MODIFICATION OF *Pichia pastoris* FOR DOCOSAHEXAENOIC ACID (DHA) PRODUCTION AND THE POTENTIAL OF OLEAGINOUS YEAST *Rhodotorula paludigena* CM33 FOR BIOFUEL PRODUCTION

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctoral of Philosophy in Biotechnology

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การปรับแต่งยีสต์ *Pichia pastoris* เพื่อผลิตกรดไขมันดีเอชเอ และความเป็นไปได้ในการใช้ยีสต์น้ำมัน *Rhodotorula paludigena* CM33 สำหรับพลังงานชีวภาพ

นางสาวโชติกา โกศัลวิตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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โชติกา โกศัลวิตร : การปรับแต่งยีสต์ *Pichia pastoris* เพื่อผลิตกรดไขมันดีเอชเอ และความเป็นไปได้ในการใช้ยีสต์น้ำมัน *Rhodotorula paludigena* CM33 สำหรับพลังงานชีวภาพ (MODIFICATION OF *Pichia pastoris* FOR DOCOSAHEXAENOIC ACID (DHA) PRODUCTION AND THE POTENTIAL OF OLEAGINOUS YEAST *Rhodotorula paludigena* CM33 FOR BIOFUEL PRODUCTION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.มารินา เกตุทัต-คาร์นส์, 72 หน้า

เนื่องจากไขมันและกรดไขมันมีความสำคัญและเกี่ยวข้องกับการดำรงชีวิตของมนุษย์ทั้งในแง่ของอาหารและการดำเนินชีวิตประจำวัน ในวิทยานิพนธ์นี้ ได้ทำการตัดต่อพันธุกรรมยีสต์ *Pichia pastoris* เพื่อให้มีการสะสมของกรดไขมันไม่อิ่มตัวสูงชนิดดีเอชเอ (docosahexaenoic acid; DHA) โดยการสกัดยีน Δ2 desaturase (Δ2D) และ Δ6 elongase (Δ6E) จากปลาม้าลาย (*Dario rerio*) และยีน Δ4 desaturase (Δ4D) จากสาหร่ายทะเลขนาดเล็ก (*Isochrysis galbana*) แล้วโคลนเข้าสู่พลาสมิด pGAP และตรวจสอบลำดับนิวคลีโอไทด์ ผลการวิเคราะห์พบว่ายีนทั้งหมดมีความถูกต้องเมื่อเปรียบเทียบกับฐานข้อมูล NCBI จากนั้นนำยีนทั้ง 3 มาตัดต่อให้อยู่ภายในพลาสมิดเดียวกัน เกิดเป็นพลาสมิดลูกผสม pGAP-Δ2DΔ6EΔ4D แล้วนำส่งเข้าสู่ยีสต์ *P. pastoris* หลังจากสิ้นสุดการเลี้ยงในสภาวะที่กำหนด พบว่ายีสต์ดังกล่าวมีการสะสมของดีเอชเอสูงถึง 1.67 มิลลิกรัมต่อกรัมยีสต์แห้ง ในขณะที่ไม่พบการสะสมของดีเอชเอในยีสต์ที่ไม่มีพลาสมิดลูกผสมและยีสต์ที่มีพลาสมิดเปล่า จากผลการทดลอง สรุปได้ว่ายีนทั้งหมดสามารถทำงานร่วมกันจนเกิดการสร้างและสะสมดีเอชเอในยีสต์ *P. pastoris*

นอกจากนี้ ยีสต์ในกลุ่มที่มีการสะสมไขมันสูง (oleaginous yeasts) กำลังได้รับความสนใจเพื่อใช้สำหรับผลิตพลังงานชีวภาพ เนื่องจากมีข้อเด่นหลายประการรวมทั้งยังสามารถเจริญได้ในของเสียหรือของเหลือทิ้งจากภาคอุตสาหกรรม จึงได้ทำการคัดเลือกยีสต์น้ำมันจากธรรมชาติโดยใช้เทคนิคการย้อมไขมันด้วย Nile Red (NR) fluorescence dye จากนั้นวัดค่าแสงฟลูออเรสเซนต์โดยใช้เทคนิค flow cytometry พบว่า ยีสต์ CM33 มีค่า relative Mean Fluorescence Intensity (MFI) และการสะสมไขมันสูงสุด จากการวิเคราะห์ลำดับนิวคลีโอไทด์บริเวณ ITS-5.8S-ITS rDNA พบว่ายีสต์ดังกล่าว คือ *Rhodotorula paludigena* และได้ทำการวิเคราะห์ลำดับนิวคลีโอไทด์ทั้งหมดของยีสต์ *R. paludigena* CM33 เป็นที่เรียบร้อยแล้ว โดยสามารถสืบค้นได้ที่ฐานข้อมูล DDBJ/ENA/GenBank ภายใต้ BioProject PRJNA491831 BioSample SAMN10089541 รหัส SWEA00000000.1 Assembled genome sequences รหัส SWEA01000001-SWEA01000078 และ Raw data sequences รหัส SRX6085390 จากนั้นเมื่อทดลองเลี้ยงยีสต์ดังกล่าวในอาหารเลี้ยงเชื้อที่มีแหล่งคาร์บอนที่หลากหลาย ได้แก่ กลูโคส กลีเซอรอล ซูโครส และไซโลส พบว่า ยีสต์ CM33 มีการสะสมไขมัน

สูงสุดถึง 23.9% จากการเลี้ยงในอาหารเลี้ยงเชื้อที่มีกลูโคสเป็นแหล่งคาร์บอนระยะเวลา 4 วัน โดยมีกรดไขมันในกลุ่ม C16 และ C18 เป็นส่วนประกอบหลัก และเมื่อนำไปเลี้ยงในอาหารเลี้ยงเชื้อที่มีกากน้ำตาลเป็นแหล่งคาร์บอนพบว่า สามารถผลิตชีวมวลและไขมันสูงขึ้นถึง 16.5 และ 6.1 กรัมต่อลิตร ตามลำดับ ซึ่งผลที่ได้จากการวิจัยนี้แสดงให้เห็นถึงความเป็นไปได้ในการประยุกต์ใช้ยีสต์ *R. paludigena* สำหรับสร้างและสะสมไขมันและกรดไขมันจากการใช้ของเหลือทิ้งที่มีราคาถูกเป็นแหล่งคาร์บอน

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2562 ลายมือชื่ออาจารย์ที่ปรึกษา _____

CHOTIKA GOSALAWIT : MODIFICATION OF *Pichia pastoris* FOR DOCOSAHEXAENOIC ACID (DHA) PRODUCTION AND THE POTENTIAL OF OLEAGINOUS YEAST *Rhodotorula paludigena* CM33 FOR BIOFUEL PRODUCTION. THESIS ADVISOR : ASSOC. PROF. MARIENA KETUDAT-CAIRNS, Ph.D., 72 PP.

DHA/OLEAGINOUS YEAST/FATTY ACID BIOSYNTHESIS/BIOFUELS

Lipid and fatty acids are known to be important molecules for human daily life both as food and fuel. In this thesis, docosahexaenoic acid (DHA) which is important for human health was produced by co-expression of desaturase and elongase genes. The Δ2 desaturase (Δ2D) and Δ6 elongase (Δ6E) genes from zebrafish (*Dario rerio*) and Δ4 desaturase (Δ4D) gene from marine microalgae (*Isochrysis galbana*) were cloned into plasmid under the constitutive GAP promoter. Sequences of the deduced amino acids of all 3 genes showed that they contained conserved regions important for their activities. The co-expression of these 3 genes using *Pichia pastoris* as expression host showed that DHA was produced in recombinant yeast but was not detected in non-transformed *Pichia* and *Pichia* transformed with empty plasmid. Cultivation under certain conditions led to maximum DHA production of 1.67 mg/g DCW. The accumulation of DHA indicated the successful co-expression of Δ2D, Δ6E and Δ4D genes in *P. pastoris*.

Moreover, oleaginous yeasts are the future of biofuel production due to their numerous advantages. They not only require a short time, are easy to manipulate, and can cultivate to high cell density, but they also are able to grow in various carbon sources and waste materials. Therefore, oleaginous yeasts were screened using Nile Red (NR) fluorescence dye staining coupled with flow cytometry techniques. Strain CM33 showed the highest relative Mean Fluorescence Intensity (MFI) value, which correlated with the maximum lipid content when

compared with other yeasts. CM33 was identified as *Rhodotorula paludigena* by sequencing the ITS-5.8S-ITS rDNA region. After that, *de novo* sequencing of *R. paludigena* CM33 was performed and the whole genome sequence was deposited in DDBJ/ENA/GenBank under the BioProject PRJNA491831, BioSample SAMN10089541, accession number SWEA00000000.1. Assembled genome sequences are provided via GenBank accession numbers SWEA01000001-SWEA01000078. Raw data sequences have been deposited in the SRA database under accession number SRX6085390. The potential to assimilate various carbon sources of CM33 was performed through glucose-, glycerol-, sucrose- or xylose-based minimal media. Glucose-based medium showed the highest lipid content of 23.9% DCW after 4 days of cultured. The major fatty acids of the total lipids were C16:0 and C18:1. Molasses-based medium, biomass, lipid content and lipid yield increased to about 16.5 g/L, 37.1% DCW and 6.1 g/L, respectively. These results suggested that *R. paludigena* CM33 is a potential candidate for the maintenance of the carbon value chain by converting renewable waste material for biolipid production.

School of Biotechnology

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Student's Signature CHOTIKA GOSALAWIT.

Advisor's Signature

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LIST OF ABBREVIATIONS

Δ4D	=	Δ4 desaturase
Δ5D	=	Δ5 desaturase
Δ5E	=	Δ5 elongase
Δ6D	=	Δ6 desaturase
Δ6E	=	Δ6 elongase
Δ7E	=	Δ7 elongase
Δ2D	=	Bifunctional Δ6&Δ5 desaturase
Δ9D	=	Δ9 desaturase
Δ12D	=	Δ12 desaturase
Δ15D	=	Δ15 desaturase
ω3D	=	ω3 desaturase
ALA	=	α-linolenic acid (C18:3n-3)
ARA	=	Arachidonic acid (C20:4n-6)
DGLA	=	Di-homo γ-linolenic acid (C20:3n-6)
DHA	=	Docosahexaenoic acid (C22:6n-3)
DPA	=	Docosatetraenoic acid (C22:5n-3)
EPA	=	Eicosapentaenoic acid (C20:5n-3)
ETA	=	Eicosatetraenoic acid (C20:4n-3)
FAMEs	=	Fatty acid methyl esters
FAs	=	Fatty acids
GAP	=	Glyceraldehyde-3-phosphate dehydrogenase
GLA	=	γ-linolenic acid (C18:3n-6)

LIST OF ABBREVIATIONS (Continued)

LA	=	Linoleic acid (C18:2n-6)
OA	=	Oleic acid (C18:1n-9)
PUFAs	=	Polyunsaturated fatty acids
SA	=	Stearic acid (C18:0)
SE	=	Steryl ester
STA	=	Stearodonic acid (C18:4n-3)
TAG	=	Triacylglycerol
THA	=	Tetracosahexaenoic acid (C24:6n-3)
TL	=	Total lipid
TPA	=	Tetracosapentaenoic acid (C24:5n-3)

CHAPPER I

INTRODUCTION

1.1 Background

Lipid and fatty acids are important for various human activities especially food and fuel. Long-chain polyunsaturated fatty acids (LCPUFAs) e.g. γ-linoleic acid (GLA), di-homo-γ-linoleic acid (DGLA), arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are important fatty acids which are synthesized by series of desaturases and elongases activities (Pereira *et al.*, 2003). These fatty acids are found in marine animals and algae. However, the fatty acids derived such sources display several disadvantages such as high value, contamination with heavy metal and diseases which resulting in stimulate the finding of alternative sources. Recently, desaturase and elongase enzymes have been studied using yeasts as host system through biotechnological and suitable cultivation methods. (Agaba *et al.*, 2004; de Antueno *et al.*, 2001; Meyer *et al.*, 2004; Nakamura and Nara, 2004; Tanomman *et al.*, 2013; Vagner and Ester, 2011; Zheng *et al.*, 2004; Zhou *et al.*, 2007). Co-expression of desaturases and elongases successfully produced long-chain fatty acid along with omega-3 and omega-6 families such as ARA and EPA (Domergue, 2002), docosapentaenoic acid (DPA) and DHA (Kang *et al.*, 2008; Li *et al.*, 2009). To our knowledge, to date no complete genetically engineered yeast for high DHA production without adding exogenous inducers and substrates fatty acids have been reported. The generally recognized as safe (GRAS) *P. pastoris* endogenously produces linoleic acid (LA) and α-linolenic acid (ALA), which are substrates for LCPUFAs productions (Yu *et al.*, 2012; Zhang *et al.*, 2005). It has been utilized as single cell protein and used as host for protein expressions. Additionally, couple this yeast with constitutive

GAP promoter presented in pGAP plasmid displayed high expression of target genes. Therefore, production of DHA by *P. pastoris* without adding external inducers and substrates (LA and ALA) is one of the aims of this thesis.

Lipid also has been used as a substrate for biofuel production especially biodiesel. These fuels are principally produced by methylation of lipid derived plant oils and animal fats which consequently increase competition between food and fuel. Oleaginous yeasts which display high lipid content up to 70 % of dry cell weight (DCW) (Chang *et al.*, 2011; Sitepu *et al.*, 2014) have similar fatty acid profiles to plant oils which are possibility to use as biofuels feedstock. Comparison with other lipid and fatty acid sources, yeasts demonstrate several advantages such as rapidly growth, easily scaled up, continuous production, less labor and land requirements, non-effect from seasonal, less requirement of cultivation (Li *et al.*, 2008; Sitepu *et al.*, 2014) and ability to recycle by-products, waste and inexpensive materials to high value biochemicals and biofuels (Bommareddy *et al.*, 2015). These reasons lead oleaginous yeasts to be alternative source for biofuels production. In recent years, numerous researches on oleaginous yeasts have been published. Several genus have been identified as oleaginous yeast such as *Candida, Cryptococcus, Rhodotorula, Trichosporon, Yarrowia, Lipomyces and Rhodosporidium* (Beopoulos *et al.*, 2009). However, the ability to produce lipid and fatty acids are influenced by yeast strain and culture conditions. Therefore, information and knowledge about oleaginous yeasts for high lipid and fatty acid production are still needed.

In this thesis, possibilities of using yeasts to produce target products are demonstrated. Δ2 desaturase (Δ2D), Δ6 elongase (Δ6E) and Δ4 desaturase (Δ4D) genes were cloned into pGAP plasmid then sequenced and analyzed. Then, the desaturase and elongase genes were co-expressed using *P. pastoris* as a host. Finally, recombinant *P. pastoris* was cultured in certain conditions afterward DHA and other fatty acids were detected using gas chromatography technique. The potential of oleaginous yeasts for biodiesel production were

accomplished by screening oily yeasts from Castor beans using Nile Red (NR) fluorescence dye staining coupled with flow cytometry techniques. NR emission was calculated as relative Mean Fluorescence Intensity (MFI) value. Strain CM33 showed the highest of relative MFI value when compared with other strains. Then, CM33 was identified as *Rhodotorula paludigena* and further *de novo* sequenced. The whole genome sequence was deposited on DDBJ/ENA/GenBank database. The potential to assimilate various carbon sources of *R. paludigena* CM33 was performed using minimal medium supplemented with glucose, glycerol, sucrose, xylose including by-products molasses and crude glycerol. Then, lipid and fatty acids were measured.

1.2 Objectives

1.2.1 Modification of *P. pastoris* for docosahexaenoic acid (DHA) production.

1.2.1.1 To clone Δ2D and Δ6E genes from *Danio rerio* and Δ4D gene from *Isochrysis galbana* into pGAP plasmid then sequenced and analyzed.

