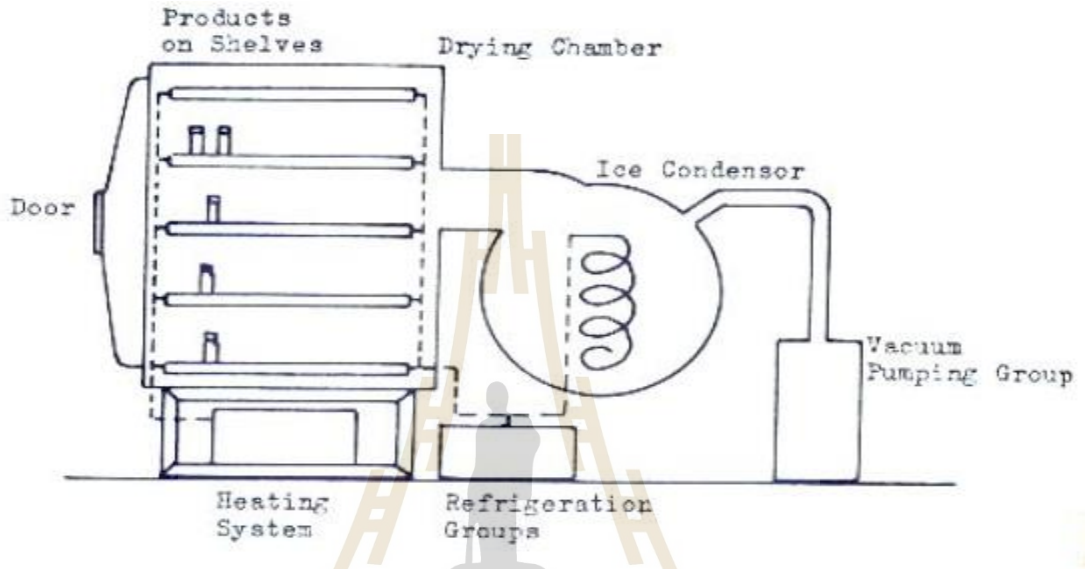


Figure 2.10 Freeze dryer

2.8 Trans-esterification of triglyceride

2.8.1 Definition and principle

The trans-esterification is the process of exchanging the organic R" group of an ester with the organic R' group of an alcohol (Otera, 1993). This reaction is often catalyzed by the addition of an acid or base catalyst (Otera, 1993). The reaction can also be accomplished with the help of enzymes (biocatalysts) particularly lipases (E.C.3.1.1.3).

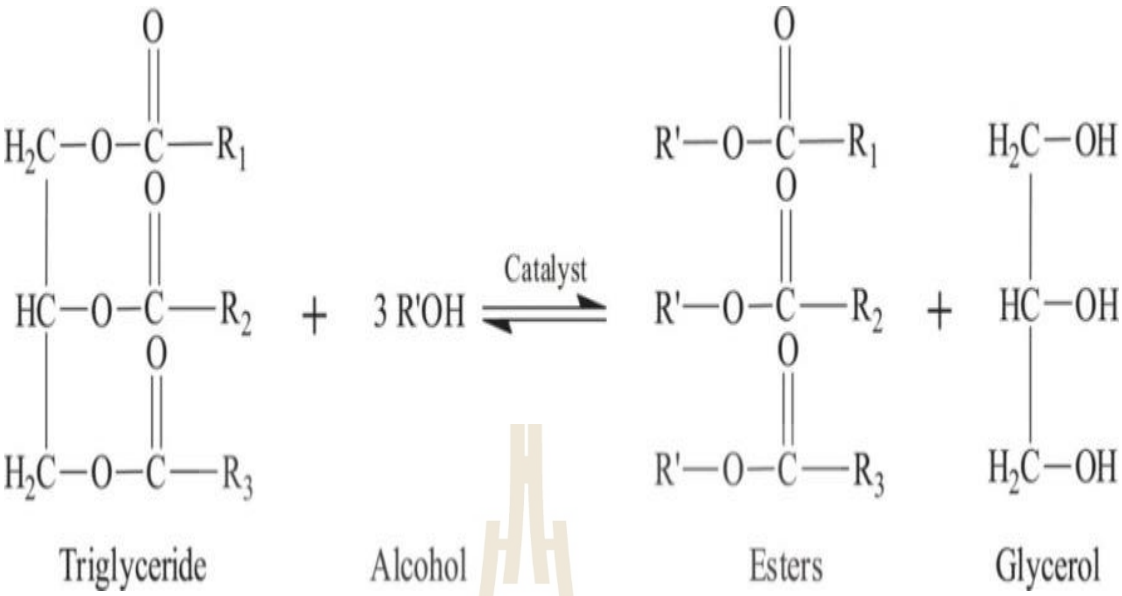


Figure 2.11 Transesterification of triglyceride

The trans-esterification of triglyceride can be done by using methanol, butanol, ethanol, ect, but the purification of omega-3 can be conducted by using ethanol or methanol to produce FAEE or the methyl ester of PUFAs. However, the glycerolysis can be reverted FAEE or FAME to triglycerides, that is quite good for human metabolism. The productivity of trans-esterification reaction depends on several factors such as time reaction, temperature reaction, the molar ratio of alcohol, water and FFA contents, catalyst concentration, and agitation speed (Mathiyazhagan and Ganapathi, 2011).

2.8.2 Factors affecting the trans-esterification reaction

- Effects of water and FFA contents

The trans-esterification is affected by water and free acid (Ayhan, 2009). If an oil samples have high FFAs, the reaction requires more alkali catalyst to neutralize the FFAs. Additionally, water can cause soap formation and frothing which can lead to

an increase in viscosity and formation of gels and foams, and hinder the separation of glycerol. Free fatty acids and water always produce negative such as causes soap formation, consumes that catalyst which leads to a reduction of catalyst effect (Mathiyazhagan and Ganapathi, 2011). That is reason produces a negative result during trans-esterification, also leads to a reduction in methyl ester (ibid.).

- Effects of molar ratio of alcohol

Normally, the trans-esterification reaction requires three mol of alcohol for one mol of triglycerides that creative three mol of fatty acid ester and one mol of glycerol. Excess amount of alcohol increases the conversion of fats into esters within a short time. Hence, the yield of FAEEs increases when the concentration of alcohol is up to certain concentration (Mathiyazhagan and Ganapathi, 2011). Furthermore, molar ratios between 3:1 and 15:1 have been experimented in the trans-esterification of Cynara oil using ethanol. The yield of FAEEs leads to when the molar ratio up to a value of 12:1. Consequently, molar ratios between 9:1 and 12:1 give the best results. In addition, incomplete reaction was observed for molar ratios below 6:1.

- Effects of reaction time

In the trans-esterification, the reaction is slow at the beginning due to the mixing and dispersion of alcohol and oil. After that the reaction proceeds very fast.

Approximately 8 hours of reaction are sufficient for the completion of acid transesterification and optimum conversion of oil to FAEEs (Mathiyazhagan and Ganapathi, 2011). The reaction temperature speeds up, the reaction time shorts, methyl ester yield will increase with an increasing in reaction time. Nevertheless, when increasing in reaction time beyond an optimum point, the reaction will subsequently

lead to a decrease in yield. However, catalyst sources effect to reaction time. Normally, lipases catalyst will be long reaction time more than use alkali or acid. In the trans-esterification reaction with lipase catalyst, reaction time is usually from 8 to 12 hour (Li et al., 2006).

- Effects of reaction temperature

Reaction temperature is another important factor that affects the yield of trans-esterification. For example, a higher reaction temperature increases the reaction rate and reduces the reaction time due to the reduction in oil viscosity. However, the reaction temperature that increases beyond an optimal level can lead to a decrease in FA EEs, because high reaction temperatures accelerate the saponification of triglycerides. The range temperature has been from 40 to 80^{0}C that investigated in their research (Kuan et al., 2019).

- Effects of catalyst concentration

FAEEs formation is also affected by the concentration of catalyst. Normally, the catalyst used for FAEEs production is sodium hydroxide, potassium hydroxide, or lipase, or acid. The catalyst can be mixed with ethanol first and then added to the oil or fats because mixing sodium hydroxide with ethanol produces a little amount of water which inhibits the formation of the end product (Mathiyazhagan and Ganapathi, 2011). In addition to this, when the concentration of catalyst increase with oil samples, the conversion of triglycerides into biodiesel also increases. However, disadvantages in alkali or acid catalyzed are potential pollution to the environment (Tan et al., 2010). Therefore, lipases can be used to catalyze the reaction in mild condition and an increasing the yield from researches have reported on its use (Ortiz et al., 2019). In

previous studies, lipase quantity can be used from 0.5 to 2.5 % (Kuan et al., 2019).

- Effects of agitation speed

Agitation speed plays a significant role in the information of the product because the agitation of oil and catalyst mixture enhances the reaction. For example, the transesterification of palm oil with four parameters of interest (agitation speed (200 – 400 rpm), catalyst loading (0.5-1.5%), methanol to oil ratio (3:1-6:1), and temperature (30-60^{0}C), agitation speed) has shown a reduction of 37-fold in the standardized effect throughout the trans-esterification process, where the effect has reached plateau around mid-way of the reaction (Wonga et al., 2019).

2.8.3 Direct transesterification of oil yeast into FAME

Direct transesterification is a reaction of cells and alcohol in one bioreactor without a solvent extraction step. The direct transesterification process uses low levels of chemical solvents, acid catalysts, and heating energy to produce the FAME in a method with increased efficiency in co-solvent system (Shinde, 2015). In previous study, transesterification have been many ways to apply include that direct transesterification can be used with high yield transesterification. For direct transesterification of oil yeast cells, the glass beads size of 0.45 to 0.55 mm has been popularly used for cell disruption that has been published (Ausubel, 1989) (Jazwinski, 1990). For example, the direct transesterification of dry algal biomass was reacted at 63^{0}C with ethanol and ethanol sulfuric catalyst (Shinde, 2015).

Figure 2.2 The glass beads

2.9 Molecular distillation

2.9.1 Definition and principles

Molecular distillation is a type of short-path vacuum distillation, characterized by an extremely low vacuum pressure. Molecular distillation has some potentials in the separation, purification and concentration of natural products. This technique is usually used for complex and thermally sensitive molecules, such as vitamins and polyunsaturated fatty acids, because it can minimize losses by thermal decomposition (Cvengroš et al., 2000). Furthermore, this process has advantages over other techniques that use solvents as the separating agent, because it can avoid problems with toxicity. The separation principles are the vacuum, which enables molecules to evaporate from the evaporator to the condenser, and the formation of a thin liquid film

which promotes effective heat and mass transfers. Falling film distillators use the gravity force to promote a thin film on the evaporating cylinder)evaporator((Fregolente et al., 2007). Two product streams are generated: distillation)D(which is rich in the molecules that escape from the evaporator and reach the condenser, and residue which is rich in heavier molecules from the evaporator.