1.2.1.2 To engineer these 3 genes to constitutively express in *P. pastoris*.

1.2.1.3 To determine amounts of DHA and other fatty acids using gas chromatography technique.

1.2.2 The potential of oleaginous yeast *Rhodotorula paludigena* CM33 for biofuel production.

1.2.2.1 To screen for high lipid producing oleaginous yeasts using NR fluorescent staining with flow cytometry techniques.

1.2.2.2 To identify oleaginous yeasts strain CM33 using ITS-5.8S-ITS rDNA region and to perform the whole genome sequencing (WGS).

1.2.2.3 To determine the ability to produce lipid and fatty acids by *R. paludigena* on various carbon sources.

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CHAPTER II

METABOLIC ENGINEERED *Pichia pastoris* TO PRODUCE DOCOSAHEXAENOIC ACID (C22:6N3, DHA)

2.1 Abstract

A bifunctional Δ6 desaturase and Δ5 desaturase (Δ6D/Δ5D), named Δ2 desaturase (Δ2D), Δ6 elongase (Δ6E) genes from zebrafish (*Dario rerio*) and Δ4 desaturase (Δ4D) gene from marine microalgae (*Isochrysis galbana*) were cloned, sequenced and analyzed. Sequences of the deduced amino acids of all genes showed that they contained conserved regions important for their activities. Thereafter, a co-expression plasmid containing these 3 genes under their constitutive GAP promoter was constructed then integrated into *Pichia pastoris* (SMD1168H) genome. Gas chromatogram results demonstrated docosahexaenoic acid (DHA) was detected in this recombinant yeast after grown in normal medium without exogenous inducers and precursors. Whereas, DHA was not detected in non-transformed *Pichia* and *Pichia* transformed with empty pGAP. The maximum DHA of ~1.60 mg/g DCW was obtained when cultured recombinant yeast through glycerol-based medium. These results indicated successful co-expression of Δ2D, Δ6E and Δ4D genes to produce DHA in *P. pastoris*.

2.2 Introduction

Docosahexaenoic acid (DHA, 22:6n3) is one of the long-chain polyunsaturated fatty acids (LCPUFAs) belonging to the omega-3 family which plays important roles in human body. For examples, they reduce the risk of cardiovascular diseases, involve in visual and brain development, and reduce anxiolytic symptoms (Chen and Su, 2012; Heird and

Lapillonne, 2005; Ruxton *et al.*, 2004; Uauy *et al.*, 2001). DHA biosynthesis starts from Δ6 desaturase (Δ6D) desaturates α-linolenic acid (ALA, 18:3n3) to stearodonic acid (STA, 18:4n3). The Δ6 elongase (Δ6E) elongates STA to eicosatetraenoic acid (ETA, 20:4n3). ETA is the substrate for Δ5 desaturase (Δ5D) to generate eicosapentaenoic acid (EPA, 20:5n3) then Δ5 elongase (Δ5E) elongates EPA to docosapentaenoic acid (DPA, 22:5n3). In the last step, the production of DHA is occurred in 2 ways. First, the DPA is further altered to tetracosapentaenoic acid (TPA, 24:5n3) by Δ7 elongase (Δ7E) then TPA converted to tetracosahexaenoic acid (THA, 24:6n3) by Δ6D activity. Subsequently, the THA is changed to DHA in peroxisome by β-oxidation process. This pathway normally happens in animal. The other pathway uses the activity of Δ4 desaturase (Δ4D) to directly convert DPA to DHA which is found in marine microalgae and vertebrates (Figure 2.1) (Lee *et al.*, 2016). The enzymes involved in the omega-3 pathway are also used in the omega-6 pathway, especially the generation of arachidonic acid (ARA, C20:4n6) which is generated by the Δ6D activity that converts linoleic acid (LA, C18:2n6) to γ-linolenic acid (GLA, C18:3n6). GLA is the substrate for Δ6E to generate di-homo-γ-linolenic acid (DGLA, C20:3n6). The DGLA desaturates to ARA by Δ5D activity (Pereira *et al.*, 2003). Both of LA and ALA fatty acids are produced by Δ12 desaturase (Δ12D) and Δ15 desaturase (Δ15D) activities which have been identified in lower eukaryotes and plants excepted animals. Therefore, both of these fatty acids are indicated as essential fatty acids and must be obtained from diets or supplementary foods (Lee *et al.*, 2016). Despite, the series of enzymes for DHA production are found in animals however, it is insufficient for target fatty acids production. Leading to direct consumption of DHA, which is majority found in marine animal and microalgae especially fish, is still need. However, there are increasing demand for omega 3 fatty acids, depleting of wild fish including contamination in the marine. Therefore, generation of clean and sustainable sources for these fatty acids are urgently required.

Desaturase and elongase genes from various organisms have been identified and functionally investigated. There are Δ6D from common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), gilthead sea bream (*Sparus aurata*), and turbot (*Psetta maximus*) (Zheng *et al.*, 2004), Δ6D and Δ5D from human (*Homo sapiens*) (de Antueno *et al.*, 2001; Nakamura and Nara, 2004), Atlantic salmon (*Salmo salar*), cherry salmon (*Oncorhynchus masou*), zebrafish (*Danio rerio*) (Vagner and Ester, 2011), and Nile tilapia (*Oreochromis niloticus*) (Tanomman *et al.*, 2013) and Δ5D and Δ4D from marine microalga *Pavlova salina* (Zhou *et al.*, 2007). Several elongase genes have also been isolated and characterized from algae (*Ostreococcus tauri* and *Thalassiosira pseudonana*), rainbow trout (*O. mykiss*), frog (*Xenopus laevis*), sea squirt (*Ciona intestinalis*) (Meyer *et al.*, 2004), and zebrafish (*D. rerio)* (Agaba *et al.*, 2004). Moreover, to produce the LCPUFAs in omega-3 and omega-6 families, desaturase and elongase genes have been co-expressed. Li *et al.*, (2009) co-expressed *Phaeodactylum triconutum* Δ6D, Δ5D, and Δ6E under AOX inducible promoter in *P. pastoris* and found that recombinant yeast could produce ARA and EPA. Kang *et al.*, (2008) co-expressed *Thraustochytrium aureum* Δ5D, Δ4D, and Elo-like enzyme also under AOX inducible promoter in *P. pastoris*. They reported that the co-expression of these genes could generate DPA and DHA when exogenous substrate GLA and stearidonic acid (SDA, C18:4n3) were added. Domergue *et al.*, (2005) reported that co-expression of Δ6D from *O. tauri*, Δ5D from *P. triconutum*, and Δ6E from *Physcomitrella patens* under GAL1 inducible promoter in *Saccharomyces cerevisiae* could produce ARA and EPA after addition of exogenous substrates LA and ALA. However, until now, there were no genetically engineered yeast for high DHA production without adding inducers and exogenous substrates.

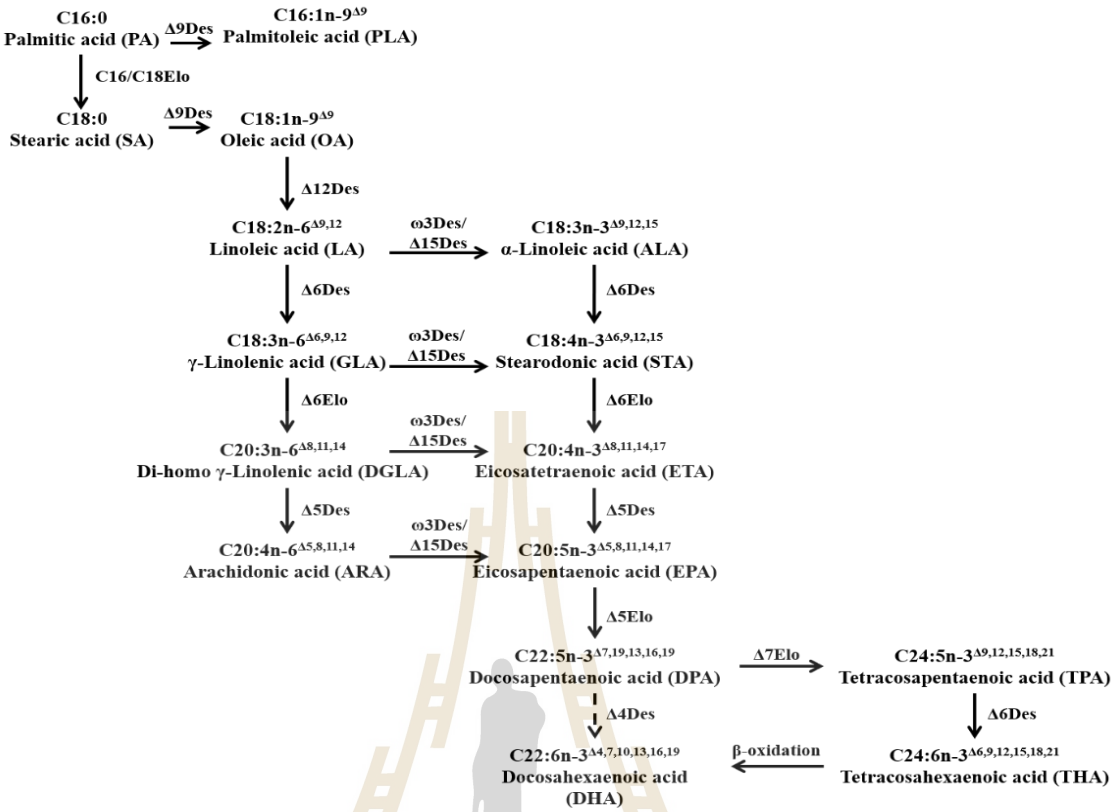

Figure 2.1 Biosynthetic pathway leading to DHA production adapted from Pereira *et al.*, (2003).

P. pastoris endogenously expresses Δ9 desaturase (Δ9D), Δ12D and Δ15D. Resulting in this yeast can produce stearic acid (SA, C18:0). Then, SA converts to oleic acid (OA, C18:1n9) by the Δ9D activity. Then, OA is converted to LA by the activity of Δ12D and by the Δ15D activity then LA is converted to ALA (Figure 2.1) (Yu *et al.*, 2012; Zhang *et al.*, 2005). Additionally, *P. pastoris* has been utilized as single cell protein and it is indicated to be generally recognized as safe (GRAS) organism. Therefore, *P. pastoris* is a suitable host for DHA biosynthesis.

In this study, Δ2 desaturase (Δ2D), which is a bifunctional Δ6D/Δ5D, Δ6E derived from zebrafish *Dario rerio* and Δ4D genes derived from marine microalgae *Isochrysis galbana* were cloned, sequenced and analyzed. Thereafter, a co-expression plasmid containing 3 genes under constitutive GAP promoter was constructed then integrated into *P. pastoris* genome.

Recombinant yeast was cultured in different media then fatty acid compositions were determined by gas chromatography (GC).

2.3 Materials and Methods

2.3.1 Δ2D, Δ6E, and Δ4D genes constructions

Since, a whole recombinant plasmid containing all Δ2D, Δ6E, and Δ4D genes, which their individual promoter and transcription terminator, will be integrated into genome of yeast at GAP promoter sequence. Moreover, to increase an efficiency of genes integration, linearized recombinant plasmid through *Avr*II restriction site which is located at GAP promoter will be accomplished prier transformation. However, digestion of normal GAP sequences with *Avr*II restriction enzyme on such recombinant plasmid leads to produce unfunctional 3 fragments. Therefore, to complete a whole fragment integration, mutation at *Avr*II restriction site on 2 GAP promoters were needed. To mutated *Avr*II restriction site, the pGAP plasmid (Invitrogen, USA) was digested with *Avr*II restriction enzyme (NEB, USA) to generate linear fragment. The sticky-end fragment was full-filled in by addition of PCR components without any primers then incubated at 72°C for 30 min. Thereafter, the blunt end pGAP plasmids were joined together by T4 DNA ligase enzyme (NEB) according to manufacturer protocol (Figure 2.2). The *Avr*II-mutated called pGAP(M) plasmids were transformed into *Escherichia coli* (DH5α) competent cell using electroporator 2510 (Eppendorf) at 1,800 volts then selected on LB contained 50 ug/ml zeocin. pGAP(M) plasmids were extracted using Plasmid Miniprep Plus Purification Kit (GeneMark, Taiwan) then the mutation was confirmed using *Avr*II digestion and sequenced (Macrogen, Korea) using pGAPZ21_F and pGAPZ654_R primers (Table 1). The GAP promoter sequences between pGAP and pGAP(M) plasmids were compared using nucleotide blast (NCBI) program.

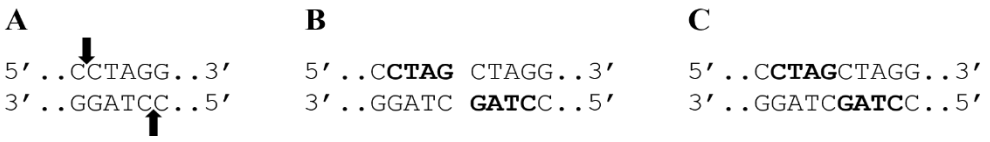

Figure 2.2 Schematic diagram to generate *Avr*II site mutation. A: *Avr*II site with digestion pattern; B: *Avr*II-digested filled in with complementary base indicated by bold; C: re-ligation of *Avr*II-mutated site.

Total RNA of zebrafish (*D. rerio*) and marine algae (*I. galbana*) were extracted then converted to cDNA using RNeasy MiniKit (QIAGEN, Germany) and iScript™ Reverse Transcription Supermix (BIO-RAD, USA), respectively. The primers for Δ2D, Δ6E, and Δ4D cDNAs amplifications were designed from GeneBank accession number NM_131645.2, NM_199532.1 and KF894987.1, respectively (Table 2.1). The Δ2D gene was amplified by primers Δ2Des_F and Δ2Des_R, and digested with *Eco*RI, and *Xho*I restriction enzymes then ligated into *Eco*RI/*Xho*I-digested pGAP plasmid (pGAP-Δ2D). The Δ6E gene was amplified by Δ6Elo_F and Δ6Elo_R and digested with *Pml*I and *Xho*I restriction enzymes thereafter cloned into *Pml*I/*Xho*I-digested pGAP(M) plasmid (pGAP(M)-Δ6E). The Δ4D gene was amplified by primers Δ4Des_F and Δ4Des_R then cut with *Kpn*I and *Sac*II then cloned into *Kpn*I/*Sac*II-cut pGAP(M) plasmid (pGAP(M)-Δ4D). The recombinant plasmids were transformed into *E. coli* competent cells using electroporation technique then cells were grown on LB contained 50 μg/ml zeocin and confirmed by colony PCR technique. The recombinant plasmids were extracted using Plasmid Miniprep Plus Purification Kit (GeneMark) then sequenced (Macrogen) using pGAPZ455_F and pGAPZ654_R primers. The nucleotide sequences of each gene were aligned with nucleotide sequences derived from GenBank. Amino acid sequences were aligned using Clustal Omega program then conserved regions were marked using UniPort database. The correct plasmids were selected for further experiments.

Table 2.1 Primers used in this study.

Primers	Sequences	Tm (°C)
pGAPZ21_F	5'-TCT-TGG-TGT-CCT-CGT-CCA-A-3'	56.7
pGAPZ654_R	5'-GTG-CCC-AAC-TTG-AAC-TGA-GG-3'	59.3
pGAPZ455_F	5'-GTC-CCT-ATT-TCA-ATC-AAT-TGA-A-3'	52.8
Δ2Des_F	5'-CC-GAA-TTC-ATG-GGT-GGC-GGA-GGA-CAG-CA-	71.0
Δ2Des_R	5'-CC-CTC-GAG-GAT-TTG-TTG-AGA-TAC-GCA-TCC-	72.0
Δ6Elo_F	5'-CC-CAC-TGA-ATG-TCG-GTG-CTG-GCT-TTG-CA-	71.0
Δ6Elo_R	5'-CC-CTC-GAG-GAT-TGG-CTT-TTC-TTG-GCT-GGC-	70.9
Δ4Des_F	5'-TGG-GGT-ACC-ATG-TGC-AAC-GCG-GCA-GTG-3'	69.6
Δ4Des_R	5'-TTT-CCG-CGG-TTA-TCC-GCC-TTG-AGC-GTC-TC-	69.1

Underline: restriction sites

2.3.2 Construction of *P. pastoris* containing Δ2D, Δ6E, and Δ4D genes

The pGAP-Δ2D plasmid was digested with *Bgl*II (Promega, USA) to generate linear fragment, whereas pGAP(M)-Δ6E was digested with both *Bgl*II (Promega) and *Bam*HI (Fermentas, USA) to release the fragment that contained *Bgl*II and *Bam*HI at the 5' and 3' ends, respectively. The linear fragment of pGAP-Δ2D and pGAP(M)-Δ6E were ligated together to construct pGAP-Δ2DΔ6E plasmid then transformed into *E. coli* competent cell by electroporation technique. Afterward, pGAP-Δ2DΔ6E plasmid was extracted and digested with *Bgl*II (Promega) to produce linear fragment, whereas pGAP(M)-Δ4D was digested with both *Bgl*II (Promega) and *Bam*HI (Fermentas) to generate fragment that contained *Bgl*II and *Bam*HI at the 5' and 3' ends, respectively. Then, both of pGAP-Δ2DΔ6E and pGAP(M)-Δ4D fragments were ligated together to generate pGAP-Δ2DΔ6EΔ4D plasmid (Figure 2.3).

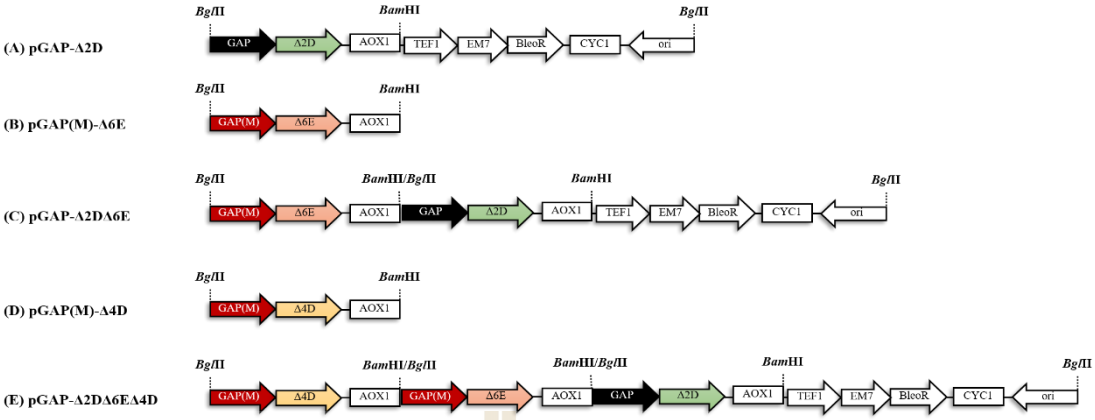

Figure 2.3 Schematic diagram for co-expression plasmid construction. (A) linear pGAP-Δ2D displaying *Bgl*II sites at the end of fragment; (B) linear pGAP(M)-Δ6E fragment showing *Bgl*II and *Bam*HI sites at the end of fragment; (C) linear pGAP-Δ2DΔ6E exhibiting *Bgl*II sites at the end of fragment; (D) pGAP(M)-Δ4D fragment presenting *Bgl*II and *Bam*HI sites at the end of fragment; (E) pGAP-Δ2DΔ6EΔ4D plasmid.

Subsequently, the co-expression plasmid was confirmed for the presence of 3 genes by *Bam*HI (Promega) digestion and PCR using pGAPZ455_F couple with the reverse primers specific to each gene. To integrate recombinant plasmids into yeast genome, the correct recombinant plasmid was digested with *Avr*II (NEB) to generate linear fragment then transformed into *P. pastoris* competent cell using electroporation technique at 1,500 volts. The transformed cells were plated on YPD contained 100 ug/ml zeocin. Then, the recombinant *P. pastoris* that harbored pGAP-Δ2DΔ6EΔ4D was confirmed by colony PCR technique using gene specific primers. Corrected clones were re-streaked on YPD contained vary concentration of zeocin (100-500 ug/ml) to predict a possibility of multiple copies inserts integrated into *P. pastoris* genome.

2.3.3 PUFAs content determination

To confirm the activity of these 3 genes, seed cultures were prepared by inoculation of single colony wild type and recombinant *P. pastoris* containing either empty

pGAP or pGAP-Δ2DΔ6EΔ4D into 5 ml YPD (1% yeast extract, 1% peptone, and 2% glucose) then incubated at 30°C with 200 rpm shaking for 2 days. Cells OD600 = 1 were transferred into 1 L flask with 250 ml working volume YPD medium then incubated at 30°C with 200 rpm shaking for 4 days. The 125 ml cultured cell was centrifuged and transferred to new 250 ml YPD medium then further cultured for 4 days as mentioned above. Thereafter, cultured cells were collected then washed twice with DI water and dried at 65°C for 2 day. The 80 mg of dried cell was added with 4 ml (5% w/v) KOH in methanol and 0.4 mg C13:0 (Sigma-Aldrich, USA) used as a catalyst and an internal standard, respectively. The samples were incubated at 80°C for 5 h then cooled down to room temperature and adjusted the pH to ~2 using conc. HCl. Two milliliters of BF_3 was added to the samples then incubated at 80°C for 90 min. The samples were cooled down to room temperature then 2 ml of DI water and hexane were added and vortex. Centrifugation at 3,000*xg* for 3 min was used to separate the fatty acid methyl esters (FAMEs) from the solution. The upper layer was collected and fatty acid compositions were determined using GC-7980A (Agilent Technology, USA) equipped with Supelco SPTM-2560 fused silica capillary column (100 m x 250 µm x 0.2 µm) (Sigma-Aldrich). Helium was used as carrier gas. The oven temperature was programmed to start at 70°C for 4 min then increased to 175°C at the rate of 13°C/min and maintain at 175°C for 27 min then increased to 215°C at the rate of 4°C/min and maintain at 215°C for 17 min and finally increased to 240°C at the rate of 4°C/min and maintain at 240°C for 10 min. FAMEs were identified by comparing with appropriate standard Supelco® 37 components FAME Mix (Sigma-Aldrich).

To investigate the influence of types and concentration of carbon sources for DHA production, recombinant *P. pastoris* was cultured in different media, each 125 ml of 4 days cultured cells previously mentioned were centrifuged at 4000*xg* for 10 min then transferred to new 250 ml YPD, YPD4% (1% yeast extract, 1% peptone, and 4% glucose), YPG (1% yeast extract, 1% peptone, and 2% glycerol) and YPG4% (1% yeast extract, 1%

peptone, and 4% glycerol) media then cultured at 30°C with 200 rpm shaking for another 4 days. Cells were collected then fatty acids were investigated.

2.3.4 Statistical analysis

All treatments were done in triplicate. Data were presented as mean ± SD. The statistical analysis was accomplished through SPSS using *t*-test for comparing between 2 groups and one-way ANOVA for more than 2 groups ($p = 0.05$).

2.4 Results and Discussion

2.4.1 Gene cloning and analysis

The cDNA of Δ2D, Δ6E, and Δ4D genes cloned from total RNA extracted from *D. rerio* and *I. galbana* were sequenced. The cloned Δ2D sequence was 1,329 bp which encoded 443 amino acids and displayed 99% identity against fatty acid desaturase 2 (fads2) zebrafish (NM_131645.2). The 1,278 bp in length of cloned Δ4D encoded 426 amino acids showed 99% identity with Δ4-fatty acid desaturase (FAD4) of *Isochrysis* sp. (JQ791105.1). Both of Δ2D and Δ4D amino acids consisted of N-terminal cytochrome *b5* containing haem-binding motif (H-P-G-G), trans-membrane regions and 3 histidine boxes (HXXXXH, HXXHH and QXXHH) which are highly conserved in membrane bound desaturases (Figure 2.4-2.5) (Lee *et al.*, 2016; Shi *et al.*, 2012; Zhu *et al.*, 2019).

Desaturase enzymes are divided into two types based on the insertion of double bonds e.g. front-end desaturase and methyl-end desaturase. The front-end desaturases (Δ2D and Δ4D) are enzymes that add a double bond between the pre-existing double bond and the carboxyl end of the fatty acid chain. The Δx desatuarse adds a double bond into the carbon position x from the carboxyl end of the fatty acid chain. Moreover, substitution of a histidine (H) by a glutamine (Q) in the third histidine box denotes it to be front-end desaturase (Domergue, 2002; Kang *et al.*, 2008; Sayanova *et al.*, 2001). Whereas, the methyl-end desaturases (ω3 desaturase, (ω3D)) insert double bond between the pre-existing double bond and the methyl-end of fatty acid chain. The

ωy desaturase adds double bond at the position y from the methyl end of the fatty acid chain (Meesapyodsuk and Qiu, 2012).

The cloned full-length 798 bp sequence of the Δ6E which coded 266 amino acids showed 99% similarity with fatty acid elongase 6 (elovl6) of zebrafish (NM_199532.1). The Δ6E amino acid contained important regions which are trans-membrane regions, a histidine (HXXHH) box which is act as active site of enzyme, a tyrosine (HXXMYXYY) box, and other 2 boxes (LFXXF and KXXEXXDT) which are highly conserve in elongase family (Figure 2.6) (Antoñanzas *et al.*, 2013; Guo *et al.*, 2019; Matsuzaka *et al.*, 2002; Zheng *et al.*, 2017). Pereira *et al.*, (2004) suggested that elongase is highly hydrophobic therefore it contains several trans-membrane domains. In this work, 7 trans-membrane domains were found in Δ6E.

Figure 2.4 Alignment of the Δ2D with desaturases from other organisms. Dotted line: N-terminal cytochrome *b5* region; Shaded: trans-membrane regions; Black frames: three histidine boxes; Dotted frame: heam-binding motif; "*" indicates identical amino acid, ":" conserved substitutions and "."semi-conserved substitutions.

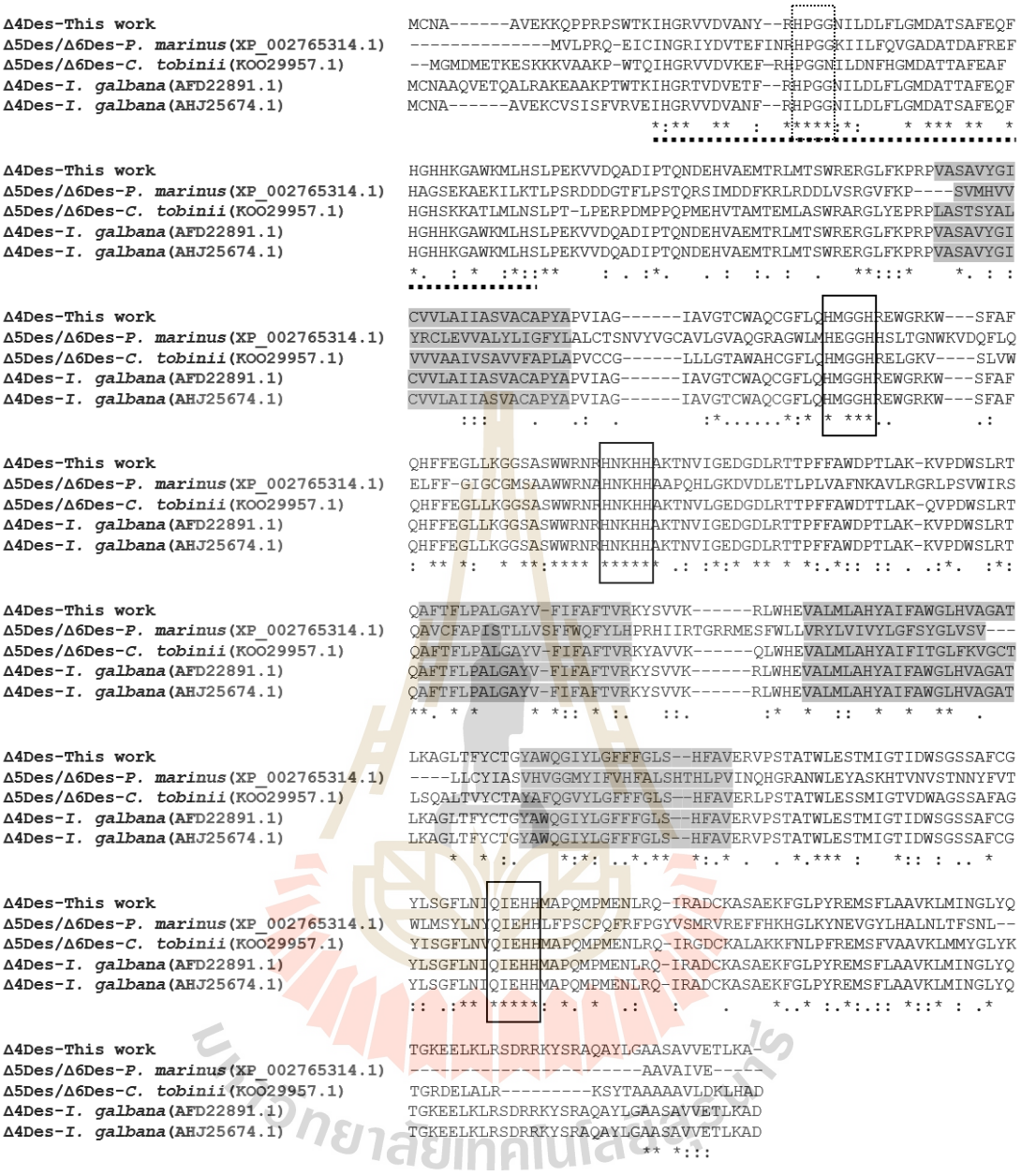

Figure 2.5 Alignment of the Δ4D with desaturases from other organisms. Dotted line: N-terminal cytochrome *b5* region; Shaded: trans-membrane regions; Black frames: three histidine boxes; Dotted frame: heam-binding motif; "*" indicates identical amino acid, ":" conserved substitutions and "."semi-conserved substitutions.