2.9.2 Optimum conditions of molecular distillation

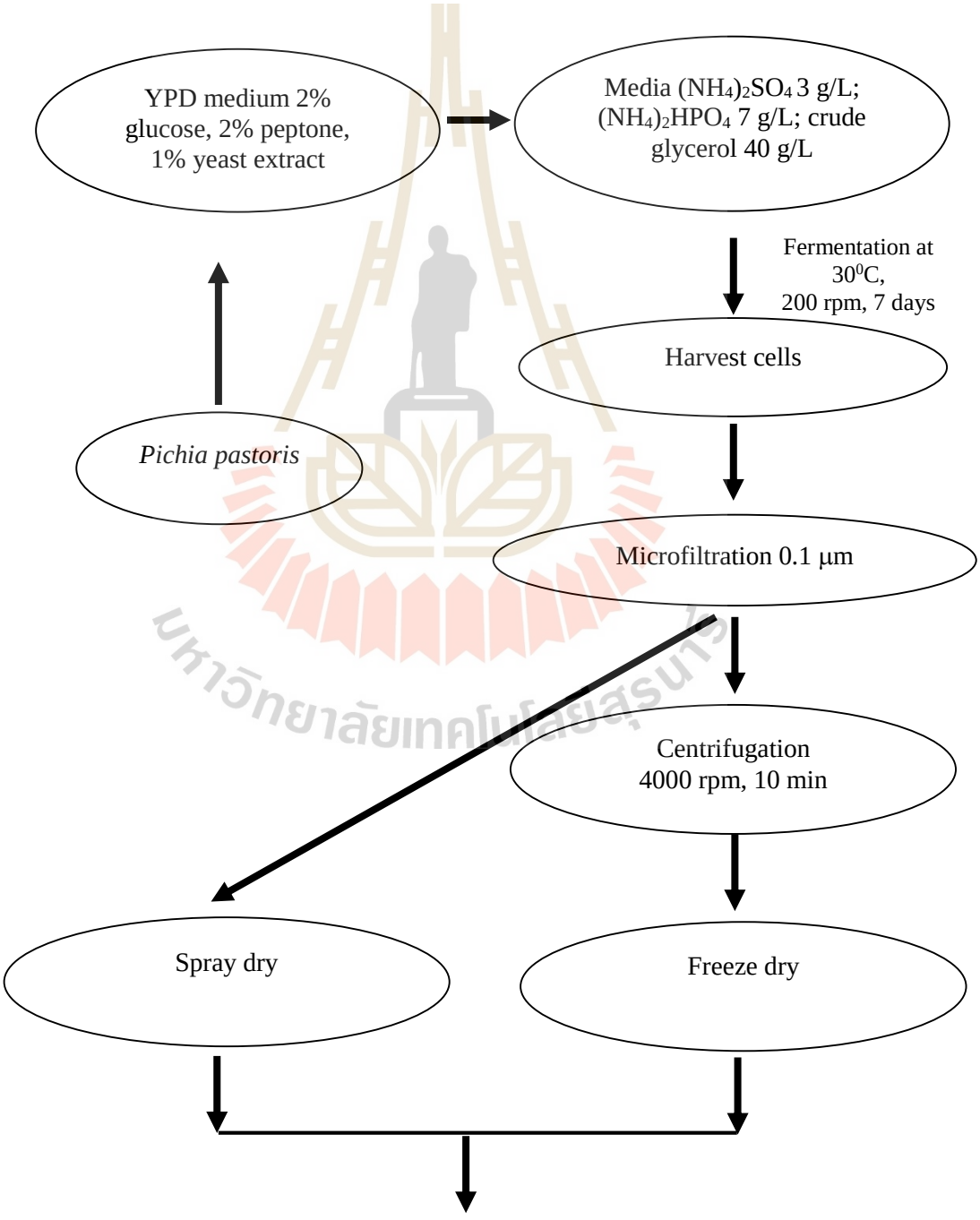
Depending on the differences existing between the carbon chair, amount of double bond, the molecular distillation can be enriched omega-3 fatty acid. In previous studies, omega-3 fatty acids can be obtained from some materials such as squid oil, sardine oil, and microalgae oil. Normally, the process of purification should have transesterifcation of triglycerides to fatty acid methyl ester or fatty acid ethyl ester. Depending on boilling point of oil molecular, the temperature distillation and pressure will set up to collect only target product.

For example, the omega-3 fatty ethyl ester obtained from squid oil can be separated by the molecular distillation at T_1= 120.5°C and T_2= 140°C (Rossi et al., 2011). However, the temperature should be less than 140°C to avoid chemical changes. Urea complexion and molecular distillation have been applied to purify omega-3 fatty ethyl ester from sardine oil ethyl ester. In addition, purification of monoacylglycerols rich in omega-3 fatty acid was successful by using SPD with two-step distillations including at 110^0C to remove glycerol and most of FFA, at 135^0C to 195^0C to distillate the MAG and other operation variables (Solaesa et al., 2016).

CHAPTER III

MATERIALS AND METHODS

Below shows the procedure for the production of omega-3-EE from *P. pastoris*



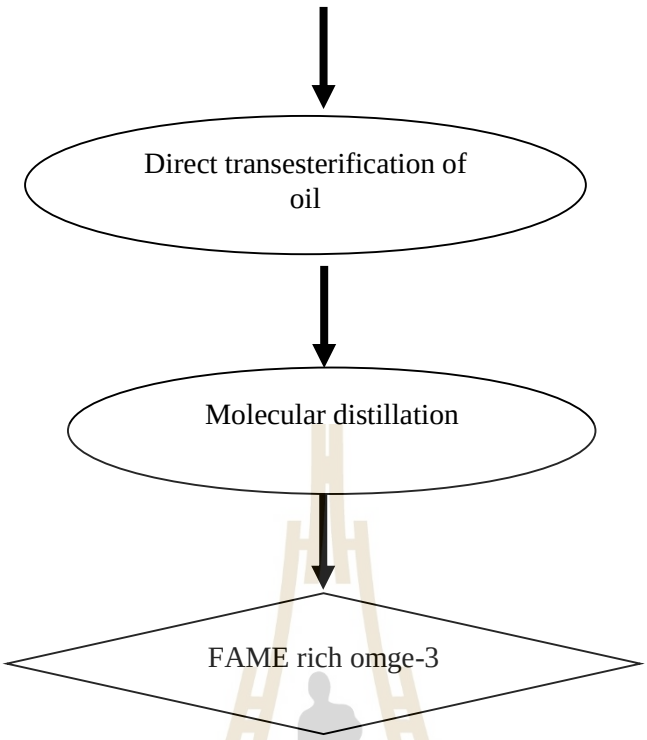


Figure 3.1 Production and purification of omega-3 fatty acid from *Pichia Pastoris*

3.1 MATERIALS

3.1.1 Microorganism

The yeast *P. pastoris* was used in this experiment (Chotika, 2013). In her research, cDNAs of Δ6 from Nile tilapia and Δ6&Δ5 bifunctional, E5, and E6 from Zebrafish were amplified and cloned into pGAPZ to generate recombinant expression plasmids (pGAPZ:Δ6, pGAPZ:Δ6&Δ5, pGAPZ:E6, pGAPZ:E6) and the co - express recombinant plasmid of Δ6&Δ5 and E6 (pGAPZ:Δ6&Δ5:E6) were transform into *P. pastoris* SMD1165 to generate P. pastoris that co-express all 3 genes. The results showed great success, the EPA and ARA accumulation were detected in recombinant *P. pastoris* containing pGAPZ:Δ6&Δ5:E6.

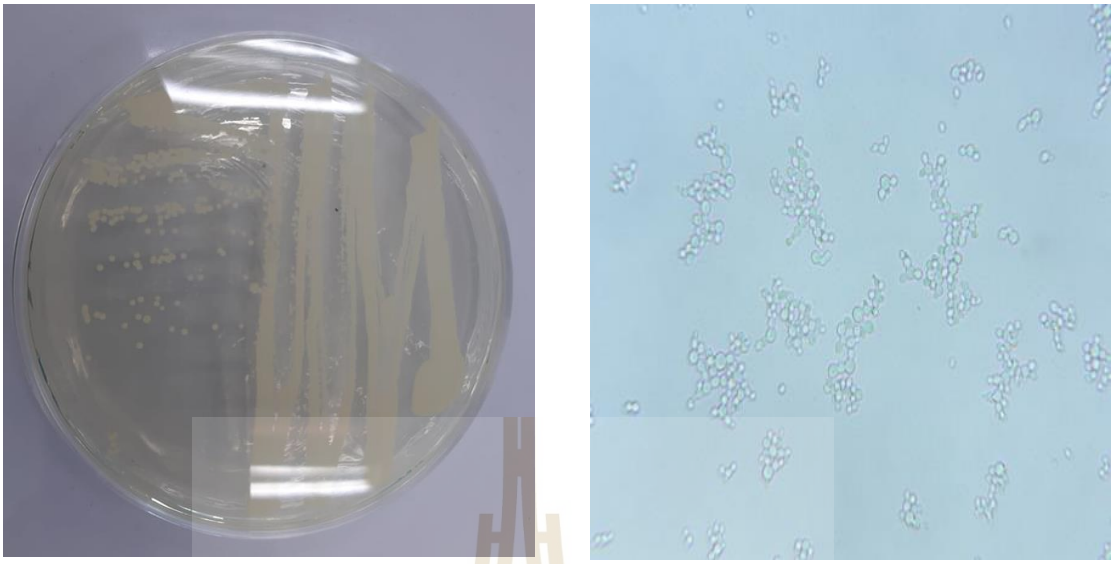


Figure 3.2 The *P. pastoris* in petri-disk and *P. pastoris* under microscope

3.1.2 Equipment

All of equipment used in the fermentation, cell harvesting, freeze-drying, spray drying, transesterification, molecule distillation, analysis process, that were shown on this table:

NAMES OF EQUIPMENT	COMPANY
ANALYTICAL BALANCE	PRESICA, USA
PH METER	OHAUS, USA
HOTPLATE AND STIRRER	SCILOGEX, USA
SHAKING INCUBATION	BENCHMARK SCIENTIFIC USA
BIOLOGICAL SAFETY CABINET	CRYSTE, KOREA
AUTO CLAVE	CRYSTE, KOREA
FERMENTER 5L	SATORIUS, GERMANY
FERMENTER 50L	APICHAT'S LABORATORY

NAMES OF EQUIPMENT	COMPANY
FERMENTER 500L	BE MARUBISHI, THAILAND
MINI SPRAY DRYER B-290	BUCHI, JAPAN
SHORT PATH DISTILLATION UNIT	ISTLAB, CHINA
GC 7890A	AGILENT, USA
FREEZE DRY	MARTIN CHRIST
MICROWAVE ETHOS ONE	MILESTONE-HELPING CHEMISTS
ICP-OIS	AGILENT, USA
CENTRIFUGAL	SORVALL RC 5C PLUS
ROTARY EVAPORATOR	BUCHI, JAPAN

3.2 METHODS

3.2.1 Medium and condition of *P. pastoris*

The yeast *P. pastoris* has been widely used as a host for heterologous protein expression. Hence, this study focuses on the omega-3 produce from *P. pastoris*. The medium optimum conditions was identified for *P. pastoris* growth fast, best biomass yield and omega-3 production. The main compositions of *P. pastoris* medium are glucose 2%, peptone 2%, and yeast extract 1% for inoculation. Carbon sources was replaced by crude glycerol because of decreasing cost of product omega-3 (Lima et al., 2016). Crude glycerol was by-product from biodiesel production that was provided by BioSynergy Company, Thailand. Crude glycerol was determined elements Na, Mg, K, Ca, Fe, P by ICP-OES (PerKinElmer, Optima 8000 ICP-OES, SUT), percent of glycerol

by HPLC. Nitrogen sources was supplanted by $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$ (Robert et al., 2017). Besides, the omega-3 aims for a high yield, so based on the metabolism engineering of yeasts, we need to add more 4.6 mL/L PTM_4 trace salt g/L (2 $CuSO_4.5H_2O$; 0.08 KI; 3 $MnSO_4.H_2O$; 0.2 $NaMoO_4.2H_2O$; 0.02 H_3BO_3; 0.5 $CoCl_2.6H_2O$; 6.7 $ZnCl_2$; 21.7 $FeSO_4.7H_2O$) (Robert et al., 2017).