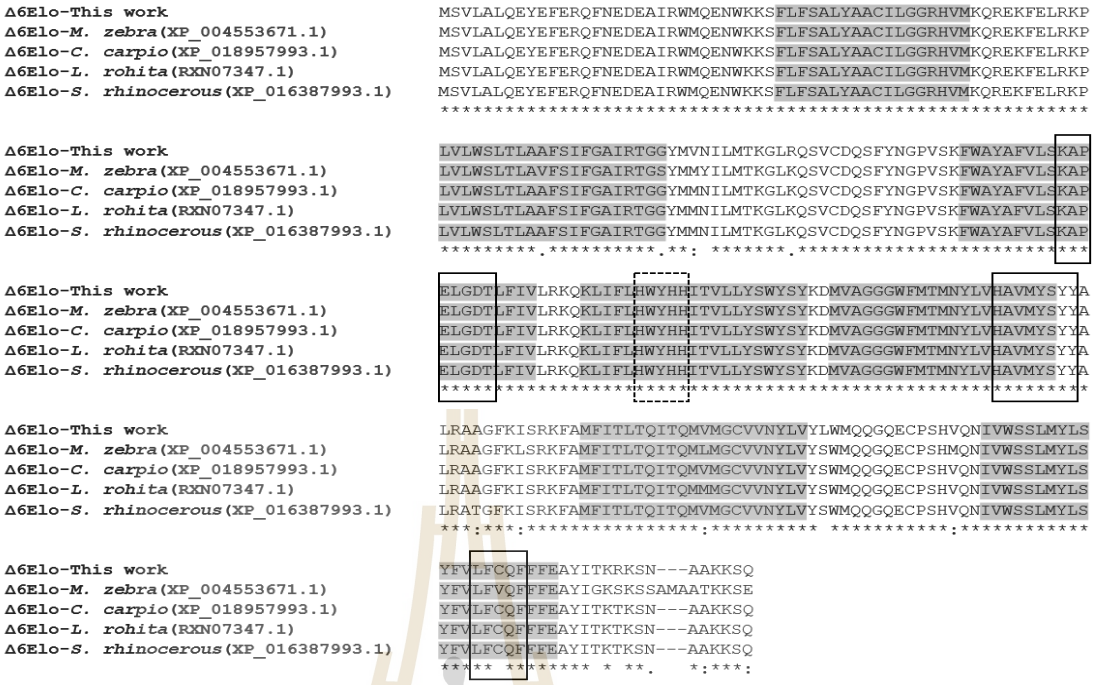

Figure 2.6 Alignment of the Δ6E with Δ6E from other organisms. Solid frames: a tyrosine and 2 other boxes; Dotted frame: a histidine box; Shaded: trans-membrane regions; "*" indicates identical amino acid; ":" conserved substitutions and "."semi-conserved substitutions.

2.4.2 Recombinant *P. pastoris* containing pGAPZ-Δ2DΔ6EΔ4D plasmid

To determine the function of Δ2D, Δ6E and Δ4D for DHA production, recombinant pGAPZ-Δ2DΔ6EΔ4D was constructed. To increase efficiency of gene integration into yeast genome, the recombinant plasmid and empty pGAP plasmid were digested with *Avr*II then separately transformed into *P. pastoris.* Target clones were confirmed by PCR technique using their specific primers and genomic DNA was used as template. The results showed all genes displayed expected size which are 1,329 bp, 798 bp and 1,278 bp for Δ2D, Δ6E and Δ4D, respectively (Figure 2.7). Recombinant *P. pastoris* was re-streaked on YPD plate containing high concentration of zeocin (500 ug/ml) to predict a possibility of multiple copies inserts integrated into *P. pastoris* genome as described in Arjmand *et al.*, (2013) work. Recombinant *P. pastoris* clone able to grow on 500 ug/ml of zeocin was selected for fatty acid investigation.

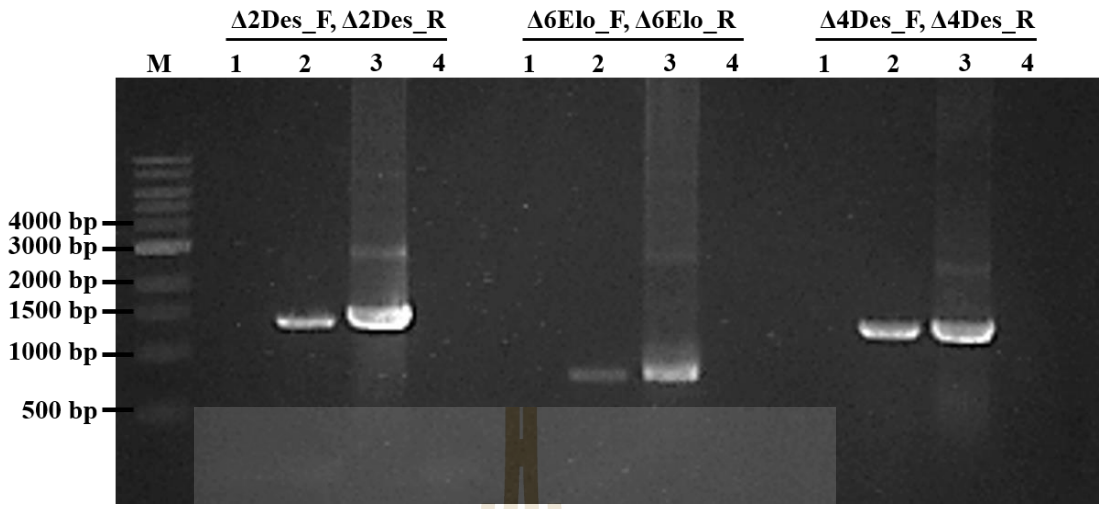

Figure 2.7 PCR of Δ2D, Δ6E and Δ4D genes. M: 1 kb marker; Lane 1: *P. pastoris* containing empty pGAP plasmid; Lane 2: *P. pastoris* containing pGAP- Δ2DΔ6EΔ4D; Lane 3: pGAP-Δ2DΔ6EΔ4D plasmid as positive control; Lane 4: negative control using water as template.

2.4.3 Fatty acid analysis

LA and ALA fatty acids, which are substrate for LCPUFAs productions in omega-6 and omega-3, are simultaneously synthesized by *P. pastoris* (Li *et al.*, 2009; Zhang *et al.*, 2005). To convert theses substrates to DHA, recombinant *P. pastoris* containing pGAP-Δ2DΔ6EΔ4D was generated. After cultivation through certain conditions, gas chromatogram results showed that recombinant *P. pastoris* containing pGAP-Δ2DΔ6EΔ4D demonstrated a novel peak of DHA when compared with FAME standard whereas none of this DHA peak was detected in both non-transformed and recombinant *P. pastoris* containing empty pGAP (Figure 2.8).

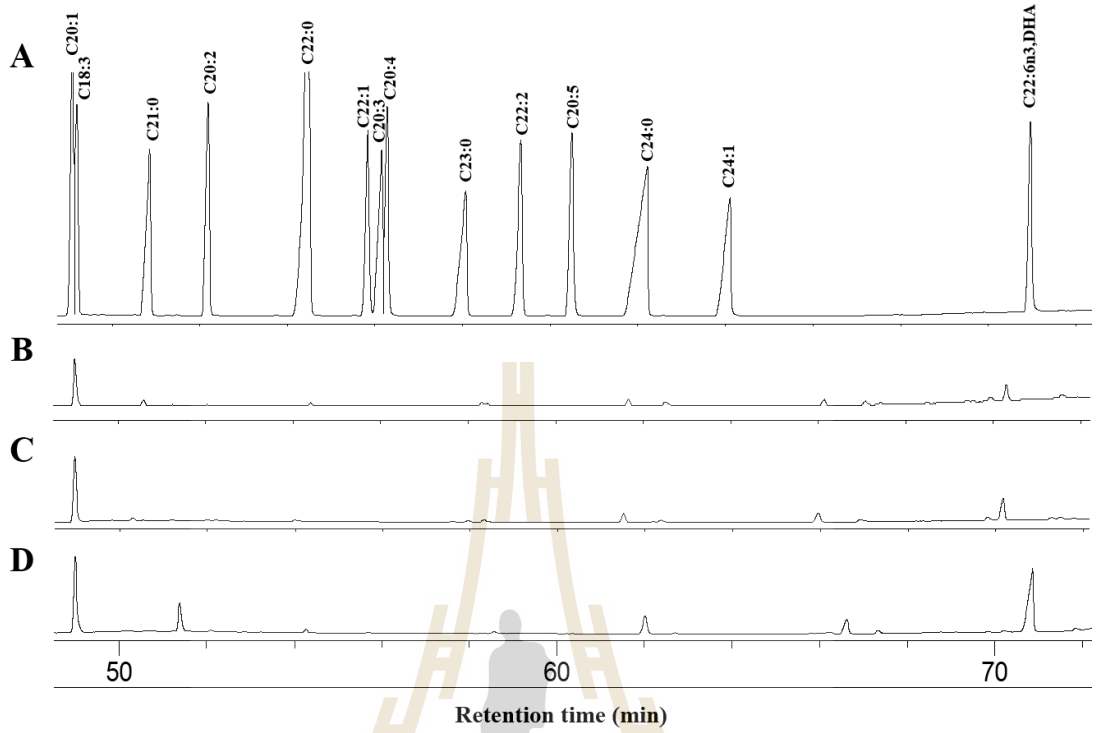

Figure 2.8 Fatty acids profile from gas chromatography. (A) Supelco® 37 components FAME Mix standard; (B) non-transformed *P. pastoris*; (C) *P. pastoris* containing empty pGAP; (D) *P. pastoris* containing pGAP-Δ2DΔ6EΔ4D.

Endogenous fatty acids of *P. pastoris* such as palmitic acid (C16:0, PA), palmitoleic acid (C16:1, PLA), stearic acid (C18:0, SA), oleic acid (C18:1n9, OA), and LA (C18:2n6) were observed among *P. pastoris* containing empty pGAP and pGAP-Δ2DΔ6EΔ4D (Table 2.2) which are similar to prior studies (Grillitsch *et al.*, 2014; Tehlivets *et al.*, 2007). C16:0, C16:1 and C18:0 were significantly higher in *P. pastoris* containing empty pGAP when compared with pGAP-Δ2DΔ6EΔ4D whereas C18:2n6 and C18:3n3 levels were lower. The C18:1n9 fatty acids revealed the highest amount of ~25 and ~27 mg/g DCW in *P. pastoris* containing empty pGAP and pGAP-Δ2DΔ6EΔ4D, respectively, which also found in study of Kajikawa *et al.*, (2004).

Table 2.2 Fatty acid composition (mg/g DCW) of *P. pastoris* containing empty pGAP and pGAP-Δ2DΔ6EΔ4D. Each value is the mean ± SD from triplicate experiments. Significant difference was calculated using Student's *t*-test method (*p<0.05).

Fatty acids	**YPD**	
	pGAP	**pGAP-Δ2DΔ6EΔ4D**
C16:0, PA	6.98±0.56*	4.82±0.1
C16:1, PLA	2.29±0.14*	1.20±0.01
C18:0, SA	2.52±0.28*	1.80±0.02
C18:1n9, OA	25.63±2.23	27.75±0.14
C18:2n6, LA	5.31±0.38	6.99±0.04*
C18:3n6, GLA	ND	0.50±0.00
C18:3n3, ALA	ND	0.03±0.05
C22:6n3, DHA	ND	0.02±0.03

ND: not detected; PA: palmitic acid; PLA: palmitoleic acid; SA: stearic acid; OA: oleic acid; LA: linoleic acid; GLA: γ-linoleic acid: ALA: α-linolenic acid: DHA: docosahexaenoic acid.

As we know, DHA is produced using ALA which is synthesized from LA and OA by Δ12D and Δ15D activities. Then, ALA is converted to STA by the Δ6D (Δ2D) then the STA is changed to ETA by the Δ6E. Next, the ETA converts to EPA by the Δ5D (Δ2D) then the EPA is further elongated to DPA by Δ5E (Δ6E). In the last step for DHA production, DPA is changed to DHA by Δ4D activity or β-oxidation process (Figure 2.1). In this work, to eliminate β-oxidation pathway shown in the last step for DHA production, Δ4D gene derived from marine microalgae were co-expressed with Δ2D, Δ6E genes derived from zebrafish. Genes functional have been investigated by works of Hasting *et al.*, (2001) showed Δ2D from zebrafish exhibited the bifunctional of Δ6D and Δ5D which converted substrates to correspond products. Shi *et al.*, (2012) and Guo *et al.*, (2013) identified and characterized the Δ4D activity derived from *I. galbana* and *I. sphaerica*, respectively, to convert DPA to

DHA. Although, the specific products of each enzyme were not elucidated in this work. However, the present of DHA could confirm the successful co-expression and co-functional of the enzymes involved in DHA biosynthesis in *P. pastoris*.

To increase the ability of recombinant *P. pastoris* containing pGAP-Δ2DΔ6EΔ4D for DHA production, recombinant yeast was cultured though either 2% and 4% of glucose- or glycerol-based media. Results demonstrated vast amount of C18:1n9 fatty acid from all media. Glucose-based media provided higher amount of C18:1n9 and C18:2n6 than glycerol-based media. Surprisingly, the highest DHA was detected in glycerol-based medium of 1.67 mg/g DCW. *P. pastoris* endogenously expresses Δ12D and Δ15D activity that able to convert C18:1n9 to C18:2n6 then C18:3n3. This work, the introduction of bifunctional Δ6D/Δ5D named Δ2D which are considered as rate-limiting step enzymes (Chuang *et al.*, 2015; Vagner and Ester, 2011) with Δ4D which directly converts DPA to DHA was accomplished in *P. pastoris*. Hasting *et al.*, (2001) work showed that the Δ2D from zebrafish preferred to convert fatty acids along omega-3 family. Moreover, glycerol and unsaturated fatty acids are indicated as precursors for neutral lipid production in yeasts (Duan *et al.*, 2015; Tang *et al.*, 2015) which was confirmed by work of Guo and Ota, (2000) and (Aoki *et al.*, 2002) that yeast incorporated EPA and DHA with glycerol to produce neutral lipid when presenting such fatty acids in culture medium. Therefore, these might be the reasons why decreasing of C18:1n9, C18:2n6, C18:3n3, non-presenting intermediate fatty acids in omega-3 family and increasing DHA (1.67 mg/g DCW) were obtained through glycerol-based condition (Table 2.3). Additionally, As shown in Table 2.4, this is the first time for the highest DHA production by genetic engineering in yeast *P. pastoris*. Moreover, such DHA is produced without adding exogenous inducers and substrates although its amount is lower than other microorganisms.

Table 2.3 Fatty acid composition (mg/g DCW) of *P. pastoris* containing pGAP-Δ2DΔ6EΔ4D when cultured in different media. Values are average of triplicate experiments and standard deviation which are presented in mean ± SD. Significant difference was calculated using one-way ANOVA (p<0.05) which is marked by different letters.

Fatty acids	**pGAP-Δ2DΔ6EΔ4D**			
	YPD	**YPD4%**	**YPG**	**YPG4%**
C16:0, PA	4.82±0.1	4.44±0.02	4.40±0.2	3.81±0.1
C16:1, PLA	1.20±0.01^{a}	1.20±0.02^{a}	0.51±0.02^{b}	0.47±0.01^{b}
C18:0, SA	1.80±0.02^{b}	2.84±0.00^{a}	3.05±0.12^{ab}	3.50±0.11^{a}
C18:1n9, OA	27.75±0.14^{ab}	30.99±0.08^{a}	21.52±1.31^{b}	19.16±0.14^{b}
C18:2n6, LA	6.99±0.04^{b}	9.28±0.01^{a}	5.44±0.31^{b}	4.62±0.08^{b}
C18:3n6, GLA	0.50±0.00^{b}	1.00±0.00^{a}	0.18±0.01^{b}	0.15±0.01^{b}
C18:3n3, ALA	0.03±0.05	ND	ND	ND
C22:6n3, DHA	0.02±0.03^{c}	0.57±0.00^{b}	1.67±0.04^{a}	1.40±0.05^{a}

ND: not detected; PA: palmitic acid; PLA: palmitoleic acid; SA: stearic acid; OA: oleic acid; LA: linoleic acid; GLA: γ-linoleic acid: ALA: α-linolenic acid: DHA: docosahexaenoic acid.

Table 2.4 Genetic engineering microorganisms for long-chain polyunsaturated fatty acid (LCPUFAs) production**.**

Organisms	Methodology	Condition	Prod	Titer	Ref.
Yeasts					
P. pastoris SMD1168H	-Expressed Δ2D, Δ6E and Δ4D genes	Flask/8 days	DHA	1.67 mg/g DCW or 17 mg/L or 3.9% TFA	This work
P. pastoris GS115 (*his4*)	-Expressed Δ5D, Δ6D and Δ6E genes -Added appropriate inducer	Flask/3 days	ARA EPA	0.3% TFA 0.1% TFA	(Li *et al.*, 2009)
Saccharomyces cerevisiae FS01699	-Expressed Δ9D, Δ12D, Δ6D, Δ5D, Δ6E and ω3desaturase genes -Added appropriate fatty acid substrates	Flask/2 days	ARA EPA	1.22%TL 0.5%TL	(Tavares *et al.*, 2011)
Yarrowia lipolytica ATCC 20362	-Expressed C16elongase, Δ12D, Δ9E, Δ8D, Δ5D, Δ17D and cholinephosphotransferase genes	Flask/6 days	EPA	56.6% TFA	(Xue *et al.*, 2013)
Y. lipolytica Po1f	-Expressed Δ9E, Δ8D and Δ5D genes	Flask/6 days	ARA	0.56% TFA	(Liu *et al.*, 2019)
Trichosporon oleaginosus ATCC 20509	-Expressed Δ9E, Δ12D/ ω3desaturase and linoleic acid isomerase genes	Flask/7 days	EDA ETA	9.7% TFA 8.9% TFA	(Görner *et al.*, 2016)
Others					
Mortierella alpina CCFM 501	-Expressed ω3desaturase gene -Added appropriate fatty acid substrates	Flask/7 days	EPA	31.5% TFA	(Tang *et al.*, 2018)
M. alpine ATCC 32222	-Expressed Δ17D gene	Flask/7 days	EPA	33.44% TFA	(Ge *et al.*, 2018)
Aurantiochytrium limacinum mh0186	-Expressed Δ5D gene -Added appropriate inducer and fatty acid substrates	Flask/3 days	EPA	2.85% TFA	(Kobayashi *et al.*, 2011)
Schizochytrium sp.	-Expressed malic enzyme and ELO3 genes	Flask/3 days	DHA	3.54 g/L	(Wang *et al.*, 2019)
Escherichia coli DH5α	-Expressed cluster of pfaA, pfaB, pfaC, pfaD and pfaE genes -Added appropriate inducer	Flask/2 days	DHA	2.2 mg/g DCW or 4.1% TFA	(Peng *et al.*, 2016)

*DHA: docosahexaenoic acid (C22:6n3); ARA: arachidonic acid (C20:4n6); EPA: eicosapentaenoic acid (C20:5n3); EDA: eicosadienoic acid (C20:2n6); ETA: eicosatrienoic acid; TFA: total fatty acid; TL: total lipid.

2.5 Conclusion

The Δ2D, Δ6E and Δ4D genes derived from zebrafish and marine microalgae were cloned, sequenced and analyzed. The Δ2D, Δ6E and Δ4D amino acids displayed conserved regions which represent among desaturase and elongase families. DHA biosynthesis was accomplished through introduction of pGAP-Δ2DΔ6EΔ4D into *P. pastoris* genome and its GC results demonstrated high amount of DHA of 1.67 mg/g DCW though glycerol-based medium. To our knowledge, this is the first study that co-expressed genes to constitutively produce DHA in *P. pastoris.* No inducers and exogenous substrates were needed since constitutive GAP promoter was used and both LA and ALA are endogenously produced by *P. pastoris.* Resulting in easier for large scale fermentation and cost effective. Since, yeasts are ability to consume various by-products and waste materials. Therefore, in the future, *P. pastoris* will be attempted to culture using such materials as carbon sources then extracted DHA could be applied for food additives, cosmetic and pharmaceutical industries. Additionally, this platform can be applied in oleaginous yeasts which are able to produce and accumulate large amount of DHA.

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CHAPTER III

THE POTENTIAL OF OLEAGINOUS YEAST *Rhodotorula paludigena* CM33 FOR BIOFUEL PRODUCTION

3.1 Abstract

Growing environmental pollutions, diminishing fuel-derived petroleum, and competing between food- and fuel-derived plant oils and animal fats stimulate the finding of alternative feedstocks for biofuels production. In this work, oleaginous yeasts were screened using Nile Red (NR) fluorescence dye staining coupled with flow cytometry techniques. NR emission was calculated as relative Mean Fluorescence Intensity (MFI) value which showed the highest in strain CM33. This value correlated with the highest lipid content of 20.75% dry cell weight (DCW) which was detected in strain CM33 when compared with others. Afterward, CM33 was identified as *Rhodotorula paludigena* by sequencing the ITS-5.8S-ITS rDNA and D1/D2 26S rDNA regions then its whole genome was done and sequences were deposited on NCBI database. The potential to assimilate various carbon sources of CM33 was performed by minimal medium using glucose, glycerol, sucrose or xylose as carbon sources. CM33 grown in glucose-based medium provided the maximum lipid content of 23.9% DCW after 4 days cultured. The major fatty acids were C16:0 and C18:1. Biomass, lipid content and lipid concentration increased by about 16.5 g/L, 37.1% DCW and 6.1 g/L, respectively when CM33 grown in molasses-based medium. These results demonstrated that *R. paludigena* CM33 has the potential to maintain the carbon value chain by converting renewable waste materials for biolipid production.

3.2 Introduction

Several problems from petroleum-derived fuel usage lead to the development of biofuel feedstocks. However, the use of plant oils and animal fats, which are the major substrates for biofuel production, give rise to various difficulties such as requirements of labors, land, time, and the productivity depends on season and climate. Microorganisms can be an alternative source to produce biofuel because of its numerous advantages such as shorter life cycle, less labor requirement, less effect from season and climate, easier to scale up, continuous production, less requirement of cultivation (Li Q. *et al.*, 2008; Sitepu *et al.*, 2014) and suitable condition lead to high accumulation of lipid content (Sant'Annal *et al.*, 2014). Furthermore, the important advantage of microorganisms is their ability to recycle by-products and waste materials to high value biochemicals and biofuels (Bommareddy *et al.*, 2015).

Various types of microorganisms such as microalgae, bacteria, fungi and yeasts are able to produce lipid (Koutinas and Raranikolaou, 2011; Sitepu *et al.*, 2014). Especially, oleaginous yeasts which have been known to accumulate lipid ≥20% of the DCW. Oleaginous yeasts have more advantages than other microorganisms e.g. cultivations are independent of climate, consume various types of carbon and nitrogen substrates, high resistant to low oxygen condition and toxicity of by-products, high cell density with lacking endotoxin and widely genetic engineering knowledges (Abghari and Chen, 2014; Bommareddy *et al.*, 2015; Chatzifragkou *et al.*, 2011; Kraisintu *et al.*, 2010; Yen *et al.*, 2012). Oleaginous yeasts can be screened from natural sources by several methods (Gao *et al.*, 2012; Kameda *et al.*, 2014; Sant'Annal *et al.*, 2014), especially, lipid staining with fluorescence dyes such as BODIPY^{TM} 505, Nile Red (NR) fluorescence dye, Sudan Black B and Oil Red O (Kameda *et al.*, 2014; Kitchaa and Cheirsilp, 2011; Pan *et al.*, 2009; Rostron *et al.*, 2015; Sitepu *et al.*, 2014).

Numerous genus have been identified as oleaginous yeasts such as *Rhodotorula*, *Rhizopus*, *Trichosporon*, *Yarrowia*, *Lipomyces and Rhodosporidium* (Beopoulos *et al.*,

2009). Some genus such as *Lipomyces* sp., *Rhodosporidium* sp. and *Rhodotorula* sp. are able to accumulate lipid up to 70% of DCW (Chang *et al.*, 2011; Sitepu *et al.*, 2014). Triacylglycerol (TAG) and steryl ester (SE) are the major neutral-lipid while glycolipids, phospholipids and free fatty acids are small fractions that accumulate in oleaginous yeasts (Abghari and Chen, 2014). Fatty acid profiles of oleaginous yeasts are C16 and C18 e.g. palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) which are comparable to plant lipid composition (Koutinas and Raranikolaou, 2011). In addition, lipid accumulation of oleaginous yeasts can be increased via genetic engineering of genes involved in lipid metabolism (Sheng and Feng, 2015; Tai and Stephanopoulos, 2013; Xue *et al.*, 2013; Zhang *et al.*, 2016; Zhou *et al.*, 2014), controlling culture condition such as trace element (Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}) concentration, C/N ratio, pH, temperature and oxygen (Chang *et al.*, 2011; Kolouchová *et al.*, 2016a; Li Q. *et al.*, 2008; Saxena *et al.*, 2014; Sitepu *et al.*, 2014).

To evaluate a potential of oleaginous yeast to be used as microbial cell factories, several factors must be considered for example; amount of lipid production, fatty acid profiles and ability to use cheap materials as nutrition source (Thevenieau and Nicaud, 2013). Moreover, production of lipid and fatty acids are influenced by yeast strain and environmental. Therefore, research for oleaginous yeasts to produce high lipid and fatty acid are still needed. In this study, oleaginous yeasts were screened from Castor (*Ricirus* sp.) beans using NR fluorescence dye staining couple with flow cytometry techniques then relative MFI values derived from NR emission were compared. Lipids were extracted from selected yeasts. The ability of the highest lipid accumulation strain (*Rhodotorula paludigena* CM33) to consume glucose, glycerol, sucrose, and xylose was evaluated. The potential of *R. paludigena* to utilize by-products as carbon sources was accomplished through cultivation on molasses- and crude glycerol-based media. Biomass, lipid and fatty acids contents were detected to determine the potential of this yeast for biofuel production.

3.3 Materials and methods

3.3.1 Yeast isolation

Yeast isolation method was modified from Lee *et al.*, (2011). Briefly, Castor beans were grinded and then resuspended with 2 ml DI water. One tenth of a milliliter from each dilution of 1 in 10 and 1 in 100 diluted samples was incubated with 100 ul of 200 mM lysozyme at room temperature for 10 min. Then, 20 ul was spread on glucose-rich YPD agar (1% yeast extract, 2% peptone, 4% glucose and 1.3% agar) contained 50 ug/ml chloramphenicol then incubated at 30°C for 2 to 3 days. Yeast colonies were picked and purified by cross streaking on YPD agar (1% yeast extract, 2% peptone, 2% glucose and 1.3% agar). Yeast morphologies were identified under microscope and maintained on YPD agar at 4°C prior to use.

3.3.2 Oleaginous yeast screening

To primary screen oleaginous yeasts that accumulate high amount of lipid and fatty acids, specific lipid staining of Nile red (NR) coupled with flow cytometry method were used following the modified method of Kameda *et al.*, (2014) and Sitepu *et al.*, (2012). In brief, after 4 days of culture in YPD medium, aliquots of cell samples (250 ul of OD600 of 1) were treated with 5 ug/ml of NR fluorescence dye (Sigma-Aldrich, USA) for 10 min at 37°C in the dark. NR fluorescence emission was investigated using BD FACSCalibur™ equipped with an air-cooled argon laser (BD Biosciences, USA). Excitation at 488 nm of argon laser resulting in NR fluorescence emission at 580 and 610 nm for neutral and polar lipids, respectively, which can be detected by FL2 (590±25 nm) and FL3 (675±20 nm) channels. A total of 10,000 cells were gated and analyzed for each sample. The data analysis was performed afterwards with FlowJo software version 7.6.1. The relative Mean Fluorescence Intensity (MFI), which was derived from NR emission level, of each cell was compared with their unstained cells. The highest and some other various levels of relative MFI cells were selected for further analysis.

3.3.3 Oleaginous yeast strain identification

The selected oleaginous yeast strains were identified based on its Internal Transcribed Spacer, 5.8S rDNA (ITS-5.8-ITS rDNA) and D1/D2 domain of the 26S rDNA sequences (de Llanos Frutos *et al.,* 2004). Genomic DNA extraction and amplifications were carried out as described previously (Looke *et al.*, 2011). The ITS-5.8S-ITS and D1/D2 variable domains were amplified from the genomic DNA using primer sets in Table 3.1. The PCR product was ligated into pTG19-T cloning plasmid (Vivantis, Malaysia) and sequenced by Macrogen Inc. (Korea). The obtained sequences were BLAST searched against NCBI database.

Table 3.1 Primers used in this study

Primers	Sequence	Tm (°C)
ITS1_F	5'-TCC-GTA-GGT-GAA-CCT-GCG-G-3'	61.88
ITS4_R	5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3'	55.75
NL1_F	5'-GCA-TAT-CAA-TAA-GCG-GAG-GAA-AAG-3'	58.55
NL4_R	5'-GGT-CCG-TGT-TTC-AAG-ACG-G-3'	59.72

3.3.4 Whole genome sequence of *R. paludigena* CM33

Genomic DNA of CM33 was extracted from a 5 ml overnight culture via the Wizard kit (Promega, WI, USA) following the manufacturer's protocols. DNA was quantified by a Qubit assay with the high-sensitivity kit (Life Technologies, CA, USA) at 0.2 ug/ul. The DNA was sequenced on an Illumina HiSeq 2000 platform using 2×150-bp paired-end reads at Novogene Bioinformatics Technology Co., Ltd (Hong Kong). Low-quality nucleotides (*Q* value of ≤38) exceeding 40 bp, N-nucleotides exceeding 10 bp, and reads overlap with adapter exceeding 15 bp were removed according to the quality control pipeline of Novogene. Low-quality paired-end and adapter sequences were eliminated with in house program of Novogene. After adapter filtering and quality trimming, *de novo* assembly of high-quality clean reads were performed via SOAPdenovo v2.04 (Li R. *et al.*, 2008; Li *et al.*, 2010). Protein-coding genes and functionally annotated were predicted using PFAM, Gene

Ontology (GO) (Ashburner *et al.*, 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa *et al.*, 2006; Kanehisa *et al.*, 2004), Clusters of Orthologous Groups (COG) (Tatusov *et al.*, 2003) and Non-Redundant protein databases (NR) (Li *et al.*, 2002) were done by Novogene company.

3.3.5 *R. paludigena* CM33 under various carbon sources

Inoculums were prepared by inoculation of 1-full loop cell into 50 ml YPD broth and incubated overnight at 30°C on a rotary shaker at 200 rpm. The OD600 = 0.5 inoculums were then added to 1 L Erlenmeyer flasks containing 250 ml minimal medium (g/L of 0.75 g yeast extract, 0.55 g $(NH_4)_2SO_4$, 0.4 g KH_2PO_4, 2.0 g $MgSO_4 \cdot 7H_2O$ and 2% each of glucose, glycerol, sucrose and xylose) adapted from Poontawee *et al.*, (2017) work then cultured at 30°C, 200 rpm. Every day, the OD600 values were measured to evaluate cell growth. In the meantime, glucose, glycerol, sucrose and xylose concentrations in the culture medium after separation of cells were analyzed by high-performance liquid chromatography (HPLC) (Agilent Technology 1200 series, Germany), which equipped with an RI detector, a HPX-87H column. H_2SO_4 0.4 mM was used as mobile phase. After culturing for 4 days, the cells were harvested for lipids content and fatty acid compositions analysis.

Since, glucose, sucrose and glycerol are found in by-products e.g. molasses and crude-glycerol. Therefore, to increase a potential of oleaginous yeast to produce high value molecules by recycling valueless substrates, molasses and crude glycerol obtained from Suranaree University and Technology (SUT) farm and Biosynergy company limited, Nakhon Ratchasima, Thailand, were used as carbon sources. Sucrose, fructose, glucose and glycerol contents were determined by HPLC. Sugars and glycerol peaks were identified based on retention time then concentrations were calculated against standard curve prior prepared. Molasses- and crude glycerol-based media were prepared in the minimal medium without removal of precipitation. Concentration of such carbon sources were varied at 5%, 10%, 15% and 20%. The pH was adjusted to 6.5 then autoclaved. Yeast was inoculated at OD600 of 0.5

in 1 L Erlenmeyer flask with working volume 250 ml then cultured for 7 days at 30°C, 200 rpm.