The composition of the medium was optimized for biomass production of recombinant omega-3 fatty acid P. pastoris by using crude glycerol as carbon source, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ as nitrogen source. The concentration of crude glycerol, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ were optimized by response surface methodology using three factor, three levels using a Box Behnken design. Three center points were chosen in this experiment. The three variables selected for the statistical analysis were designated as X_1, X_2, X_3 and predicted response in the form of biomass production was designated as Y (Table 3.1). The experimental design protocol for response surface methodology was developed using design-expert software. A total of fifteen experiments with different composition of three factors were carried out (Table 3.1). As a dependent variable, biomass production was measured at the end of cultivation, 7 days for cultivation, when glycerol completely consumed.

For optimum medium condition, the procedure was conducted in 250 mL flask with 100 mL solution at temperature 30 °C, pH 5.0, agitation 200 rpm. For dry cells weight estimation, samples after 7 days of cultivation were centrifuged at 4000 rpm for 10 min. The cell pellet was washed with DI water and then dried by freeze dryer to a constant weight. DCW was calculated that showed in Table 3.1.

Table 3.1 Box-Behnken design matrix for the three variables and experimental and predicted results under different composition and same condition

Runs	$(NH_4)_2SO_4$ (X_1) (g/L)	$(NH_4)_2HPO_4$ (X_2) (g/L)	Crude Glycerol (X_3) (g/L)	Biomass (Y) (g/L)	
				Experimental	Predicted
1	9	4.5	20	4.142	4.159
2	3	7	30	5.416	5.406
3	6	4.5	30	4.614	4.609
4	6	7	20	4.137	4.159
5	9	7	30	4.678	4.66
6	3	2	30	4.042	4.028
7	9	4.5	40	4.289	4.299
8	9	2	30	4.209	4.223
9	3	4.5	20	4.121	4.129
10	6	2	40	4.408	4.569
11	6	4.5	30	4.502	4.569
12	6	7	40	4.797	4.567
13	3	4.5	40	5.05	5.042
14	6	4.5	30	5.285	5.303
15	6	2	20	3.912	3.889

3.2.2 Fermentation

The fermentation parameters are important to monitor and control throughout the fermentation process. Thus, *P. pastoris* was grown at temperature 30 °C, pH 5.0, agitation 200 rpm, and avoid foam)reduces headspace). For dissolving oxygen, the normal air was used for fermation process. However, in previous study,

DO extremely effect to reproduction of *P. pastoris* and lipid produce inside cells (Adelantado et al., 2017). The target collect a high biomass and a high amount of oil inside yeast cells. Therefore, the DO was be controlled at 30 - 40% until day 3 that support to produce high biomass and then reduce DO to 4-8% until the end that focus to produce oil inside yeast. Production of fermentation was be done following some steps. Firstly, the seed culture was prepared in a 500 mL, 5L, 50L medium containing 20% glucose, 20% peptone and 10% yeast extract. Cell cultivation began in a 5-L bioreactor at 30 °C for 24 h. Secondly, the inoculum was transferred into a 50-L bioreactor at 30 °C for 24 h. And finally, the batch experiment was performed in a 500-L bioreactor with 350-L of working volume (BE Marubishi, Thailand). For 500-L bioreactor, the optimum medium was used including crude glycerol, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$.

(a) 5-L bioreactor

(b.) 50-L bioreactor

(c.) 500-L bioreactor

Figure 3.2 Bioreactors in the experiment (a) 5-L bioreactor (b.) 50-L bioreactor (c.) 500-L bioreactor

3.2.3 Cells harvesting

After fermentation, the P. pastoris must be collected by using membrane filtration and centrifuge separation. In this study, cross-filtration membrane was used to separate water from broth to 350L to 30L. After membrane filtration, the cells solution was divided two ways: 1 - continuous to spray dryer; 2 - separation of cells by centrifuge machine.

1. Membrane separation

The cross-flow membrane filtration was shown in Figure 3.4. The cross-flow MF experiment was investigated using a spiral-wound MF element with the nominal pore size of 0.1 μm (Synder's Filtration, USA) installed in a stainless steel housing. The diameter, and the length of the membrane element were measured at 10.2 cm, and 96.5 cm, respectively. The feed spacer was 3 mm, and the total filtration area was 4.27 m^2. Microfiltration is crow flow filtration with pore 0.1 μm. The size of *P. pastoris* is around 4-6 μm.

Figure 3.4 Cross-flow membrane filtration in experiment.

Before each run, the membrane was firstly rinsed with RO water. It was then circulated with 1 wt% NaOH solution for 20 minutes, and rinsed to neutrality with RO water. Finally, it was cleaned with 1.5 wt% phosphoric acid solution before rinsed to pH 7.0 with RO water.

2. Centrifugal

After membrane filtration, the solution was centrifuged at 5000 rpm, 10 min to collect the concentrate cells. The yeast cells were filled to petri dishes that was preparing for freeze dry next step.

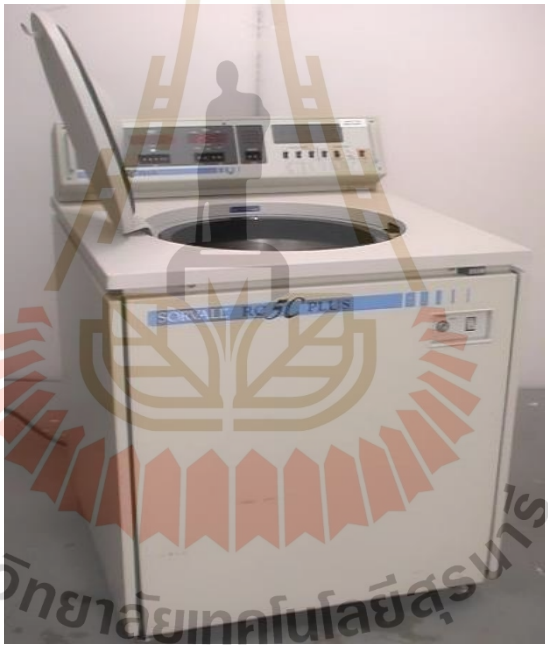


Figure 3.5 Centrifuge machine

3.2.4 Spray dry

The *P. pastoris* was spray dried at air inlet temperatures 110 °C (Pongsatorn, 2019). The spray dryer (Buchi's mini spray dryer B-290, Germany) was showed in Figure 3.6. The operating parameters in this work were 470 L/h of air flow rate, 3.5 mL/min feeding rate, and cell concentration of 50 %, nozzle size 1.4 mm, aspirator

pressure drop -50 mbar. The yeast powders were collected in the auto desiccator with a moisture content within specifications of <10%.

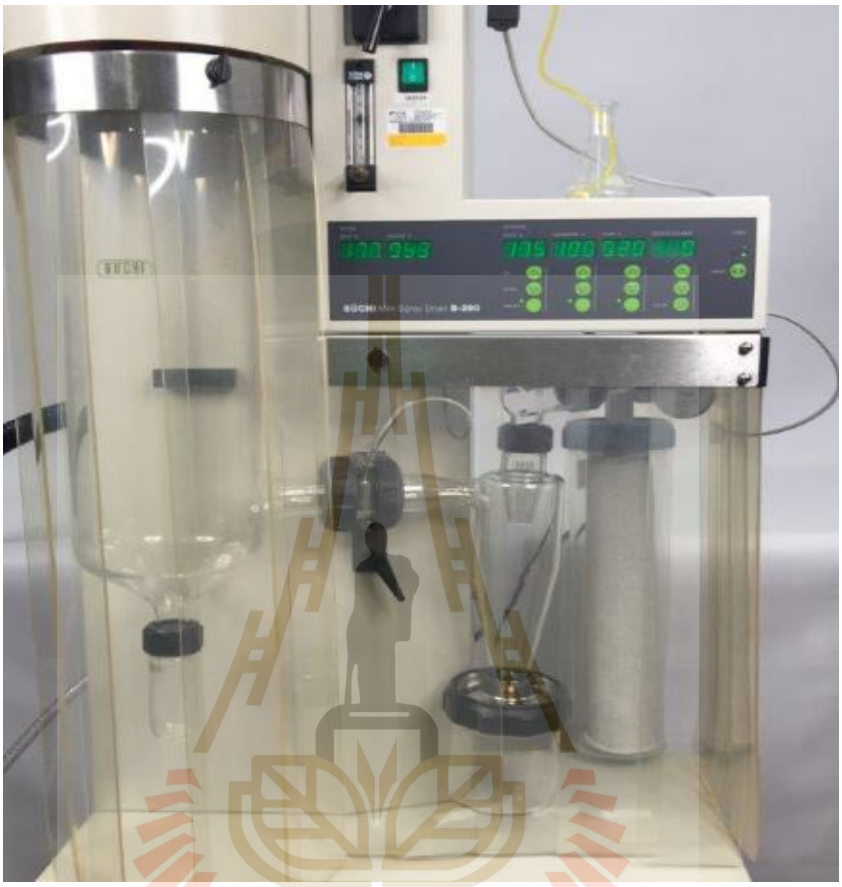


Figure 3.6 A mini spray dryer

3.2.5 Freeze dry

After preparing P. pastoris cells into the petri-dishes, the sample was frozen at -80^{0}C in refrigerator overnight. The parameter was used at -65^{0}C, vacuum around 0.01 mbar. After 58 hours, yeast cells were collected and kept in refrigerator.

Figure 3.7 DCW after drying

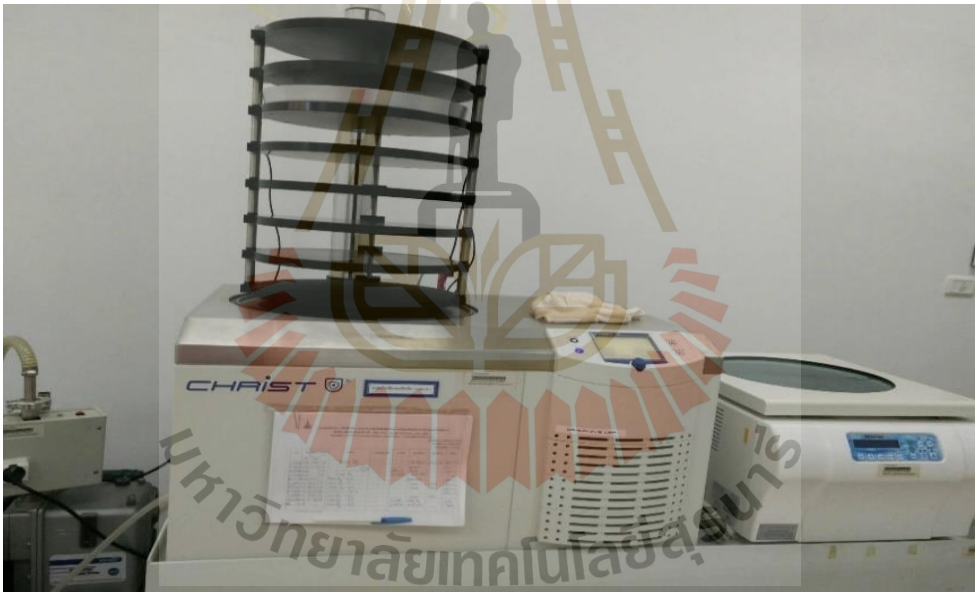


Figure 3.8 Freeze dryer

3.2.6 Direct trans-esterification

Supporting for molecular distillation, trans-esterification is an important step transfer *P. pastoris* oil to fatty acid methyl ester. In this study, the 5% KOH in methanol and H_2SO_4 is catalyst for this reaction. The protocol was followed: DCW and glass beads (ratio 1:1 (w/w)) (Louhasakul et al., 2018) were added 5% KOH in

methanol with 1:20 w/v ratio and then stired at 300 rpm, 70^0C, kept it overnight. The reactor was designed with a pressure gauge, a condenser, a rotor in figure 3.9. The pressure gauge can control the pressure inside that make sure for safe reaction. For condenser, it can collect methanol vapor during reaction. After that let it cool around 2 or 3 hours, then added H_2SO_4 with 1:0.66 (w/v) ratio. Any steps, the sample was removed oxygen in the reactor by feeding nitrogen gas and taken quickly close. The sample was kept at 70^0C around 1.5 hours. And adding n-hexane with 1:10 (w/v) ratio was done to collecting FAME. The saturated NaCl was supplemented to easily separated the phase. The lower n-hexane and FAME was collected and casted out n-hexane by using evaporator at 40^0C. Before this step, using filter for removing cells in solution was did. In adding, the nitrogen gas was used to support for dried FAME. The sample was kept in refrigerator for next step.