3.3.6 Lipid extraction and fatty acid composition analysis

Yeast cells were harvested by centrifuge at 4,000 rpm for 10 min and washed with sterile water twice then centrifuged again. The cells were dried at 65°C until a constant weight was achieved. Dry biomass was ground as powder then 300 mg DCW was mixed with 50 ml 2:1 chloroform:methanol then incubated at 30°C and 200 rpm overnight (Bonturi *et al.*, 2015). The sample was added with 10 ml DI water then shake vigorously. Lower layer was evaporated then the lipid weight was measured. After that, fatty acids were methylated by modifying the method of Qiao *et al.*, (2015). Briefly, 25 mg lipid was mixed with 2 ml BF3 in methanol (12%) (Sigma-Aldrich, USA) and 0.2 mg/ml C13:0 (Sigma-Aldrich, USA) then incubated at 80°C for 3 h. Samples were cooled to room temperature then added 1 ml each n-hexane and DI water. The resultant fatty acid methyl esters (FAMEs) were collected then detected using GC-7980A (Agilent Technology, USA) installed with Supelco SPTM-2560 fused silica capillary column (100 m x 250 μm x 0.2 μm) (Sigma-Aldrich). Helium was used as carrier gas. The oven temperature was programmed to start at 70°C for 4 min then increased to 175°C at 13°C/min for 27 min then increased to 215°C at 4°C/min for 17 min and finally increased to 240°C at 4°C/min for 10 min. FAMEs were identified by comparing with appropriate standard Supelco® 37 components FAME Mix (Sigma-Aldrich). The fatty acid contents were calculated as percentage total lipid (TL).

3.3.7 CRISPR/Cas9 for elongase (ELO) genes knockout

Oleaginous yeasts normally contain high amount of C16 and C18 fatty acids, which are unsuitable for bio-jet fuel production. Thereby, genetic engineering tools will be used for lipid profile modification. This experiment, CRISPR/Cas9 system pML104 plasmid (Figure 3.1), which is simple plasmid for genome editing in *Saccharomyces cerevisiae*, was applied to knockout elongase (ELO) genes.

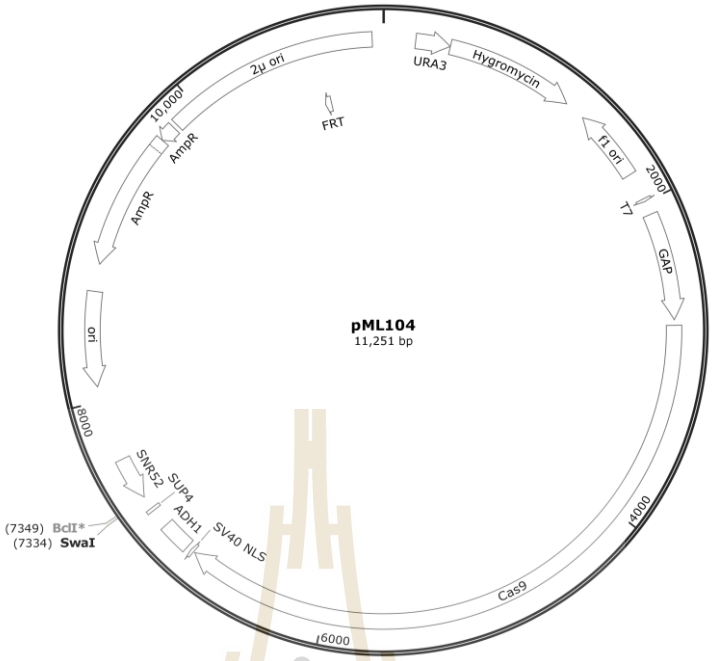

Figure 3.1 pML104 plasmid map**.**

3.3.7.1 CM33 competent cell preparation

To prepare fresh competent cells, single colony was inoculated into 5 ml YPD then incubated at 30°C 200 rpm overnight. Two percentage overnight cultured cell was transferred into 50 ml YPD then incubated 30°C 200 rpm for 3 h. Cells were centrifuged at 4,000 rpm then resuspended with 20 ml solution containing 10 ml 1M LiAC, 1 ml 10x TE buffer and 9 ml DI water. Cells were incubated at 25°C with 90 rpm for 45 min then 250 ul of 1M dithiothreitol (DTT) was added and incubated for 15 min. Cells were centrifuged at 4,000 rpm at 4°C for 10 min then twice washed with each of cool DI water and 1M sorbitol. Cells were resuspended with 1M sorbitol then 200 ul competent cells were kept at 4°C until used.

3.3.7.2 gRNA constructions for knockout elongase genes

To produce short-chain fatty acids, elongase (ELO) genes were attempted to knockout. There were fatty acid elongase 1 and 2 which are specific to extend medium- and long-chain fatty acids. In this study, numerous gRNA fragments which complemented to target genes were generated.

(1) To remove whole fatty acid elongase 1 (ELO1) gene, gRNA fragments were designed based on exon 1 and 4 sequences of ELO1 gene (accession number EMS18352.1) derived from *Rhodosporidium toruloides* (accession number KB722681.1) using CRISPRdirect program (Naito *et al.*, 2014). The high score gRNA sequences which related with specificity to target sequence were chosen. Designed gRNA complementary sequences were shown at Figure 3.2.

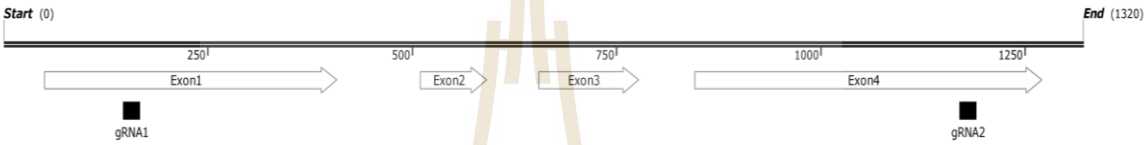

Figure 3.2 Schematic for gRNA complementary with exon 1 and 4 sequences of ELO1 gene. Black boxes: gRNA binding sites.

gRNA11 was annealed with gRNA12 fragments while gRNA21 was annealed with gRNA22 fragments (Table 3.2). Annealing program was started at 95°C then temperature was decreased 1°C per cycle with 10 sec. holding. Thereafter, fragments were ligated into *Bcl*I/*Swa*I digested pML104 plasmid (Figure 3.1) to produce pML104-gRNA1 and pML104-gRNA2 plasmids. Then, plasmids were transformed into *E. coli* (DH5α) using electroporation technique at 1,800 volts and selected using LB agar containing 50 ug/ml ampicillin. Corrected clones were checked by colony PCR technique using CheckgRNA_F with either gRNA12 or gRNA22 for pML104-gRNA1 and pML104-gRNA2, respectively. Recombinant plasmids were extracted then sequenced by Macrogen Inc. (Korea) using CheckgRNA_F and CheckgRNA_R primers. The 2 ug either pML104-gRNA1 or pML104-gRNA2 plasmids were transformed into *R. paludigena* CM33 competent cells using electroporation technique at 700 volts. Transformed cells were selected on YPD agar containing 50 ug/ml each of chloramphenicol and hygromycin. Knockout ELO1 gene of CM33 was confirmed through colony PCR technique using ELOKO2_F and ELOKO2_R

primers. After confirmations, it was not successful which might be non-complementary between gRNA and target gene because of such gRNA fragments were designed using ELO1 gene derive *R. toruloides* as template. Then, whole genome sequencing of CM33 was done then gene functionals were predicted.

Table 3.2 gRNA sequences used in this study.

gRNA	Sequences	Tm (°C)
gRNA1		
gRNA11	5'-TCGCTTCGACCTACGACCTAT-3'	57.58
gRNA12	5'-GATCATAGGTCGTAGGTCGAAGCGA-3'	60.12
gRNA2		
gRNA21	5'-TTGACGTACCTCTTCGTCGCT-3'	58.39
gRNA22	5'-GATCAGCGACGAAGAGGTACGTCAA-3'	60.77
CheckgRNA_F	5'-CTCGAAGACATAAAAAACAA-3'	44.42
CheckgRNA_R	5'-TTCTGTTCAAAAGATTTTGG-3'	44.91
ELOKO2_F	5'-AGCGGCGTCGAGGAGTCTGGCGATG-3'	70.38
ELOKO2_R	5'-GGGAAAGCGACGGACTACGGAGAGG-3'	66.07

(2) To remove whole fatty acid elongase 2 (ELO2) gene**,** exon 1 and 7 sequences of ELO2 gene derived from whole genome sequence of *R. paludigena* CM33 were used for designing gRNA fragments though CRISPRdirect program (Figure 3.3). gRNA sequences shown in Table 3.3 were used for pML104-gRNA1 and pML104-gRNA2 plasmids construction according to methods mentioned earlier then transformed into *R. paludigena* CM33 competent cells. Colony PCR technique was applied for confirmation ELO2 gene knockout using CgRNARP_F and CgRNARP_R primers. After confirmations, it was not successful which might be non-complementary between gRNA and target gene or large size of gene.

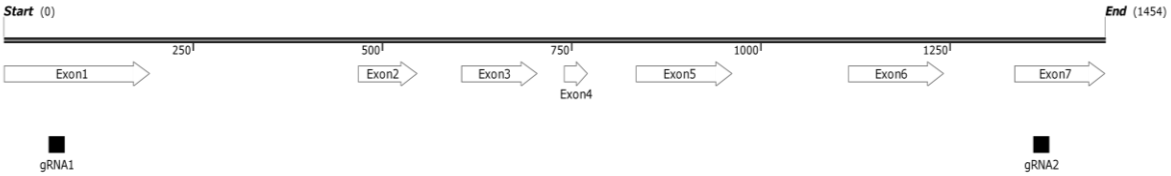

Figure 3.3 Schematic for gRNA complementary with exon 1 and 7 sequences of ELO2 gene. Black boxes: gRNA binding sites.

Table 3.3 gRNA sequences used in this study.

gRNA	Sequences	Tm (°C)
gRNA1		
gRNA1RP_F	5'-GATCCCCCTACAACATCGAGCACT-3'	61.09
gRNA1RP_R	5'-AGTGCTCGATGTTGTAGGGG-3'	57.18
gRNA2		
gRNA2RP_F	5'-GATCCTTCTACCAGAAGACGTACA-3'	55.43
gRNA2RP_R	5'-TGTACGTCTTCTGGTAGAAG-3'	50.50
CgRNARP_F	5'-ATGGCTCCTCCTCCCGGCCC-3'	68.82
CgRNARP_R	5'-CTTAAACTGCGGGAGTGCGG-3'	59.57

(3) To destroy functional of fatty acid elongase (ELO1) gene, gRNA fragments were designed using exon 2 and 3 sequences of ELO1 gene derived from whole genome sequence of *R. paludigena* CM33 (Figure 3.4). The gRNA was generated using CRISPRdirect program. gRNA sequences shown in Table 3.4 were used for pML104-gRNA1 and pML104-gRNA2 plasmids construction according to methods mentioned earlier then transformed into *R. paludigena* CM33 competent cells. Colony PCR technique was utilized for confirmation ELO1 gene knockout using ELO1641bp_F and ELO1641bp_R primers. However, it was not successful which might be low efficiency of CRISPR/Cas9 system in natural screened yeast. Therefore, to increase knockout efficiency, donor plasmid and DNA fragments were constructed.

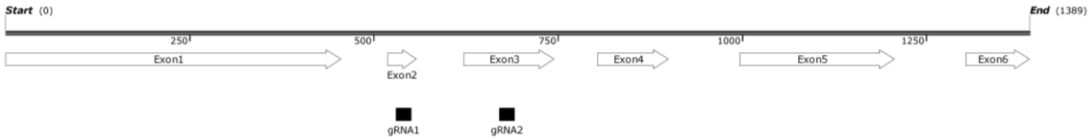

Figure 3.4 Schematic for gRNA complementary with exon 2 and 3 sequences of ELO1 gene. Black boxes: gRNA binding sites.

Table 3.4 gRNA sequences used in this study.

gRNA	Sequences	Tm (°C)
gRNA1		
ELO1gRNA1_F	5'-GATCACGGCCATTCTCCTTCTCAA-3'	58.9
gRNA1RP_R	5'-TTGAGAAGGAGAATGGCCCT-3'	56.3
gRNA2		
gRNA2RP_F	5'-GATCGTACTCGGCCTACGTGACGC-3'	62.9
gRNA2RP_R	5'-GCGTCACGTAGGCCGAGTAC-3'	60.1
ELO1641bp_F	5'-CGACTCGTCGTTCTCGATCTGGTCG-3'	62.3
ELO1641bp_R	5'-AGAGGTACGAGGCGGCGAGGGAGCC-3'	69.6

(4) To construct pGAP-GFP donor plasmid and DNA fragment, pET32-GFP plasmid previously constructed by lab's postdoc was digested with *Hind*III and *Not*I then ligated into *Hind*III/*Not*I cut pGAP plasmid. Recombinant plasmid was transformed into *E. coli* using electroporation technique then selected on LB agar containing 50 ug/ml zeocin and corrected clones were confirmed by colony PCR technique using pGAP_F: 5'-GTCCCTATTTCAATCAATTGAA-3' and pGAP_R: 5'-AAGTGCCCAACT TGAA-3' primers. Then, plasmids were extracted and sequenced.

To construct donor pGAP-GFP plasmid, 549 bp and 658 bp fragments which are located up and down, respectively, of PAM sequence of gRNA1 derived from previous experiment, were amplified using BstBIE1UP_F; 5'-CTTCGAAATGGAC TACGAGATGTTCGCCGC-3'; HindIIIE1UP_R: 5'-CAAGCTTTGAGAAGGAGAATG CCGTG-3' and NotIE1DOWN_F: 5'-CGCGGCCGCAGGGAAAGAAGGTG CGCACG-3'; ApaIE1DOWN_R: 5'-CGGGCCCTGAGAGGGACAAGGTAGATGC AG-3' primers,

respectively. *Bst*BI/*Hind*III digested 549 bp fragment was ligated with *Bst*BI/*Hind*III cut pGAP-GFP to construct pGAP-549upGFP plasmid. Thereafter, *Not*I/*Apa*I digested 658 bp fragment was cloned into *Not*I/*Apa*I cut pGAP-549upGFP to generate pGAP-549upGFP658 down plasmid which was utilized as donor plasmid (Figure 3.5). Such recombinant plasmid was digested with *Apa*I restriction enzyme to generate linear fragment which was used as donor DNA fragment. pML104-gRNA1 plasmid with either donor plasmid or DNA fragment were transformed into *R. paludigena* CM33 competent cells using electroporation technique then clones were selected on YPD containing 50 ug/ml each of chloramphenicol and hygromycin. Unfortunately, it was not successful after several transformation.

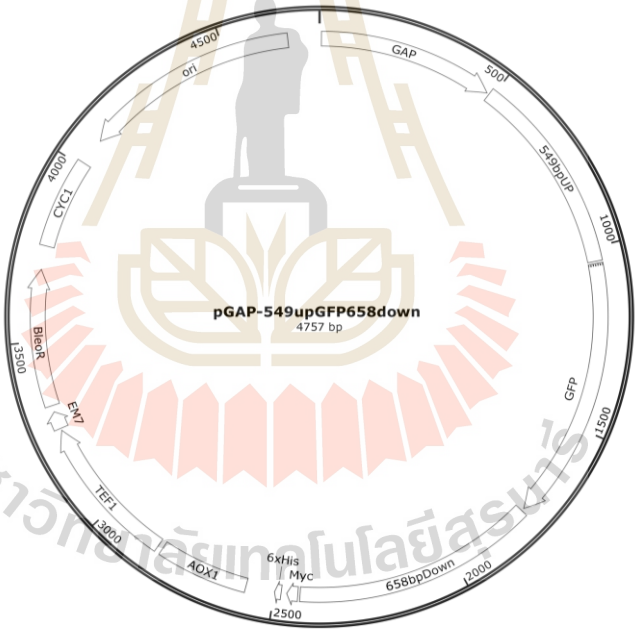

Figure 3.5 pGAP-549upGFP658down plasmid map.