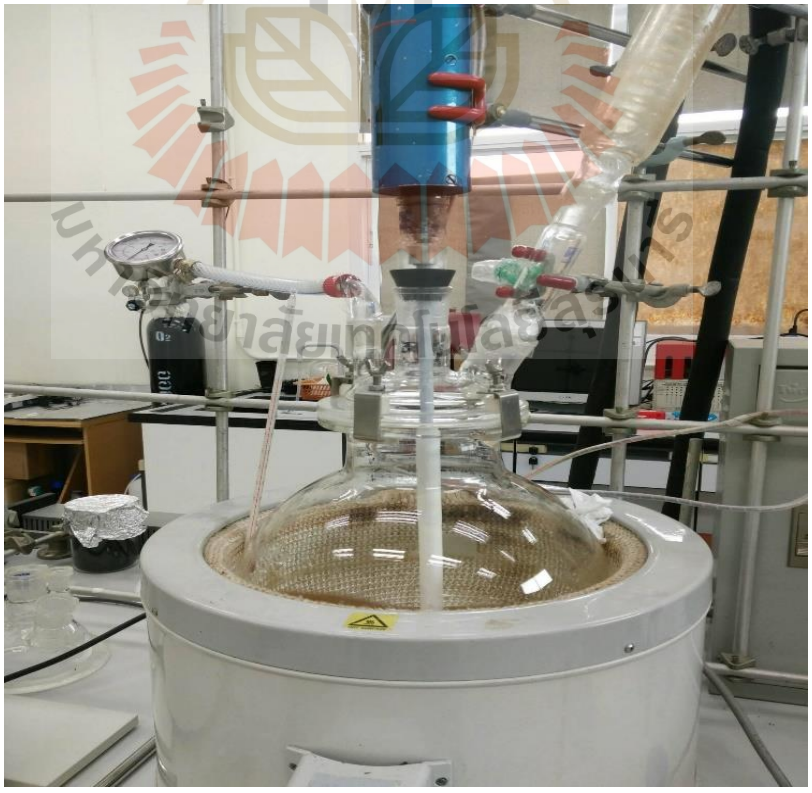


Figure 3.9 Trans-esterification reactor

3.2.7 Molecular distillation

Molecular distillation can be separated methyl ester under vacuum. However, it is depending on the differences existing between the carbon chair, amount of double bond. The DHA, EPA methyl ester could be enriched from PUFA methyl ester through the control of feed temperature, evaporation temperature, pressure, feeding flow rate.

In this study, the molecular (short path) distillation with unit model of LAB 1st MD-150 was used for distillated product omega-3 methyl ester. The short path distillation (SPD) unit was shown in Figure 3.9. The feed was heated to 60^{0}C before feeding to SPD that supporting for distillation. The sample after transesterification was tested at 150^{0}C, the vacuum around 0.2 to 1 pa, the temperature inside column around 40^{0}C, the flow rate 0.5 mL/min, the condenser outside around -20^{0}C. Due to small amount of sample after trans-esterification, the soybean oil was added to make up the volume of sample to 100 mL.

Figure 3.10 Molecular distillation

3.2.8 Analyses

1. Crude glycerol

Crude glycerol was determined elements Na, Mg, K, Ca, Fe, P by ICP-OES (Optima 8000 ICP-OES, SUT). Crude glycerol (0.207 – 0.2114g) was digested using 10 mL of HNO_3 in a microwave digester (Ethos One, SUT). The digester temperature was ramped to 200^0C in 15 min, maintained at 200^0C in 15 min, and cooled down to 25^0C. The digested crude glycerol solution was transferred into a 100mL volumetric flask and filled to mark using DI water. The solution was mixed and analyzed using ICP-OES. Amount of Glycerol from crude glycerol was performed by HPLC using Aminex HPX-87H column, RID-10A (Anastácio et al., 2014). The

parameters were used including the mobile phase H_2SO_4 5mM, the flow rate 0.6 mL/min, the column temperature at 50^0C (Anastácio et al., 2014).

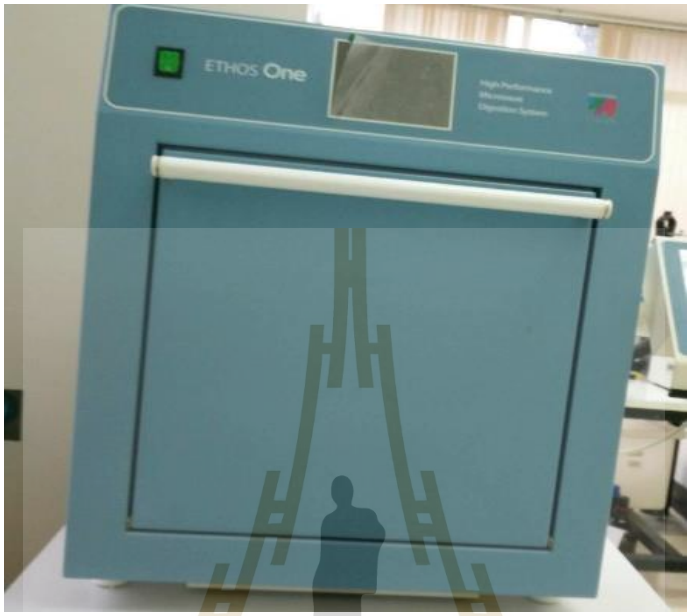


Figure 3.11 The microwave digester

Figure 3.12 The ICP-OES machine

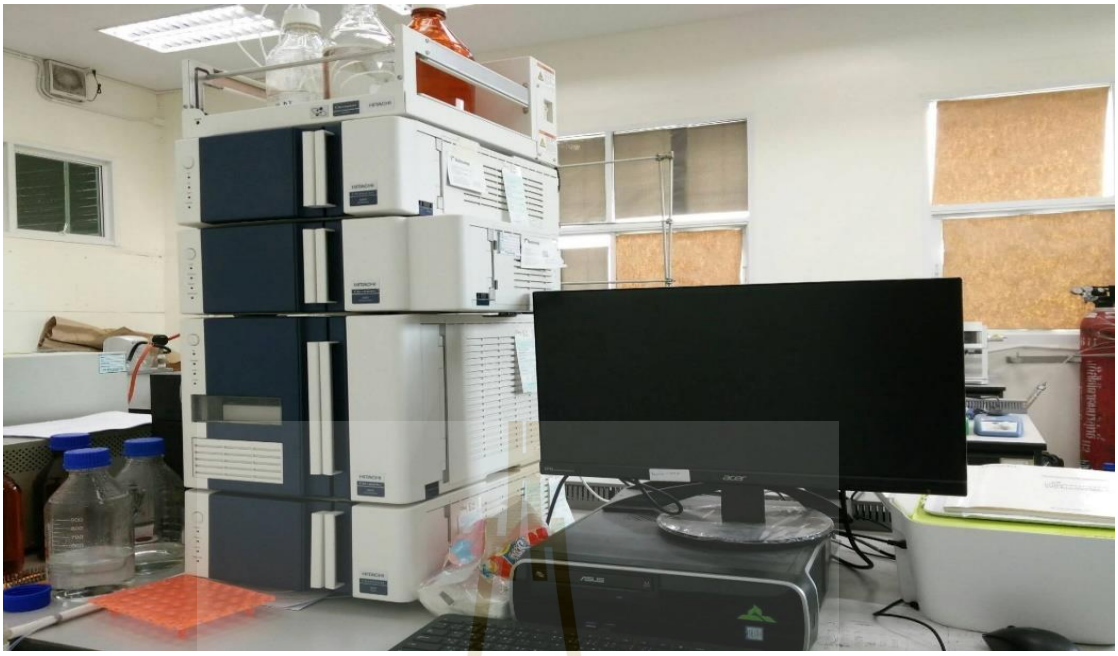


Figure 3.13 The HPLC with Aminex HPX-87H column, RID-10A

2. Gas chromatography analysis

Gas chromatography is a common type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition. The *P. pastoris* oil was analyzed by GC-FID. However, before inject to GC-FID, *P. pastoris* oil was changed to fatty acid methyl ester by direct transesterification. It was followed: 300 mg DCW and glass bead (ratio 1:1 DCW) were added 3 ml 5 % KOH methanolic and then shaken 5 minutes with vortex at 2000 rpm. Then 50 µl 4 mg/ml C13:0 was used as internal standard that was used to calculated yield of transesterification reaction. After that, 3 ml 5 % KOH methanolic was added and shaken it and then the sample was removed oxygen in the tube by feeding nitrogengas and it was quickly closed. The sample was kept in oven at 80^0C overnight. Before continuous with next step, the sample was left cool around 15 minutes, and then was reacted with 200 µl H_2SO_4 concentrate. After that the sample was removed oxygen

in the tube by feeding nitrogen gas and it was quickly closed too. The sample was kept oven 80^0C around 1.5 hours. And adding 3 ml hexane to collecting FAME was done and 2 ml 36 % NaCl saturated was supplemented to easily separated the phase. The lower phase hexane and FAME were dried to 1 ml by nitrogen gas and kept -20^0C waiting for injection.

The sample around 1 µL was injected in GC Agilent 7890A with FID – Detector. The instrument was illustrated in Figure 3.8 The Agilent J&W *CP-Sil 88* column (100m × 200 µm × 0.25 µm) provides a greater efficiency and resolution for FAME compounds. The injector temperature of 240 °C, split 50:1, detector temperature of 250 °C, and flow rate of 0.4 mL/min. Helium was employed as the carrier gas.

Figure 3.14 The GC Agilent 7890A

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Medium and condition of *P. pastoris*

The mount of glycerol in crude glycerol was measured around 72.6% (v/v) by using HPLC, Aminex HPX-87H column, RID-10A, H_2SO_4 5mM mobile phase (Figure 4.1). The results indicated that amount of glycerol in crude glycerol was relatively similar with other crude glycerol (Anastácio et al., 2014) (Tang et al., 2009) (Robert et al., 2017), appeared at 12.870 min reaction time.