However, the contamination with *Saccharomyces cerevisiae* which displayed green fluorescence when detected by UV light was found in this experiment. Since, this pML104 plasmid also have been designed for genome editing of *S. cerevisiae*. Therefore, it concluded the CRISPR/Cas9 pML104 plasmid derived from *S. cerevisiae* is not suitable to knockout genes in *R. paludigena* CM33.

3.4 Results and Discussion

3.4.1 Oleaginous yeast screening and identification

Lipid content of yeasts detection could be immediately accomplished by solvent-dependent extraction however there is huge problem when applying to numerous yeast samples. Nile Red (NR) fluorescence dyes or 9-dimethylamino-5H-benzo[α]phenoxazine-5-one have been reported to dissolve in lipid but poor solubility in water (Greenspan *et al.*, 1985) and can provide fluorescent when activate with argon laser. It has been widely used to detect intracellular lipid in several microorganisms such as microalgae (Bertozzini *et al.*, 2011; Halim and Webley, 2015; Siegler *et al.*, 2012), yeasts, fungi (Jacquier *et al.*, 2011; Kimura *et al.*, 2004; Kobae *et al.*, 2014) and bacteria (Mrunalini and Girisha, 2017). Couple with flow cytometry technique at 488 nm excitation using argon laser, NR fluorescence is emitted at 580 nm (FL2 channel) and 610 nm (FL3 channel) which according to neutral and polar lipids, respectively (Kameda *et al.*, 2014). In this work, 67 yeast strains that isolated from Castor beans were stained with NR fluorescence dye then 10,000 cells from each sample were accomplished by flow cytometer (Table 3.5). The results showed that strain Castor 33 named CM33 displayed the highest relative MFI value approximately of 108.2 and 109.1 after detected by FL2 and FL3 channels, respectively (Table 3.5).

Table 3.5 Relative MFI of yeasts from Castor (CM) beans when detect for neutral and polar lipid using NR and flow cytometry techniques.

Strains	Relative MFI of FL2 channel	Relative MFI of FL3 channel
CM1	42.44	56.36
CM2	25.93	29.76
CM3	22.36	27.93
CM4	11.76	14.39
CM5	21.09	26.45
CM6	56.56	66.56
CM7	27.63	38.88
CM8	43.43	52.92
CM9	37.75	43.77
CM10	36.46	43.19
CM11	39.04	53.20
CM12	40.67	56.25
CM13	38.09	50.22
CM14	19.71	22.07
CM15	42.03	60.09
CM16	41.65	59.78
CM17	50.79	63.24
CM18	58.37	69.47
CM19	28.78	42.54
CM20	49.50	74.24
CM21	44.79	37.92
CM22	63.43	65.00
CM23	19.87	25.19
CM24	53.54	57.91
CM25	34.11	47.50
CM26	41.17	54.89
CM27	36.26	48.87
CM28	38.85	53.08
CM29	40.45	46.70
CM30	33.94	46.64
CM31	44.87	57.82
CM32	34.55	46.25
CM33	108.18	109.08
CM34	9.56	13.71
CM35	18.80	22.07
CM36	12.65	15.58
CM37	56.91	68.51
CM38	22.35	25.85
CM39	14.57	19.65

Table 3.5 (continued).

Strains	Relative MFI of FL2 channel	Relative MFI of FL3 channel
CM40	25.24	30.62
CM41	24.48	40.16
CM42	26.58	34.32
CM43	19.34	21.75
CM44	23.03	26.57
CM45	61.33	63.19
CM46	15.03	18.70
CM47	26.33	33.90
CM48	15.73	19.66
CM49	85.82	88.95
CM50	28.46	33.01
CM51	3.64	2.57
CM52	2.07	1.53
CM53	42.11	43.59
CM54	44.11	56.18
CM55	40.52	49.77
CM56	61.26	71.57
CM57	33.19	38.53
CM58	70.79	90.36
CM59	20.04	19.21
CM60	47.28	58.61
CM61	37.12	37.30
CM62	67.51	76.16
CM63	34.23	35.97
CM64	26.70	28.68
CM65	36.92	40.53
CM66	25.24	25.78
CM67	25.81	24.42

Stained CM33 was further confirmed the NR accumulation into intracellular lipid droplets using confocal microscope (Figure 3.6). To confirm the efficiency of this method for oleaginous yeasts screening, lipid quantification of CM33 and 3 randomly selected strains, CM15, CM59 and CM64, which demonstrated various relative MFI values of 42.0, 20.0 and 26.7, respectively, was validated. Correspondingly with the relative MFI values, the highest lipid and fatty acids contents was found in strain CM33 as 20.5% DCW followed by 5.6%, 3.6% and 3.4% DCW of strains CM15, CM59 and CM64, respectively (Table 3.6).

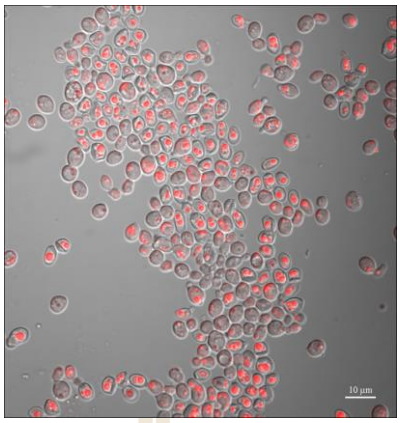

Figure 3.6 Visualization of CM33 cell morphology and lipid bodies. The lipid bodies were stained with Nile red and observed under a confocal microscopy (Nikon/Ni/E, Nikon®Nikon Instruments Inc.).

Table 3.6 Biomass, lipid, and fatty acids (FAs) composition of selected yeasts after culturing in YPD for 4 days. Data are mean ± standard deviation (SD) of triplicate.

Strains	Biomass (g/L)	Lipid content (%)	FAs profile (% of TL)					
			C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
CM15	2.53±0.1	5.62±1.2	11.31±2.9	0.24±0.1	4.05±0.9	12.88±3.4	11.96±3.1	ND
CM33	4.53±0.0	20.75±0.3	7.76±0.1	0.36±0.0	7.66±0.1	41.70±0.6	7.63±0.1	0.17±0.0
CM59	2.70±5.2	3.63±1.0	9.62±1.0	0.32±0.2	5.95±0.9	11.14±0.8	7.60±0.7	ND
CM64	7.20±0.1	3.59±0.3	10.30±1.3	0.59±0.1	3.09±0.2	22.71±2.8	8.96±1.1	0.01±0.0

ND: Not detected, TL: total lipid

Since, unknown natural-derived yeasts display different morphologies, which are difficult to adjust the cell-based number by optical density (OD) absorption. Therefore, using dye staining couple with individual cell automatically detection by flow cytometry is a more accurate way to evaluate endogenous lipid. This method is more convenient, require few of cells, less time, labor and solvents consuming when compare to numerous other methods. Additionally, flow cytometry provides more applicable 2 light scatters; (i) forward and (ii) size scatters which increase a potential of this technique to detect size and granularity of cells (Sonowal *et al.*, 2019).

Naturally fatty acid profiles of yeasts are composted of C16:0, C16:1, C18:0, C18:1 and C18:2 (Beopoulos and Nicaud, 2012; Klug and Daum, 2014; Mattanna *et al.*, 2014) which were also found in the selected strains (Table 3.6). C18:1 fatty acid was predominant among 4 selected strains, especially, CM33 displayed the highest C18:1 of 41.7% total lipid (TL). In addition, C22 and C24 long-chain fatty acids, which are important substrates for plastic, nylon, lubricant industries and cosmetic production (Park *et al.*, 2017) were also detected in this strain (data not shown). Small proportion of short-chain fatty acids (C10-C14) were also detected which Abghari and Chen, (2014) speculated that such fatty acids are easier to degrade and use for cell function while long-chain fatty acids (C16 and C18) are preferred for lipid production in oleaginous yeasts.

The yeasts were identified based on ITS-5.8S-ITS rDNA and D1/D2 26S rDNA nucleotides alignments on NCBI database. The highest percent identity each yeast was shown in Table 3.7. Therefore, CM33 identified as *Rhodotorula paludigena* was further investigate in this propose.

Table 3.7 Yeast identification based on ITS-5.8S-ITS and D1/D2 26S rDNA region.

Strain	Highest relative	Identity (%)	Accession number
CM15	*Cryptococcus flavescens*	99.69	FJ743610.1
CM33	*Rhodotorula paludigena*	99.00	FJ743628.1
CM59	*Cryptococcus rajasthanensis*	99.84	AM262981.2
CM64	*Aureobasidium melanogenum*	99.84	MH875142.1

3.4.2 Whole genome sequence of *R. paludigena* CM33

In the future, to increase ability of *R. paludigena* CM33 for biofuel and biomolecule productions using genetic engineering, *de novo* sequencing of this yeast was completed. Elimination of undesired nucleotide sequences was done according to methodologies of Novogene company which then yielded genome size of 20,657,327 bp and the G+C content of 64.3%. There were 82 contigs with a maximum contig length of 1,647,824 bp and minimum contig length of 20,441 bp. Genome sequence which is 20,657,327 bp was predicted to contain 6,497 protein-encoding genes, 127 tRNA genes, 6 snRNA genes and 570 non-coding RNAs genes. The whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the BioProject PRJNA491831, BioSample SAMN10089541, accession number SWEA00000000.1. Assembled genome sequences are provided via GenBank accession numbers SWEA01000001-SWEA01000078. Raw data sequences have been deposited in the SRA database under accession number SRX6085390.

3.4.3 *R. paludigena* CM33 using various carbon sources

It is well known that culturing under nitrogen limitation conditions induces oleaginous yeasts to accumulate high lipid content (Kolouchová *et al.*, 2016a, 2016b). During nutrient limitation, adenosine monophosphate (AMP), which is activator of isocitrate dehydrogenase, is decreased causing digestion of AMP desaminase to produce inosine monoophosphate, and ammonium ions. These ammonium ions are used for amino acid biosynthesis (Fakas, 2017). Consequently, isocitrate dehydrogenase cannot convert isocitrate to α-ketoglutarate. Leading to accumulate isocitrate that is equilibrated with citrate, which excess in mitochondria. The excess citrate is transported into cytosol using citrate-malate shuttle then cleaved by ATP:citrate lyase to produce oxaloacetate and acetyl-CoA. This enzyme is exhibited only in oleaginous yeast and normally located in cytoplasm where claves citrate to oxaloacetate and acetyl-CoA. Whereas, acetyl-CoA comes from conversion of acetic acid by acetyl-CoA synthase activity in glycolytic pathway has been found in non-

oleaginous yeast such as *S. cerevisiae* (Fakas, 2017). Therefore, the ability of *R. paludigena* CM33 to utilize different carbon substrates to produce lipid was accomplished through cultivation on minimal medium supplemented with glucose, glycerol, sucrose and xylose for 4 days. As shown in Figure 3.7, all carbon sources were assimilated and exponential phase was shown from day 0-3 then followed by stationary phase. It appeared that glucose-based medium gave higher OD600 while other carbon sources were similar. Not only mentioned carbon sources, genus *Rhodotorula* sp. also able to consume arabinose and fructose (Gong *et al.*, 2019) .

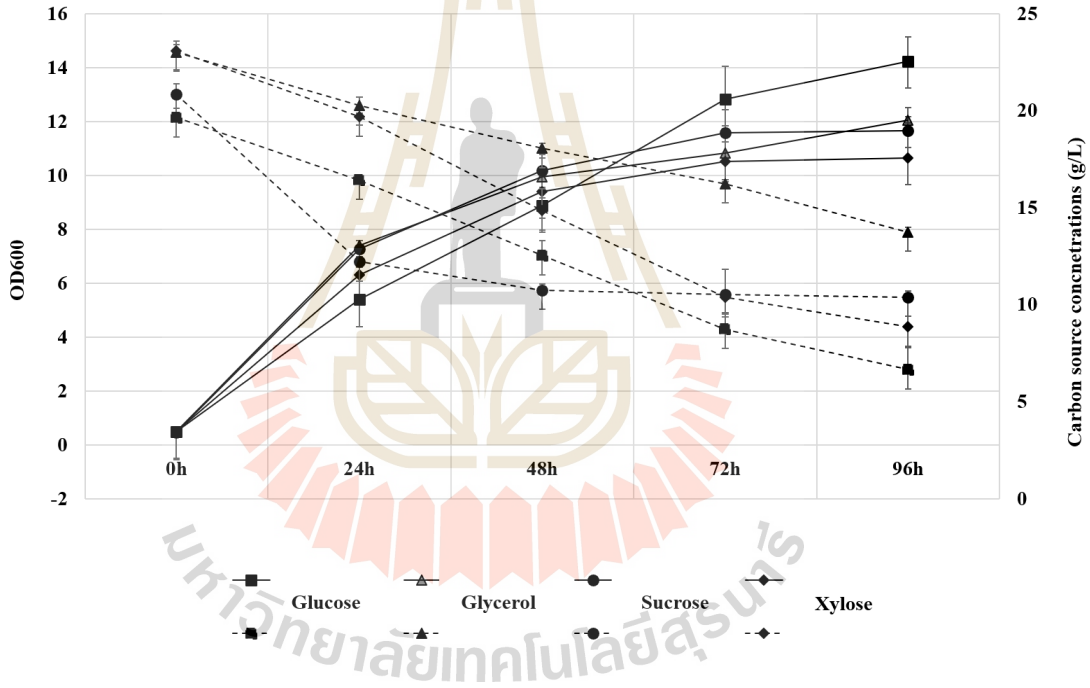

Figure 3.7 Time course of carbon sources concentration and OD600 of cell during culture of *R. paludigena* CM33 in the minimal medium containing glucose, glycerol, sucrose, and xylose as carbon sources.

Growing CM33 in glucose-based medium provided the maximum biomass and lipid content of 4.7 g/L and 23.9%, respectively when compared with other carbon sources (Table 3.8). Disaccharide molecule sucrose can also be utilized by CM33 to give biomass and lipid content of 4.3 g/L and 14.8%, respectively, which greater than glycerol and xylose. These results corresponds to the report of VijayaKumar *et al.*, (2010) that glucose provided

largest biomass and lipid yields of *Rhodotorula glutinis*, *Rhodosporidium toruloides*, and *Lipomyces starkeyi* when compared with fructose and sucrose. Glucose-based medium yielded higher lipid than sucrose-based medium was also observed in *Cryptococcus* sp. SM5S05 and *Moesziomyces eriocauli* SJ3L01 which Chang *et al.*, (2015) suggested that simplicity of glucose could be directly use while yeast has to break down sucrose into constituent sugars before using. The yeast provided higher biomass and lipid accumulation in glucose than xylose. Tiukova *et al.*, (2019) suggested that it might be downregulation of genes involved beta-oxidation and higher efficiency of transporters when yeast culturing in starvation condition and glucose used as carbon source. The similar results was reported in *Rhodotorula toruloides*, *Rhodosporidium fluviale*, *Rhodosporidium paludigenum* and *Rhodotorula taiwanensis* (Poontawee *et al.*, 2017). Both C16:0 and C18:1 were the major fatty acids in all 4 carbon sources. Glucose-based medium gave the highest of C16:0 and C18:1 of 23.79% and 53.6% TL, respectively, followed by sucrose and glycerol and xylose. It is noteworthy that C18:2 fatty acid was increased up to 9.7%, when glycerol was used as carbon sources. This result coincides with the report of Yen *et al.*, (2012) that C18:2 unsaturated fatty acids in *Rhodotorula glutinis* increased about 25%-27% and 14%-22% (Gong *et al.*, 2019) when cultivation through glycerol-based medium. Although, there were remaining carbon sources in the medium after cultured for 4 days. However, it could be concluded that the CM33 was able to consume and grow on all substrates. Additionally, the ability of CM33 to use glucose, sucrose, xylose and glycerol to produce lipid is a platform for economic applying agricultural hydrolysates and by-product derived biodiesel plant such as molasses and crude glycerol as carbon sources.

Table 3.8 Biomass, lipid, and fatty acid compositions of *R. paludigena* CM33 compared between different carbon sources.

Carbon sources	Biomass (g/L)	Lipid content (%)	FAs profile (% of TL)								
			C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0	C24:0
Glucose	4.68±0.2	23.87±2.6	23.79±1.9	0.98±0.1	4.39±0.3	53.57±5.3	4.72±0.5	0.42±0.1	0.08±0.1	0.16±0.1	0.95±0.0
Glycerol	3.83±0.2	12.37±0.2	10.44±0.9	0.89±0.2	2.32±0.2	18.41±1.6	9.67±1.2	0.92±0.1	0.01±0.0	0.22±0.0	0.70±0.1
Sucrose	4.27±0.2	14.82±0.3	17.02±0.4	0.65±0.1	4.32±0.1	34.74±1.3	7.10±1.6	0.75±0.1	0.08±0.1	0.42±0.0	1.32±0.2
Xylose	3.79±0.4	12.55±3.1	11.68±3.7	0.47±0.2	3.00±0.9	26.68±8.3	5.07±1.9	0.60±0.3	0.12±0.1	0.25±0.2	1.02±0.4

TL: total lipid

3.4.4 *R. paludigena* CM33 using molasse and crude glycerol

Reducing cost and polluted wastes are also points to produce microbial oil as biofuels feedstock. As a potential of *Rhodotorulu* sp. to utilize various carbon sources, waste materials as substrate and tolerate to inhibitory molecule derived from the hydrolysates (Enshaeieh *et al.*, 2015; Enshaeieh *et al.*, 2012-2013; Liu *et al.*, 2015; Yen *et al.*, 2012), in this work, different concentration of molasses and crude glycerol were preliminary attempted for the production of microbial lipids through 7 days cultivation of *R. paludigena* CM33. Sucrose, glucose, fructose and glycerol concentration at 0h and 168h were measured by HPLC. Biomass productions were ranged from 4.60-16.45 g/L. The highest biomass production of 16.5 g/L and 16.1 g/L were achieved with 10% and 15% molasses, respectively (Figure 3.8A). At such concentration of molasses, the maximum lipid yields were up to 6.10 and 6.07 g/L. These results related with there were sugar remaining e.g. sucrose, glucose and fructose, after cultured for 7 days. Lower amount of biomass and lipid concentration were obtained in 5% and 20% molasses-based media which might be the affected of exhausting and substrate inhibiting, respectively (Figure 3.8B). In contrast, biomass production was gradually decreased with increasing of crude glycerol concentrations, which gave lipid yields ranged from 1-1.4 g/L (Figure 3.9A). As shown in Figure 3.9B, small amount of crude glycerol was utilized which might be due to the existing inhibitors that inhibited growth and lipid production. Crude glycerol derived from biodiesel plant without pretreatment may contains free fatty acids, inorganic compounds and methanol that can interfere cell metabolism (Polburee *et al.*, 2015; Yen *et al.*, 2012). As shown in Table 3.9, biomass and lipid productions of *Rhodotorula* sp. were achieved through cultivation using molasses, crude glycerol and agricultural hydrolysates as carbon sources. Kot *et al.*, (2016) reviewed that biomass, lipid, and fatty acid profile can be affected by several factors such as yeast strain, culture condition and medium composition. Therefore, such factors must be considered for target molecules production. Quality of biodiesel depended on fatty acid profiles which

highly contain C16:0, C18:0, C18:1 and C18:2 fatty acids with suitable amount of polyunsaturated fatty acids (Bessadok *et al.*, 2019; Tanimura *et al.*, 2014). In this work, FAMEs result of CM33 grown in 10% molasses-based medium showed the largest amount of C18:1 (40.98% TL) followed by C16:0 (15.98% TL), C18:0 (11.51% TL) and C18:2 (2.8% TL) which were suitable for biodiesel production. Other fatty acids such as C16:1, C20:0, C22:0 and C24:0 which are substrates in plastic, nylon, lubricant and cosmetic industries, were also detected.

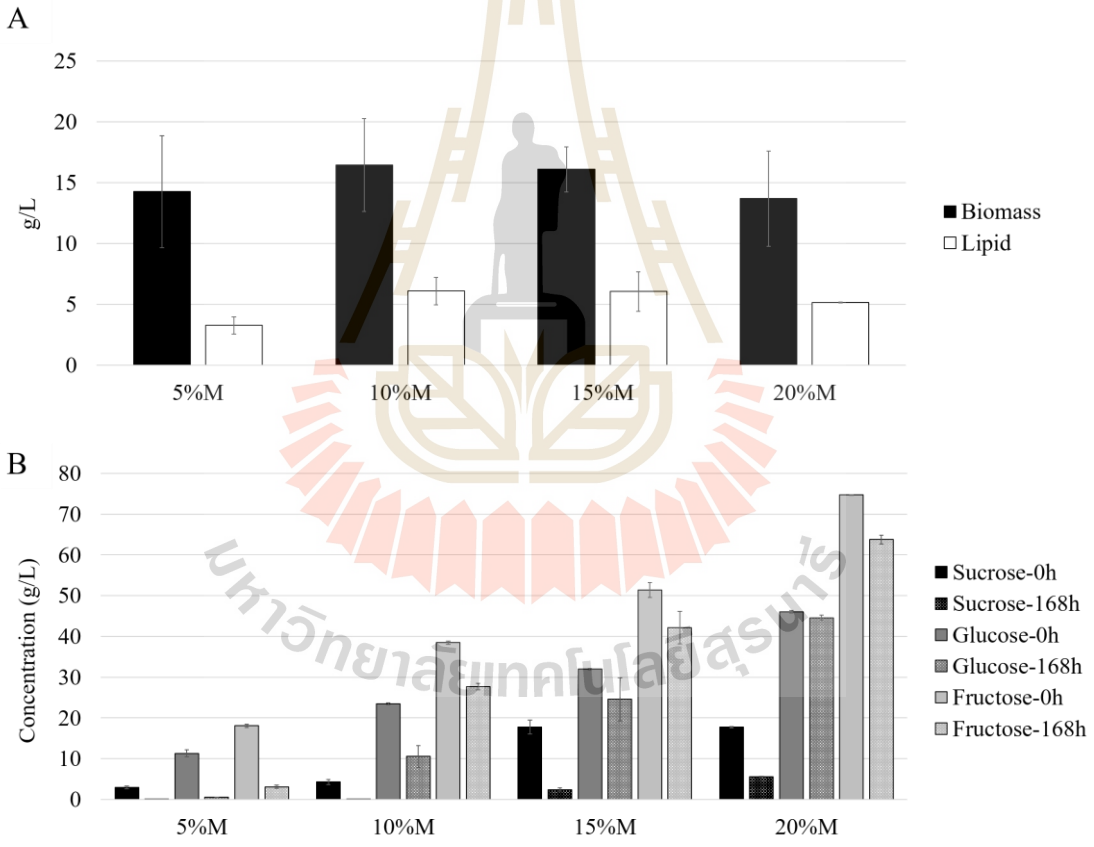

Figure 3.8 Biomass concentration (g/L), lipid concentration (g/L) (A) and three sugar remains (B) of *R. paludigena* CM33 in minimal medium containing different concentration of molasses (M) at 30°C, 200 rpm for 7 days.

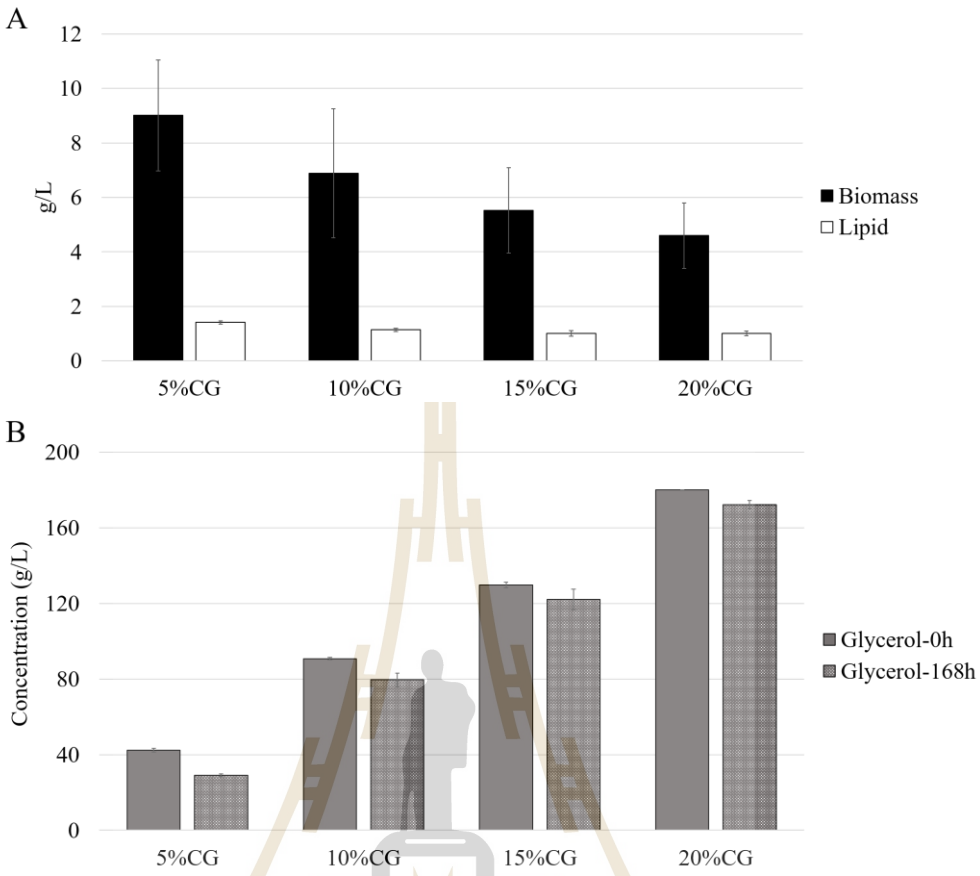

Figure 3.9 Biomass concentration (g/L), lipid concentration (g/L) (A) and glycerol remains (B) of *R. paludigena* CM33 in minimal medium containing different concentration of crude glycerol (CG) at 30°C, 200 rpm for 7 days.

Table 3.9 Lipid content, lipid yields, biomass and cultivation modes of *Rhodotorula* using different industrial and agricultural wastes as carbon source.

Oleaginous yeasts	Carbon sources	Lipid content (%DCW)	Lipid concentration (g/L)	Biomass (g/L)	Cultivation mode	Ref.
R. paludigena CM33	Molasses	37.08	6.10	16.45	7-days, Flask	This work
	Crude glycerol	15.70	1.41	9.01		
R. kratochvilovae	Molasses	38.25	4.82	13.25	7-days, Batch fermenter	Jiru *et al.*, (2018)
R. glutinis	Non-detoxified liquid hydrolysate	25.00	3.50	13.8	6-days, Flask	Yu *et al.*, (2011)
	Detoxified liquid hydrolysate	20.70	2.40	11.8		
R. toruloides	Non-detoxified liquid hydrolysate	ND	NA	ND	6-days, Flask	
	Detoxified liquid hydrolysate	24.60	2.40	9.90		
R. glutinis BCRC 22360	Crude glycerol	36.50	5.40	14.80	4-days, Batch fermenter	Yen *et al.*, (2012)
Rhodotorula sp. 110	Wheat bran hydrolysate	43.40	6.50	14.97	4-days, Flask	Enshaeieh *et al.*, (2012-2013)
	Corn stalk hydrolysate	38.90	5.84	15.01		
R. mucilaginosa	Xylose	51.33	8.10	15.78	4-days, Flask	Enshaeieh *et al.*, (2015)
	Wheat straw hydrolysate	42.63	5.9	13.84		
	Rice bran hydrolysate	38.24	6.2	16.21		
	Grass hydrolysate	49.02	7.5	15.30		
	Leaves hydrolysate	45.12	6.8	15.07		

*ND: not detected; NA: not available

3.5 Conclusion

In this work, MFI values from NR emissions were compared among isolated yeast strains. The CM33 displayed the highest MFI value and lipid accumulation. It was identified as *Rhodotorula paludigena*. Ability of *R. paludigena* CM33 to consume various carbon sources were explored. Minimal medium supplemented with glucose, glycerol, sucrose, and xylose were tested. The highest biomass and lipid content were obtained from glucose- and sucrose-based medium. Fatty acid profiles consisted of C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 which were similar to plants. In addition, biomass and lipid content of CM33 was very high when cultured using agricultural by-products molasses as carbon source. Several factors should be considered when microorganisms are expected to be used for oil production e.g. amount, quality, and yeasts ability to use cheap substrate. All such factors are demonstrated in CM33. It produced high cell mass, lipid, fatty acids contents including its ability to assimilated waste materials. This work indicated the usefulness of natural screened *R. paludigena* CM33 for the use as yeast-based biofuel feedstock. Moreover, we performed whole genome sequencing of *R. paludigena* and deposited nucleotide sequences on NCBI database. The WGS could be used to metabolic engineer to improve CM33 to produce lipid, fatty acid and valuable molecules. Metabolic engineered methods combining with modification of cultured conditions using various industrial wastes of CM33 can be done in the near future.

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CHAPTER IV

CONCLUSION

Docosahexaenoic acid or DHA is important and valuable. It is generated by a series of desaturase and elongase activities. In this thesis, Δ2D, Δ6E and Δ4D genes involved in DHA biosynthesis were cloned and then co-expressed in *P. pastoris*. Results demonstrated that the recombinant yeast produced and accumulated high amount of DHA which is not detected in non-transformed *Pichia* and *Pichia* transformed with empty plasmid. These results confirmed the activities of these enzymes to produce DHA the target product.

The potential of oleaginous yeasts to use for biofuels and biochemical production were screened. The screened yeast *R. paludigena* CM33 was grown in various carbon sources including waste materials to increase the potential of this yeast to use as feedstock. Finally, *de novo* sequencing of *R. paludigena* CM33 was done then whole genome sequence was deposited on DDBJ/ENA/GenBank.

The potential of yeasts for lipid and fatty acids production has been shown. Its several advantages including recycles by-products and wastes material as carbon and nitrogen sources to produce target products lead these yeasts to be biofuels and biochemicals feedstock. Widely genetic engineering tools can be applied to yeasts for increasing and producing specific lipid and fatty acids. Then, these yeasts could be directly applied for food, feed or lipid and fatty acid will be extracted for more applications.

BIOGRAPHY

Miss Chotika Gosalawit was bone on June 13, 1986 in Sakaeo, Thailand. She graduated with a Bachelor of Science in Marine Science from Kasetsart University in 2008. Then, she studied Master of Science in school of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology (SUT) with Assoc. Prof. Dr. Mariena Ketudat-Cairns. She received a Graduate scholarship from SUT to support her tuition and fee. In 2014, she applied for Ph.D. program. Her research consisted of docosahexaenoic acid (DHA) and lipid and fatty acids production using yeasts as cell factories. She received a Graduate scholarship from SUT to support her tuition and fee and Canada-ASEAN Scholarships and Educational Exchanges for Development SEED scholarship from the Government of Canada for an opportunity to do part of her research in Canada and SHELL Centennial Education Fund, Shell Companies in Thailand for financial supported.