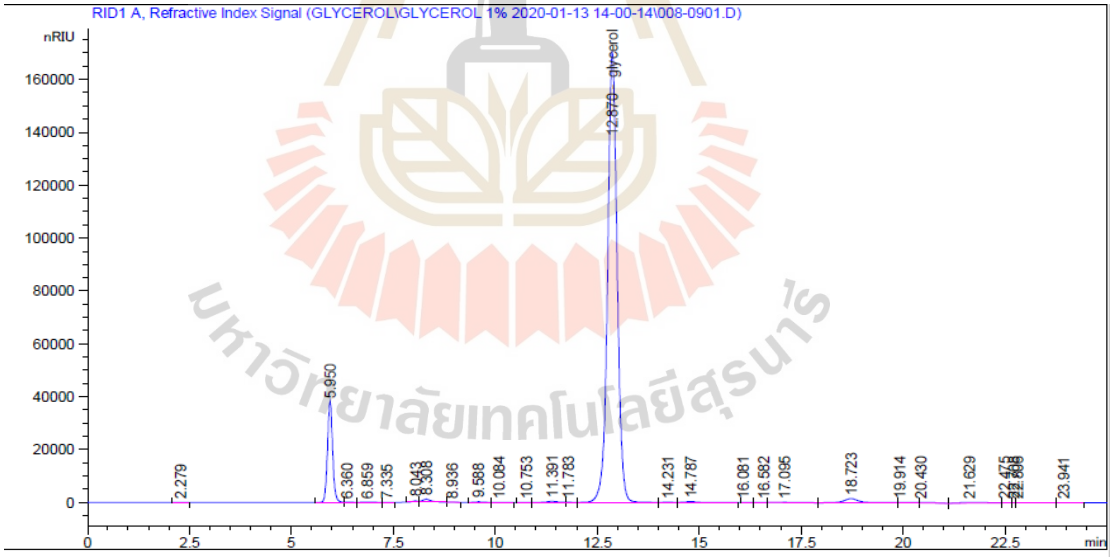


Figure 4.1 HPLC profiles of glycerol from crude glycerol

For elements analyst of crude glycerol, the results were shown in Table 4.1. Nevertheless, the biodiesel reaction of BioSynergy Company was used acid catalysis, elements of crude glycerol were quite lower more than base catalysis (Hu et al., 2012).

Table 4.1 Elements Analyses of crude glycerol

Elements	**Crude glycerol**
Na (ppm)	11.9 ± 1.5
K (ppm)	19.8 ± 1.2
Ca (ppm)	7.0 ± 1.3
Mg (ppm)	8.6 ± 6.5
Fe (ppm)	4.8 ± 5.2
P (ppm)	0.6 ± 0.1

Before investigation of biomass production, the growth curve was determined at flask during 7 days. The findings indicated that clearly four phase living of *P. pastoris* in Figure 4.2. Based on that, the log phase was in from day 1 to day 4 with specific growth rate $\mu = 0.2619\ day^{-1}$ (Figure 4.3).

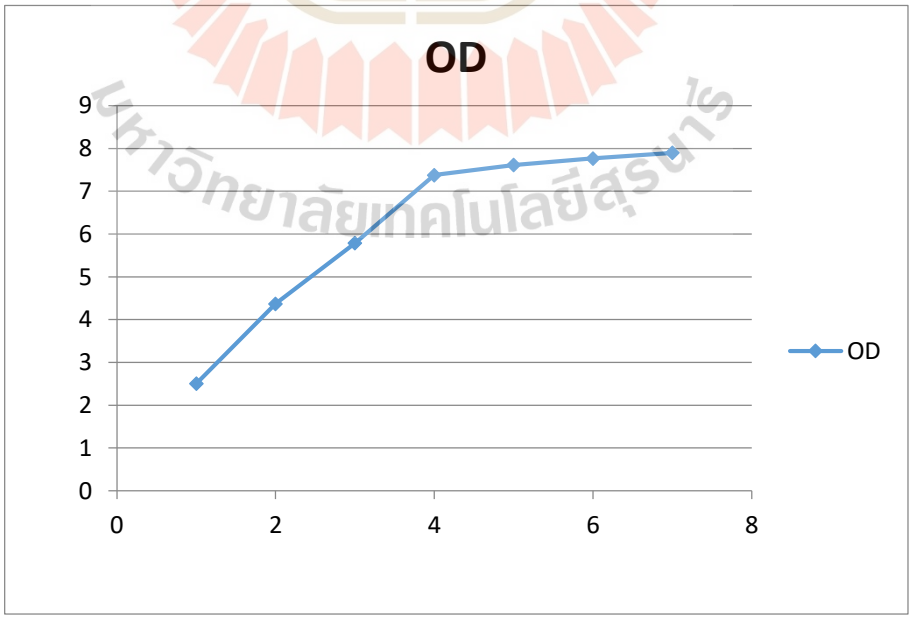


Figure 4. 2 Growth curve of *P. pastoris* in flask 250 mL with 35 g/L crude glycerol, 6 g/L $(NH_4)_2SO_4$, 4.5 g/L $(NH_4)_2HPO_4$

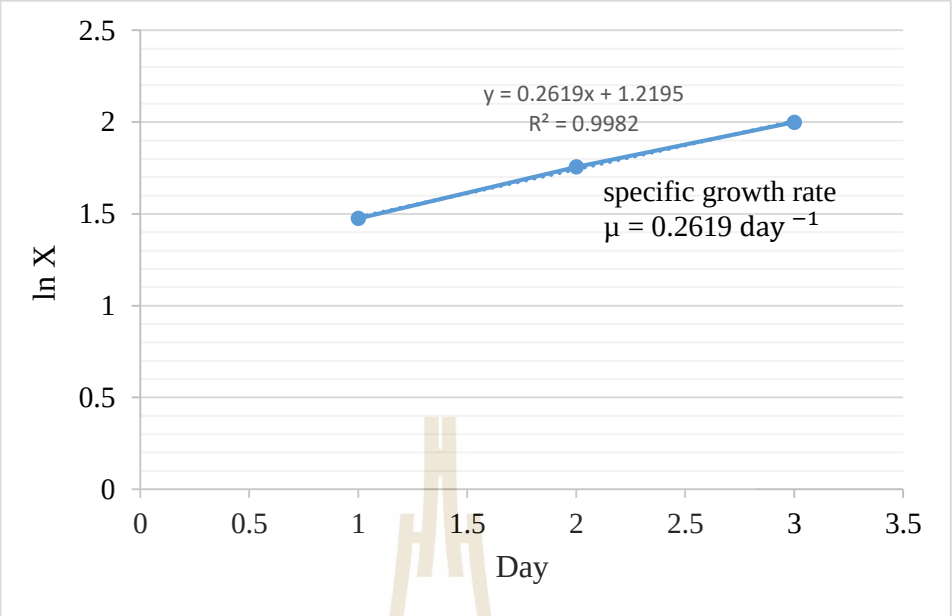


Figure 4.3 Measurement of specific growth rate in exponential phase

Response surface methodology for biomass production

The amount of dry cells weight was measured in fifty-five experiments with different composition and corresponding results were shown in Table 3.1. The results were exhibited in quadratic model using a multiple regression analysis (see equation 1).

Biomass = $4.36428 + 0.372789X_2 - 0.0558X_3 - 0.053358X_1 - 0.0552X_2X_3 - 0.003467X_2X_1 + 0.01711X_3X_1 - 0.011255X_2^2 - 0.004727X_3^2 + 0.000140\ X_1^2$ (1).

The ANOVA for quadratic model was demonstrated in Table 4.2. The coefficient of multiple determinations, R^2 was found to be 0.9716, which was shown that the model could be explained 97.16% of the variability in the system. For a good statistical model, the R^2 value should be close to 1.0. The relatively high value of R^2 indicated that second order polynomial equation is capable of representing the system under the given experimental domain. The significance of the model terms was evaluated by applying the analysis of variance (ANOVA) test using a design expert software. The P-value of less than 0.05 indicated that the model term was significant. The contour plot was

created as Figure 4.4 to evaluated the combined effect of two ammonium salts on biomass production.

Table 4.2 ANOVA for quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.90	9	0.3223	19.03	0.0023	significant
A-(NH4)2SO4	0.9467	1	0.9467	55.91	0.0007	
B-(NH4)2HPO4	0.3507	1	0.3507	20.71	0.0061	
C-CRUDE GLYCEROL	0.1006	1	0.1006	5.94	0.0589	
AB	0.6856	1	0.6856	40.49	0.0014	
AC	0.0433	1	0.0433	2.56	0.1708	
BC	0.7319	1	0.7319	43.23	0.0012	
A^2	0.0379	1	0.0379	2.24	0.1949	
B^2	0.0032	1	0.0032	0.1903	0.6808	
C^2	0.0007	1	0.0007	0.0425	0.8448	
Residual	0.0847	5	0.0169			
Lack of Fit	0.0024	3	0.0008	0.0194	0.9952	not significant
Pure Error	0.0823	2	0.0411			
Cor Total	2.99	14				

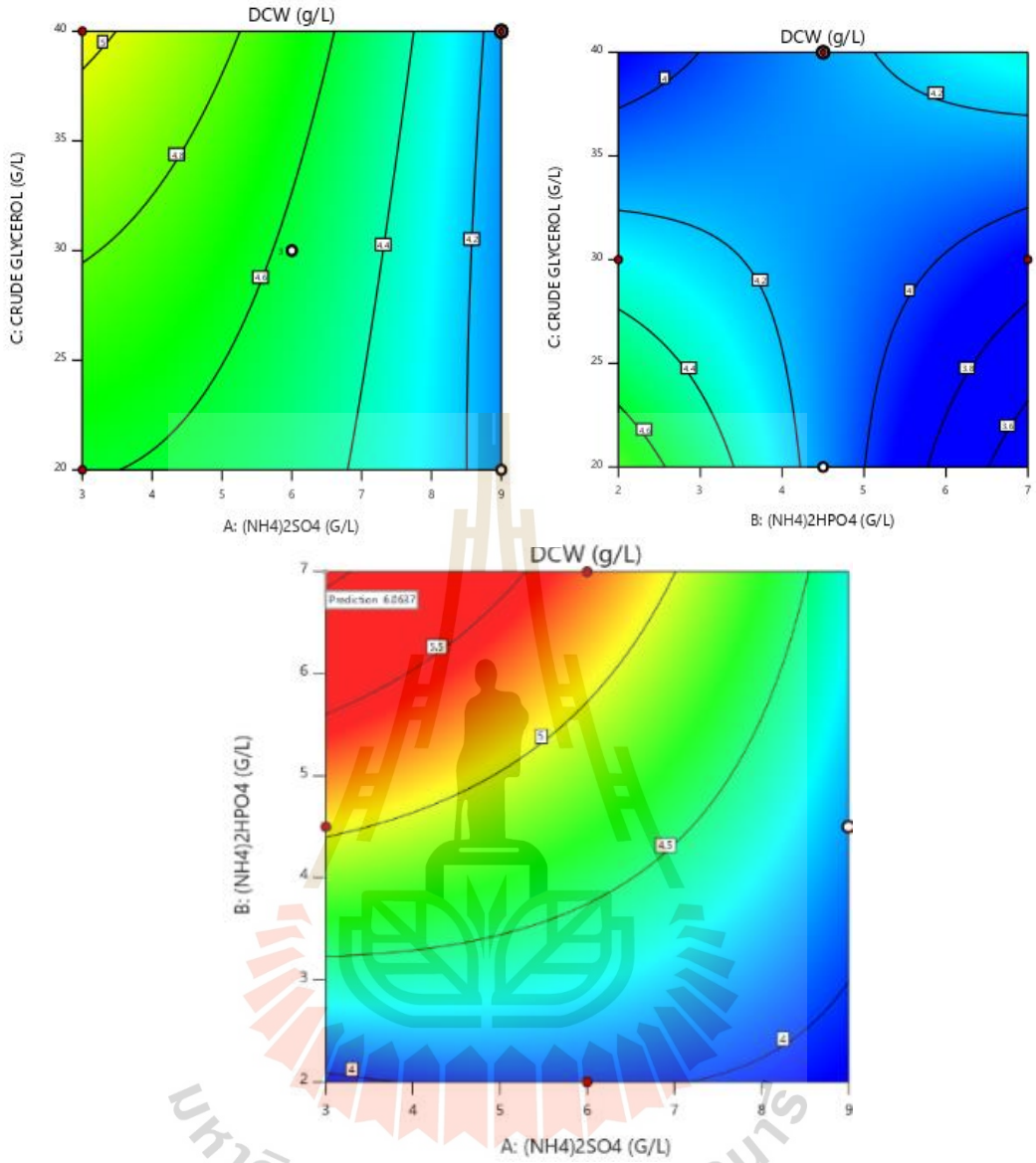


Figure 4.4 Contour plots of the combined effects of $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, and crude glycerol

The optimum medium condition was 40 g/L crude glycerol, 3 g/L $(NH_4)_2SO_4$, and 7 g/L $(NH_4)_2HPO_4$. The highest biomass in flasks cultivation after 7 days production was 6.064 g/L DCW and 0.1516 g biomass/ g crude glycerol.

4.2 Fermentation

After optimum condition of medium, the fermentation of *P. pastoris* was done at the bioreactor 500 L. The profile of *P. pastoris* was shown in Figure 4.5 including concentration of glycerol, OD, DCW, respectively. The fermentation with 350 L volume medium in 500L bioreactor was controlled at 30^0C, 200 rpm, initial pH 5 adjusting by citric acid. For DO, on 3 days first, the dissolve oxygen was feed 30-40% DO by normal air and then reduced to 8 - 10% DO.

When the *P. pastoris* feed was transferred to 350 L new medium, the cell concentration was extremely changed in period approximately 24 hours. The exponential phase was shown from day 1 to day 3 at Figure 4.5. The specific growth rate (Figure 4.6) was calculated around $\mu = 0.249$ day^{-1} in exponential phase.

The samples were collected a day per time and then centrifuge to collect concentrated cells. Due to small amount of samples, freeze dryer was employed to drying *P. pastoris* cells. Comparing between cultivation of flask and 500L bioreactor, the OD and DCW in flask was quite higher than in 500L bioreactor. The DO has significantly affected to biomass production (Adelantado et al., 2017). In the flask 250 mL with 100 mL medium containing, covering by cotton cap, the big space was enough for living cells. And also, the shaker at 200 rpm that was supported to mixing the oxygen into medium. In the 500L bioreactor, the dissolved oxygen was controlled after day 3. Therefore, the biomass production was prevented the speed in log phase and made the OD and DCW lower than in the flask. The specific growth rate in exponential phase in both flask and bioreactor was not different. The DCW of *P. pastoris* in this study was quite lower than DCW of other recombinants *P. pastoris* that was shown in Table 2.1. However, the high amount of omega-3 fatty acid product is main target in this study.

Thus, controlling the dissolved oxygen (Baumann et al., 2010) and nitrogen source is necessary.

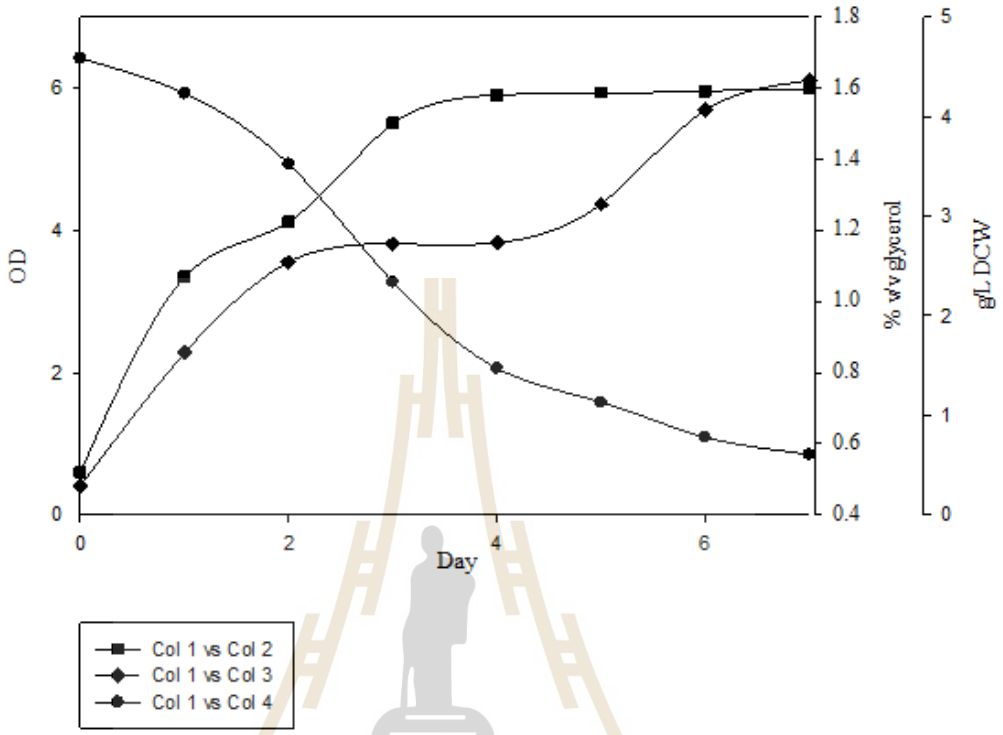


Figure 4.5 Growth curve of P. pastoris, DCW and glycerol concentration in fermentation of *P. pastoris* at 500L bioreactor

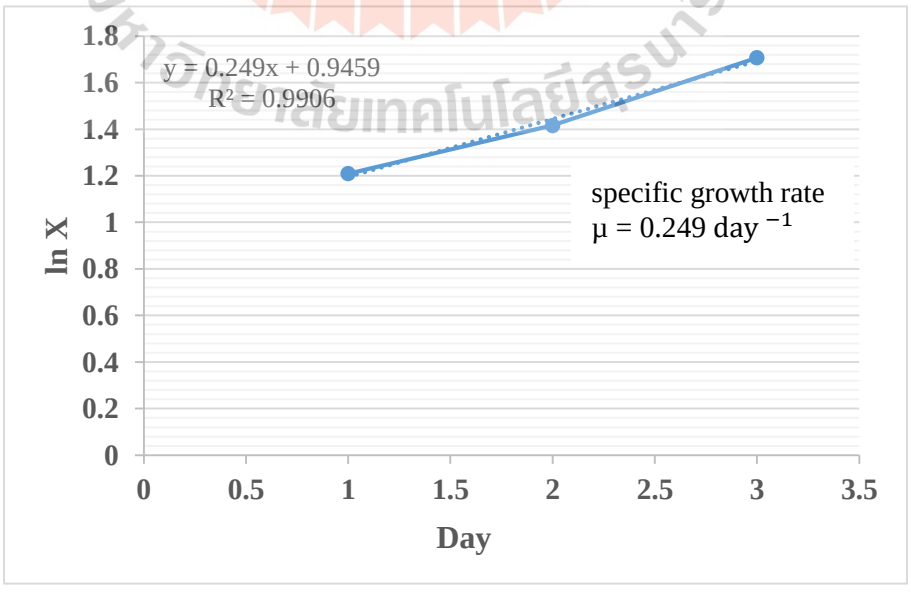


Figure 4.6 Measurement of specific growth rate in exponential phase

4.3 Cells harvesting

4.3.1 Membrane filtration

The fermentation broth around 350L was filtered through microfiltration membrane with pore 0.1 μm to separate the cells. The membrane area was 24 m^2, retentate pressure 1 bar. Each 12.5 L of the permeate was determined and the time was recorded. The time course of the flux was shown in Figure 4.7. The flux of pure water was identified around 60 L/m^2h. From Figure 4.7, the finding shown that the characteristic of flux decline was extremely changed from the flux of pure water to broth fermentation. Cross-flow membrane is an effective filtration method that can control the yeast cells deposition of the membrane surface. The outcome indicated that maintaining of the permeate flux at a constant level. Therefore, the cross flow mode prefers to use in separation more than death end mode in maintaining term of cake thickness. And then the retentate was collected around 35 L containing yeast cells. After that, the cells solution was harvested by using spray dryer. Another way, the retentate was continuous centrifuged to collect high concentrated, and then using freeze drying, the dry cells were obtained. Due to large volume of broth, the microfiltration supported to move a high amount of water for concentration of cells using centrifugal. Another hand, increasing the homogenous sample, the tank 5L retentate was mixed and separated 2.5L for spray dry and 2.5L for freeze dry.

Finally, a cleaning method was employed to the machine by RO water followed by 1% NaOH. The recovered flux of water was recorded of each cleaning step until it reached to the original flux value.

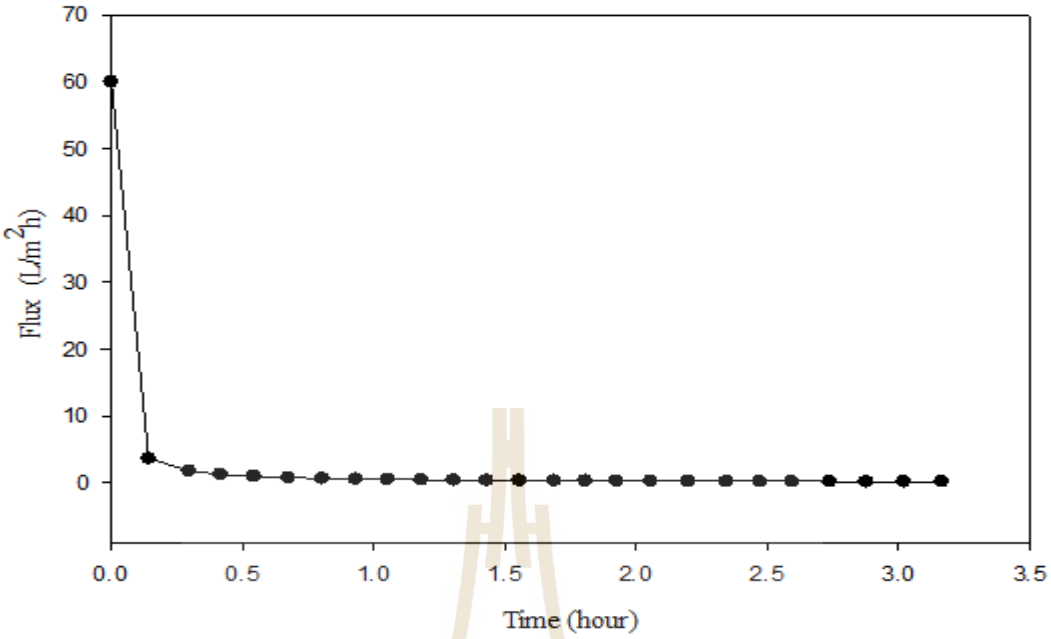


Figure 4.7 Time course of the flux in cross flow microfiltration in fermentation broth

4.3.2 Centrifugal

For helping freeze dry step, the centrifugal was used to eliminate substance and collected high concentrated cells in Figure 4.8. The parameters at 5000 rpm, 10 min was applied.

Figure 4.8 The yeast cells after centrifuge

4.4. Spray dryer, freeze dryer

The *P. pastoris* broth after running membrane filtration was dried by using a spray drying technique. The nozzle size of 1.4 mm and vacuum pressure of -50 mbar was employed (Pongsatorn, 2019). The feed rate of broth was 3.5 mL/min, air flow rate 470 L/hour was flown through a heater. At 110^0C air inlet and around 60^0C air out, the yeast cells were completely dried below 10% moisture (Aponte et al., 2016) (Pongsatorn, 2019).

Another way for drying, the freeze dryer is a good technology for drying transport the lipid to vapor that is a reason to keep high quality of samples yeast cells. In this study, both method for drying was applied and compared the results that was shown in Table 4.3 below.

Figure 4.9 The dry yeast cells after drying

Based on the results, the freeze drying was had a lot of advantage more than spray dry. Additionally, amount of omega-3 in spray dryer sample was quite lower more than amount of omega-3 in freeze dryer. The outcomes indicated that the freeze dryer should

be used for drying. The yeast broth after filtration by membrane had mature yeast cells and new yeast cells. However, when drying in spray dryer, the yeast cells passed to the chamber and then followed air to the cyclone and then fill out the sample bottle, new yeast cells were quite small and easily moved to sample bottle. On the other hand, the high amount of mature yeast was lost in the chamber. Therefore, the difference in the amount oil of both samples was quite different. Moreover, the color of dry yeast cells was different between both methods. The crude glycerol was used in this study such as carbon source. The color of crude glycerol was affected to the color of the samples. The short time for drying and the high quality cells, the freeze dryer was chosen main dry method in this step.

Table 4. 3 Comparing spray dryer and freeze dryer

Methods	**Freeze dryer**	**Spray dryer**
Time	50 hours	3.5 ml/min
Color	Quite dark, same color of wet cells	Ivory-white
Yield	collection 100%	Lost in chamber, cyclone
Total oil	3.6816 ± 1.56 % w/w	2.892 ± 1.56 % w/w
DHA (µg)/ 1g DCW	3.483 ± 4.925	4.357 ± 0.45
EPA (µg)/ 1g DCW	0.963 ± 1.362	0

4.5 Direct trans-esterification

Collecting the dry cells after freeze drying, the direct trans-esterification was applied to obtain FAME before injecting GC-FID. For lab scale, the sample after centrifuging was dried by freeze dry due to small amount of yeast cells. In this step, the methodology was separated 2 ways including lab scale and industry scale.

With 300 mg DCW samples for lab scale, the results were shown amount of omega-3 during 7 days in Figure 4.8. The results indicated that DHA appeared in the day 1. Although, EPA did not appear in day 1, DHA was in broth. Maybe, DHA was synthesized in the cells at 50L fermenter. On the another hand, the amount of DHA during 7 days higher more than EPA that was shown in Figure 4.8. With 30-40% dissolved oxygen, the amount of omega-3 fatty acid increased from 6.45 ± 1.994 µg/ 1g DCW to 8.99 ± 2.557 µg/ 1g DCW. However, the omega-3 fatty acid decreased after reducing the DO to 8-10%. *P. pastoris* is a facultative anaerobe, so can live with or without oxygen. Therefore, with oxygen limitation, *P. pastoris* leads to by-product fermentation and can use product itself due to reducing omega-3 fatty acid. Normally, the lipid have been synthesized in some organelles such as the plasma membrane, peroxisomes, mitochondria, lipid droplets or endoplasmic reticulum (Adelantado et al., 2017). Extracting pass from plasma membrane to broth that was a way to lose an amount of omega-3 fatty acid. After reducing DO to 8 – 10%, increasing the oil was shown and reached highest point at day 5 with 3.9 % dry cells. Comparing with other oleaginous yeast, for example, this yeast had quite lower the amount of EPA more than *Yarrowia lipolytica* that were reported EPA around 15 % dry weight (Xue et al., 2013). However, *P. pastoris* is non-oleaginous yeast that why amount of oil lower more than other oleaginous yeast. Comparing with another *Pichia pastoris* (Li et al., 2009), this

yeast was improved to produce both EPA and DHA with industrial medium Table 4.4. The results indicated that reducing DO just increased the amount of oil inside yeast, but that did not affect the production of omega-3 fatty acid. Therefore, maintenance of DO at 30-40% can be obtained high omega-3 fatty acid product and the yeast cells should collect at last day of exponential phase because it can use product itself when limitation of nutrient in medium.

In scale up, the reactor 10L was designed with a gauge pressure, a condenser, a rotor. The pressure gauge can control the pressure inside that make sure for safe reaction. For the condenser, it can collect methanol vapor during reaction. With 500 gram DCW, the sample was done after trans-esterification reaction in Figure 4.9. The findings were shown 37.2% FAME and 62.79% impurity in Table 4.5. The profile of FAME was shown the amount of oil in Table 4.5 and the retention time of GC-RID in Figure 4.10. The amount of omega-3 fatty acid was 0.019%. Continuously, the sample was purified by molecular distillation (short path distillation) to collect PUFAs rich omega-3.

Table 4.4 Recombinant yeast for long-chain polyunsaturated fatty acids production

Strain	Carbon sources	Nitrogen sources	Product	References
Pichia pastoris pGAPZ:Δ6&Δ5:E6	Crude glycerol	$NH_4)_2SO_4$; $(NH_4)_2HPO_4$	2.6 µg DHA/g DCW, 6.39 µg EPA/g DCW at day 3 fermentor 500L	In this study
Pichia pastoris pGAPZ:Δ6&Δ5:E6	20 g/L glucose	10 g/L yeast extract, 10 g/L peptone	ARA 0.4%, EPA 0.5% TFA	(Chotika, 2013)
Yarrowia lipolytica	20 g/L glucose	6.7g/L yeast nitrogen base, 5 g/L yeast extract and some sources.	EPA 15% DCW	(Xue et al., 2013)
Pichia pastoris GS115 (his4), expressed Δ5D, Δ6D, Δ6E	No report	Yeast Nitrogen Base	EPA 0.1% TFA	(Li et al., 2009)
Yarrowia lipolytica (RH_4) expressed Δ9E, Δ8D	20 g/L glucose	10 g/L yeast extract, 10 g/L peptone	ARA 118.1 mg/L	(Liu et al., 2019)

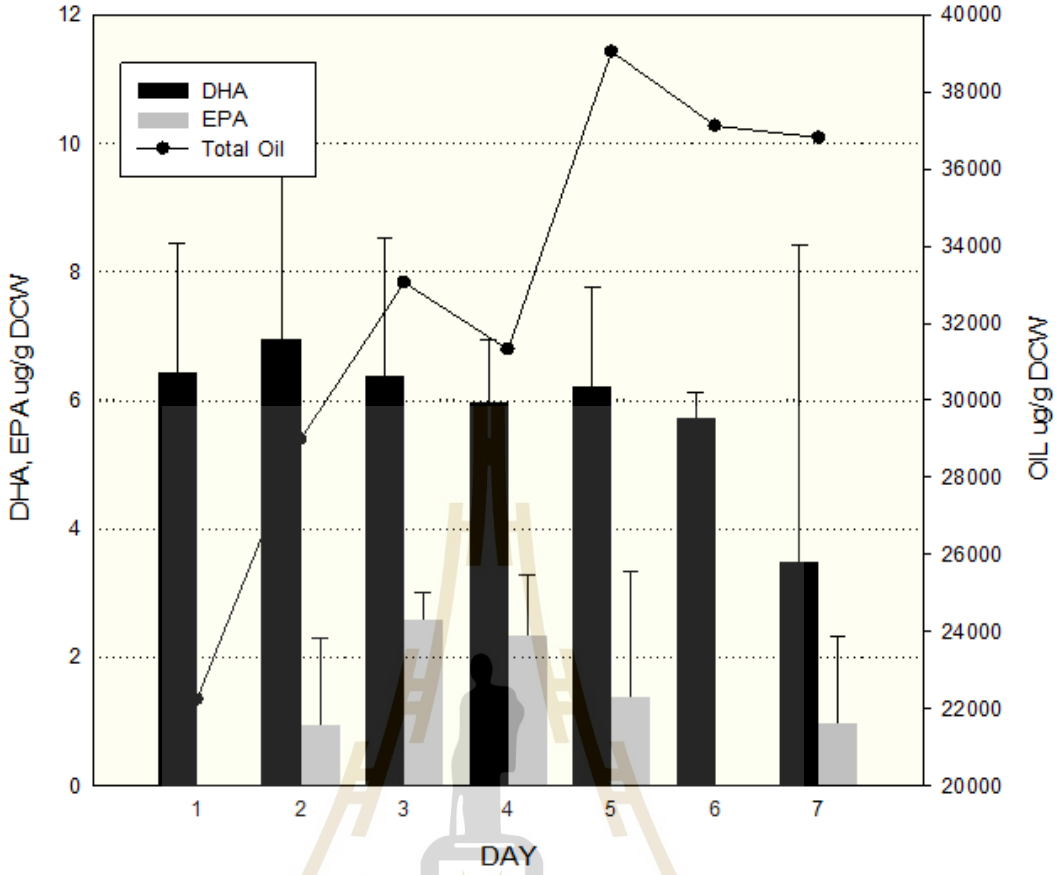


Figure 4.10 Amount of omega-3 during 7 days at 500L fementer

Figure 4.11 The sample after trans-esterification of 500 gram DCW

Table 4.5 Profile of the sample before run SPD

Name	**Formula**	**% w/w**
Lauric acid	C12:0	0.084
Myristic acid	C14:0	0.621
Myristoleic acid	C14:1	0.621
Palmitic acid	C16:0	8.958
Palmitoleic acid	C16:1	1.404
Cis-10-heptadecenoic acid	C17:1	0.097
Elaidic acid	C18:1n9t	9.265
Oleic acid	C18:1n9c	0.192
Linolelaidic acids	C18:2n6t	7.153
Arachidic acid	C20:0	6.828
Gamma-linoleic acid	C18:3n6	0.091
Eicosenoic acid	C20:1	0.062
Alpha-linolenic acid	C18:3n3	3.089
Conjugated linoleic acid	T9,T11	0.049
Heneicosylic acid	C21:0	0.015
Behenic acid	C22:0	0.083
Erucic acid	C22:1n9	0.084
Eicosapentaenoic acid	**C20:5n3**	**0.009**
Lignoceric acid	C24:0	0.347
Docosahexanenoic acid	**C22:6n3**	**0.012**
Impurity		60.93

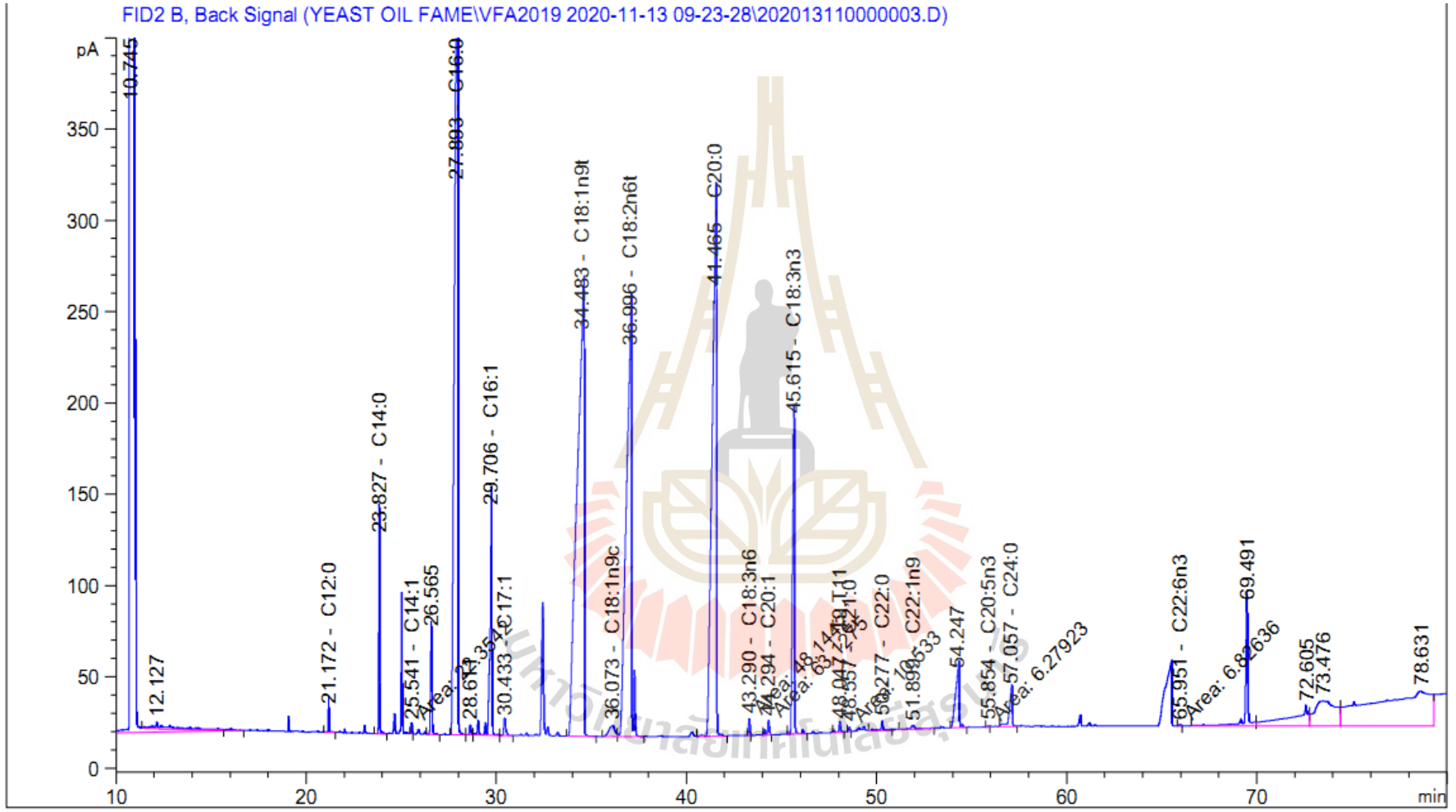


Figure 4.12 Profile of the sample with GC-RID

4.6 Molecular distillation (short path distillation)

The parameters at 150^0C distillation, 0.2 mPa – 1 Pa, 40^0C of condenser inside column, -20^0C of condenser outside, feed temperature 60^0C, 0.5 mL/min were investigated for the sample after trans-esterification. Profile of the sample after running SPD was shown in Table 4.5. The outcomes indicated that the impurity extremely decreased from 60.93 % to 6.7%. However, the EPA was lost that did not appear in the sample after running SPD. Alternatively, the DHA increased from 0.012% to 0.0135%. Because the time for running SPD was quite long around 7 hours with 100 mL sample, that effected to EPA and the mount of EPA was really small due to lost EPA. From all results, SPD had useful for purification of the yeast oil. After SPD, the sample can revert to triglycerides for human diet. Methanol is a toxic alcohol that can effect to human, so the methanol should replace by ethanol.

Comparing with the sample before running SPD (Figure 4.9), this sample (Figure 4.13) had clear color more. However due to small amount of sample, the SPD cannot value an optimum condition for omega-3 methyl ester.

Table 4.6 Profile of the sample after running SPD

Name	**Formula**	**% (w/w)**
Myristoleic acid	C14:1	0.116
Palmitic acid	C16:0	26.309
Cis-10-heptadecenoic acid	C17:1	0.0431
Stearic acid	C18:0	24.755
Oleic acid	C18:1n9c	0.497

Table 4.6 Profile of the sample after running SPD (Continued)

Name	**Formula**	**% (w/w)**
Elaidic acid	C18:1n9t	18.216
Lindeic acid	C18:2n6c	18.589
Gamma-linoleic acid	C18:3n6	0.186
Alpha-linolenic acid	C18:3n3	2.646
Arachidic acid	C22:0	0.471
Dihomo-gamma-linolenic acid	C20:3n6	0.659
Erucic acid	C22:1n9	0.081
Tricosylic acid	C23:0	0.712
Docosahexaenoic acid	**C22:6n3**	**0.0135**
Impurity		6.706

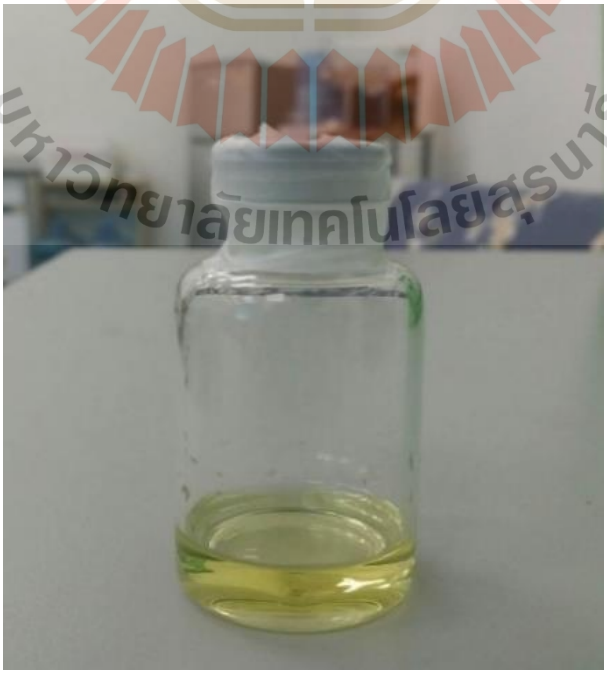


Figure 4.13 The sample after running SPD

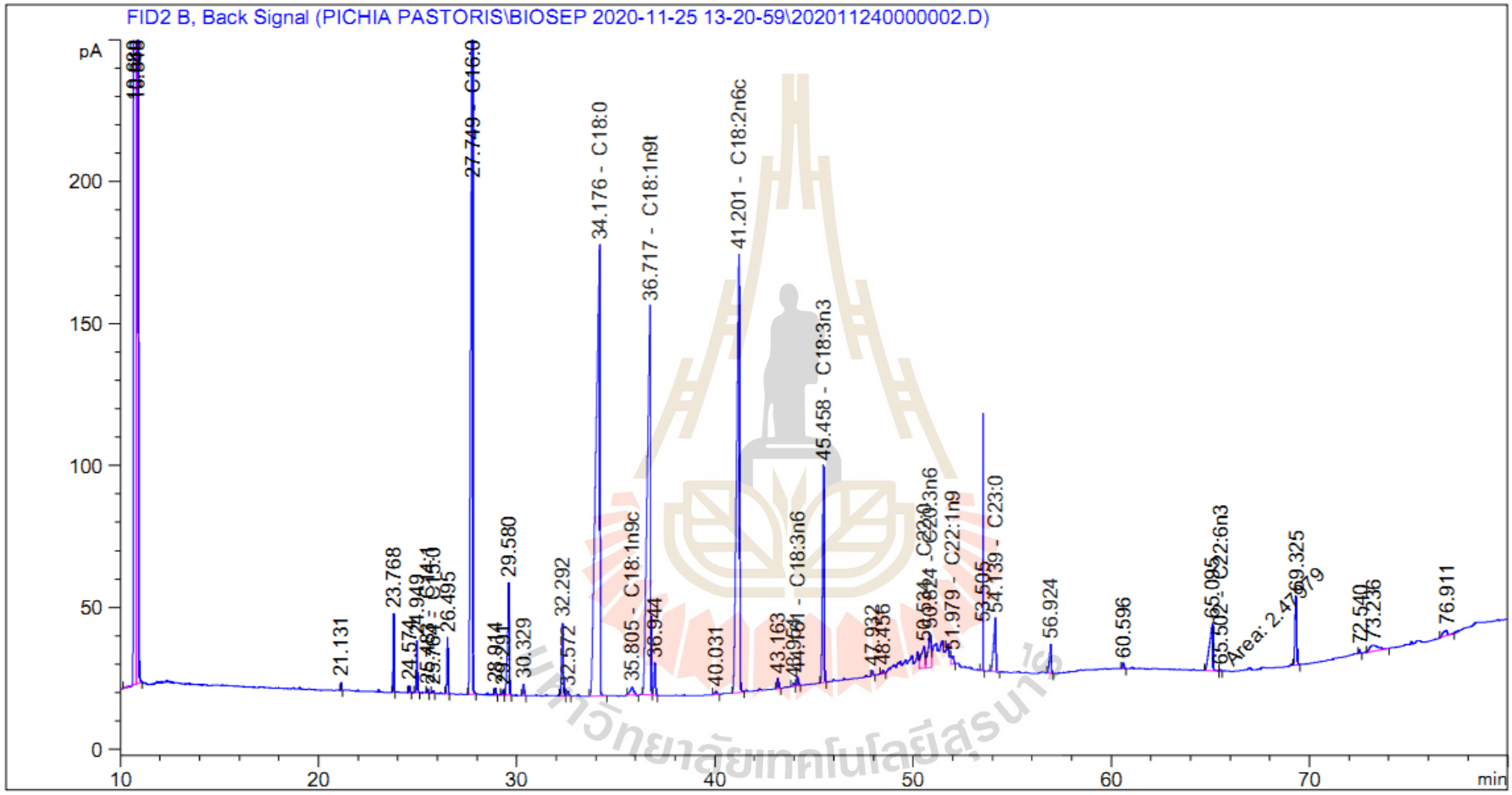


Figure 4.14 Profile of the sample with GC-RID

CHAPTER V

CONCLUSION

In this study, the process of optimization of medium and condition of fermentation was done. The optimization of medium was 40 g/L crude glycerol, 3 g/L $(NH_4)_2SO_4$, and 7 g/L $(NH_4)_2HPO_4$. The parameter of fermentation was at 30^0C, 200 rpm, initial pH 5 adjusting by citric acid, and 4.6 mL/L PTM_4 trace salts. Applying this above results, the cultivation of *P. pastoris* was studied at 500L bioreactor. With DO 30-40%, the omega-3 fatty acid was synthesized more than reducing to DO 8-10%. After that using microfiltration and centrifugal, the cells were obtained and dried by both methods spray dry and freeze dry. With high quality of dry *P. pastoris* cells, the freeze dry was chosen in this study. The direct trans-esterification was done with methanol to transfer triglycerides to FAME that was supported for purification next step. Finally, the molecular distillation was done to release impurities from 60.93% to 6.706%. The amount of omega-3 fatty acid was 0.0135 % after purifying.

In the future work, the ethanol can be alternative methanol for trans-esterification that is safe for human healthy. Another way, the glycerolysis can be applied to transfer FAME to triglycerides for human diet. *P. pastoris* can produce omega-3 fatty acid but the amount of lipid was quite low that was shown in Table 4.4. Therefore, producing omega-3 fatty acid should be chosen another microorganism that can replace for *P. pastoris*.

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BIOGRAPHY

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